Characterization Of *In Vitro* Generated Autologous Osteoid Tissue

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Abstract

Bone disease and injury is expected to increase in the near future due to an ageing population, causing social and economic burden due to disability and lowered quality of life. Critical sized defects, the smallest bony injury that will not heal completely over the lifetime of an individual, are significant orthopedic and oral-maxillofacial issues, as they require bone grafts to facilitate repair. As the gold standard autograft is in short supply with additional disadvantages, bone tissue engineering seeks to alleviate this demand. It combines cells isolated from patients with biomaterials to produce bone tissue. This research looks into the feasibility of bone banking: the cryopreservation and storage of a patient’s cells that can be reanimated at the time of injury or disease and the engineering of a bone graft in vitro.

Chitosan and nano-hydroxyapatite composite scaffolds were fabricated to produce appropriate pore diameters and porosities. A final concentration of 8% (w/v) chitosan and 5% (w/v) nano-hydroxyapatite were used in the final scaffold, where imaging studies (scanning electron microscopy, micro-computed tomography) showed pore diameters with a 106 µm average with 79% porosity. These scaffolds were subjected to testing, such as degradation, pH, sterilization, swelling studies, Fourier transform infrared spectroscopy, and energy dispersive X-ray spectroscopy in order to characterize physical and chemical properties.

Using an osteogenic sarcoma cell line (SAOS-2), scaffolds were then subjected to in vitro cell studies including the LIVE/DEAD® viability assay and the MTS cell proliferation assay to characterize biocompatibility. Results indicate scaffolds are biocompatible and non-cytotoxic. Further three-dimensional in vitro testing was performed using SAOS-2 in a custom-made perfusion bioreactor for 14 days. After this period isolated cells were detected on the scaffold using histological and
fluorescent microscopy techniques. Further in vitro testing was investigated using mesenchymal stem cells isolated from rats. However, these cells reached senescence rapidly, therefore, the supply of cells were limited and differentiation down the osteoblastic lineage and three-dimensional culture was unable to be accomplished.

This research demonstrated the development of a biocompatible scaffold with appropriate structural parameters that promote viable cells when cultured in a bioreactor. Future study should investigate the scaffolds mechanical and structural properties to determine suitability for bearing load and the production of a homogenous scaffold matrix that results in homogenous osteoid tissue. Cell culture optimization in terms of mesenchymal stem cell proliferation and extracellular matrix production also needs to be investigated in terms of modifying tissue culture factors such as shear stress and seeding density.
Acknowledgements

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Thanks to Ed for doing the initial work on the bone banking concept. Yet again, no animal trials but whoever is carrying on this work I hope my contribution helps, if you need help get in contact with me, ask George. I’ll be in Dunedin for at least another 3 years.
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<th>Abbreviation</th>
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<tr>
<td>µCT</td>
<td>Micro-computed tomography</td>
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<tr>
<td>µHA</td>
<td>Micro-hydroxyapatite</td>
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<tr>
<td>3D</td>
<td>Three-dimensional</td>
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<tr>
<td>AA</td>
<td>Acetic acid</td>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>BMP-2</td>
<td>Bone morphogenetic protein two</td>
</tr>
<tr>
<td>BMU</td>
<td>Basic multicellular unit</td>
</tr>
<tr>
<td>BTE</td>
<td>Bone tissue engineering</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>CS</td>
<td>Chitosan</td>
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<tr>
<td>CSD</td>
<td>Critical size defect</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
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<tr>
<td>EDS</td>
<td>Energy dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMTECH</td>
<td>Electromechanical Technology for Teaching and Research</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblastic growth factor</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
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<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
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<tr>
<td>KBr</td>
<td>Potassium bromide</td>
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<tr>
<td>LEI</td>
<td>Lower secondary electron image</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
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</table>
nHA  Nano-hydroxyapatite
PBS  Phosphate buffered saline
PPI  Pounds per inch
PTH  Parathyroid hormone
SAOS-2  Osteogenic sarcoma cell line
SEM  Scanning electron microscopy
SHED  Human exfoliated deciduous teeth stem cell
TE  Tissue engineering
TGB-β  Transforming growth factor beta
α MEM  Alpha-minimal essential media
1 Introduction

Due to demographic changes, clinicians are exposed to increasing numbers of geriatric diseases such as osteoarthritis and osteoporosis (1). According to Chang and Xiao, bone diseases affect 10 to 12 million people in the United States and there are an estimated 1.5 million fractures every year. In the United Kingdom, a fracture occurs every 30 seconds due to osteoporosis. In China, 3 million individuals suffer from bone defects or injury every year. More than 500,000 bone graft procedures are performed in the United States, with approximately 2.2 million performed worldwide, and estimated to cost $2.5 billion per year. These figures illustrate the impact bone disease and injury has on society. It is expected to get worse, as bone grafting procedures will increase while the challenge to repairing bone defects remains unsolved (2,3). Regenerative medicine can solve these detrimental problems in humans, though further research is required (2).

Although the body has a high healing potential, bone loss remains an unsolved clinical problem. A critical size defect is bone loss of a certain size that does not heal and remains a huge problem with more than 40% of cases having inadequate autograft supplies. The ‘gold standard’ autograft has the best clinical outcome, but its shortcomings are limited availability and the need of a second surgical site (1). Although allografts, xenografts and synthetic bone substitutes are widely available there are many risks including rejection, disease transmission and suboptimal healing (4). There is increasing demand for superior bone substitutes because of the disadvantages with current ones. To improve the healing potential of these synthetic bone graft substitutes, scaffolds should be seeded with osteogenic cells obtained from the patient (1), which is a premise of engineered tissue.
Tissue loss is a common theme in surgery, and organs for transplantation are always in demand, with immunosuppressive agents causing additional problems. The goal of tissue engineering is to develop new biotechnologies in order to restore structure and function of damaged tissue (5). Bone is the second most transplanted tissue in the human body after blood (3) and has the highest potential for regeneration in the body (5). Simply put, tissue engineering is the use of cells and supporting structures and/or biomolecules to regenerate tissue (6). Scaffolds with three-dimensional, interconnected, porous architectures are commonly used to provide a template for tissues to regenerate. Osteoprogenitor cells can be taken by bone marrow biopsy sampling, expanded in vitro and seeded onto the scaffold. This cell-scaffold construct can be cultured in three-dimensions in a bioreactor until an appropriate graft is formed. This final 3D cell-scaffold construct can be implanted into the patient without rejection and is theoretically unlimited in supply (5).

In the research undertaken for this thesis, the focus is placed on the development of an appropriate scaffold for cell viability and proliferation, so when cultured in a bioreactor, produces a 3D sample of osteoid. A major limitation of bone tissue engineering is the development of mechanically sound scaffolds that have porous interconnectivity, allowing appropriate nutrient and waste perfusion, and cell proliferation. By using a custom-made bioreactor, we can develop a homogenous sample of tissue, while also improving seeding efficiency, mechanically stimulating cells, and overcoming diffusional limitations present in static culture. This thesis will further investigate the feasibility of a “bone bank” that was investigated by Stace 2011 (40). Similar to blood banking, bone banking will involve the storage of tissue for future use. This will assist those at risk of bone damage and disease, including but not limited to: soldiers, athletes, elderly and congenital defects such as cleft palate.

The aims of this study were:

1. To manufacture a structurally appropriate and biocompatible chitosan and hydroxyapatite scaffold.
2. The generation of osteoid tissue, by three-dimensional cell culture of cells seeded onto the above scaffold within a bioreactor.
2 Literature Review

2.1 Overview
This literature review encompasses four main sections. The first section examines bone biology, its structure and function so we can apply this knowledge to bone tissue engineering (BTE). The second section examines various ways of bone regeneration with both intrinsic and extrinsic elements. And the third section focuses on BTE and its various components: cells, scaffolds and bioreactors.

2.2 Bone Tissue

2.2.1 Overview
Bone is a specialized connective tissue that serves the functions of locomotion, protection and metabolic functions (7). There are three main cells present in bone tissue: osteoblasts, osteocytes and osteoclasts (8) within an extracellular matrix (ECM) called osteoid that is dominated with fibrillar proteins, providing a flexible trait. Upon the deposition of hydroxyapatite the osteoid becomes mineralized, providing properties of rigidity and strength (8).

2.2.2 Structural Organisation
The architecture of bone is organized for maximal strength and the lowest possible weight. The majority of bone has a dense, rigid exterior of compact (cortical) bone, and an interior of cancellous (spongy or trabecular) bone. There are two types of bone: woven, which is immature osteoid with disorganized collagen fibres; and lamellar, where collagen is arranged in sheets organized into regular parallel bands arranged in sheets (8). Lamellar bone is considered to be mature or adult bone (7).
Compact bone is predominantly strong along the diaphysis and becomes progressively thinner towards the metaphysis and epiphysis (7). It is composed of units called osteons that are columns arranged in parallel to the line of stress applied to bone. Each individual column is made up of concentric bony layers, or lamellae, arranged around a Harversian (central) canal that contains neural and vascular elements. Osteons are produced by osteoclasts breaking down cortical bone to form a canal that blood vessels, lymphatics and nerves penetrate. Osteoblasts internally line this canal and begin producing lamellae. After deposition of a series of lamellae, the diameter of the Harversian canal decreases and osteoblasts are trapped in lacunae to become osteocytes. Small canals called canaliculi interconnect other lacunae and contain cytoplasmic processes of trapped osteocytes. Volkman canals interconnect the neurovascular bundles within each Harversian canal (8). Compact bone is highly mineralized (up to 90%) allowing it to achieve its mechanical requirements (7).

Cancellous bone consists of myriads of highly interconnected bony trabeculae and is mostly apparent in the epiphysis of bone. The trabeculae number, thickness and orientation depend on how stress is applied to bone. For example, there are thick intersecting trabeculae in weight bearing bone such as vertebrae, but fewer in ribs as they are exposed to less stress. Trabeculae are composed of lamellar bone with scanty lacunae containing osteocytes. Nutrient exchange occurs by canaliculi, with blood sinusoids in the marrow space that also allows osteocyte communication with one another. The interior of the trabecular surface has a coating of endosteum with inactive osteoblasts. Cancellous bone, in contrast with compact bone, is only 20% mineralized. The other 80% of bone is covered by marrow, vasculature and stem cells. This allows it to achieve its metabolic function by allowing high communication between bone and other tissue (7). Spaces in the cancellous bone trabeculae contain haematopoietic bone marrow (8).

2.2.3 Extracellular Matrix

The extracellular matrix of bone is called osteoid and consists of organic and inorganic portions. Inorganic salts make up approximately 70% whilst organic matrix makes up the remaining 30% of bone (8).
Collagen almost makes up the entirety of the organic component at 90%, with the remaining 10% made by ground substance proteoglycans and non-collagen molecules (8). Collagen in bone is predominantly in the form of type I fibres, that follow specific directions to form the basis of the lamellar structure of bone (7). The ground substance serves to control water content in bone and regulate the formation of collagen fibres (8). The non-collagenous proteins, such as osteocalcin, osteopontin, and fetuin serve to balance mineralization by inhibition and exert important metabolic functions, such as control of energy metabolism by osteocalcin (7). Osteonectin provides a bridging function between collagen, mineral component and bone sialoproteins (8).

The inorganic matrix serves as an ion reservoir. Ions form crystalline structures predominantly in the form of calcium hydroxyapatite \( \text{Ca}_{10}[\text{PO}_4]_6(\text{OH})_2 \) in ‘hole regions’ of the triple helical fibrils of collagen I. The addition of minerals to bone is controlled by non-collagenous proteins to balance flexibility and stiffness requirements (9,10). Hydroxyapatite formation occurs in membrane-bound vesicles secreted by osteoblasts, which accumulate calcium and phosphate ions. The vesicles ultimately rupture and hydroxyapatite crystals spill into the extracellular fluid to become part of the extracellular matrix. The alkaline phosphatase enzyme secreted by osteoblasts cleaves large phosphate molecules (such as the mineralization inhibitor pyrophosphate) to individual inorganic phosphate ions, removing inhibition and further increasing phosphate concentration (10). Around 20% of the mineral component remains in an amorphous form, allowing the rapid exchange of ions for calcium homeostasis (8).

2.2.4 Cellular Structure
Bone cells develop from mesenchymal stem cells (MSC) or haematopoietic stem cells (HSC), to give rise to the principle cells that produce bone (osteoblasts), resorb bone (osteoclasts) and other cells associated with bone formation such as osteocytes, osteoprogenitor cells, and other bone lining cells (9). Osteoblasts and osteoclasts are important for the constant turnover and remodelling of bone (8).
Osteoblasts are cuboid-shaped cells that form clusters over a bone surface. They synthesize and excrete collagenous and non-collagenous bone matrix proteins onto bone before becoming either bone lining cells or trapped as osteocytes (7,9). This new non-mineralized matrix is called osteoid and takes about 10 days to be mineralized (7). Osteoblasts develop locally from MSC proliferation in bone marrow and periosteum. Differentiation is dependent on two key transcription factors, Runx2 and its target Osterix 1, which cause the differentiation of MSCs into osteoblasts in response to various stimuli, such as bone morphogenic proteins (BMPs) and parathyroid hormone (PTH) (7).

Osteoclasts are large, multinucleate cells containing up to 20 nuclei and have the unique ability to resorb bone. They have a characteristic ‘ruffed border’, which is a highly folded plasma membrane designed to secrete and resorb proteins and ions into an isolated space where bone resorption occurs. This region is sealed by contractile proteins and tight junctions to localize the acidity (7). Energy demanding proton pumps acidify the contained region to mobilize the mineralized bone. The remaining organic matrix is subsequently broken down with proteases. This results in Howship’s lacunae, a characteristic shallow cavity. Osteoclasts are derived from HSCs that have generated mononuclear cells, which further develop into preosteoclasts that leave into the blood. These cells leave the circulation at sites that require resorption and fuse with one another to form a multinucleated immature osteoclast. Mature osteoclasts are differentiated from immature osteoclasts by attachment to bone matrix by integrins (9).

The most numerous cells are osteocytes and are found in lacunae throughout the matrix. Osteocytes develop from osteoblasts that are trapped in bone matrix during bone formation (7). Lacunae are interconnected by canaliculi where osteocyte cytoplasmic processes pass through. Neighboring osteocytes communicate via gap junctions to convey intracellular messages, and for nutrient transport. Their principal role appears to be the sensing of mechanical stimuli, to regulate bone remodelling and development in response to shear or strain forces. Their possible role in calcium and phosphate homeostasis remains unclear (9,11).
The endosteum is the inner surface of compact bone, whereas the periosteum is a layer of condensed fibrous tissue covering the outer aspect of bone. Both contain osteoprogenitor cells that are activated for healing and remodelling (7,8). These processes will be discussed in more detail in 2.2.6 and 2.2.7.

2.2.5 Bone Development
Bone development occurs by two main processes, intramembranous and endochondral ossification. Intramembranous ossification forms flat bones. It is the formation of primary ossification centers by MSCs that have differentiated into osteoblasts, which directly produce osteoid. These centers then fuse to form an interconnected network of trabeculae made of woven bone. The periosteum forms on the surface, further mineralization occurs and the connective tissue within the trabeculae is transformed into haematopoietic tissue. Remodelling finally follows to produce lamellar bone. Endochondral ossification occurs in long bones, pelvis and sacrum and is the ossification of cartilage that is produced by chondrocytes that have differentiated from MSCs. Hypertrophic chondrocytes release various molecules that promote vascularization and primary ossification, calcifying cartilage. Secondary ossification centers form after birth in long bones to allow growth and development (7,12).

2.2.6 Remodelling
Growth, maintenance, and regeneration of bone involve the high regulation of mechanical integrity and calcium metabolism. Bone remodelling is dynamic, occurs continuously throughout life and consists of bone resorption coupled with simultaneous formation (7,13). It is tightly controlled by a complex arrangement and number of cell types including osteoblasts, osteoclasts and osteocytes, which are collectively called basic multicellular units (BMUs) (14). Remodelling occurs in response to mechanical forces, microdamage and systemic hormones, that are likely sensed by osteocytes to initiate remodelling at a specific site. This mostly occurs in cancellous bone, building an optimum architecture to adapt to an individual’s demands (7), to obtain maximum strength with minimal weight (14). Wolff’s Law directs remodelling. If a mechanical force is exerted onto bone, bone will remodel itself to resist that loading (15). Coupling, the phenomenon of tight regulation of
resorption and formation is regulated on three different levels: direct interaction between osteoblasts and osteoclasts, local interactions between the immune system and bone cells, and neuroendocrine systemic factors that control bone metabolism (7). This ensures that removed bone is always replaced by new bone (9).

2.2.7 Regeneration
Fractures are one of the most common injuries to the musculoskeletal system (16). In response to injury, bone heals by regeneration instead of repair. This allows tissue and mechanical properties to be restored at the site of injury, allowing bone to continue meeting its mechanical demands (9).

There are two types of bone healing: primary and secondary. Primary, or direct healing requires fracture ends to be rigidly fixed and immobile by early orthopedic intervention (9). This allows the omission of an external callus for stability. Osteoclasts tunnel into bone, allowing blood vessel penetration and osteoblast precursor recruitment, creating new osteons that connect ends of the fracture. Primary bone healing is the synthesis of lamellar bone in the same direction as the bone’s longitudinal axis (9). This type of healing is slow and considerable time is necessary for the healed fracture to weight bear after the removal of the orthopedic interventions (16).

Secondary, or indirect healing is more frequent (9). The fracture is stabilized using plaster of Paris, braces, and external fixators or bridging plates. These allow considerable movement and stimulate the development of a soft callus. Secondary healing has three overlapping steps: inflammation, reparation, and remodelling (16). A fracture results in a hematoma, a hemorrhage that is infiltrated by neutrophils and macrophages, and marks the start of inflammation. This initiates angiogenesis, chemotaxis of neutrophils, exudation of plasma, and fibroblast synthesis of collagen, which form granulation tissue that replaces the hematoma (9,16). The reparative phase involves osteoprogenitor cells of the periosteum to differentiate into cartilage producing chondroblasts, and osteoblasts that synthesize woven bone. The fibroblasts within the granulation tissue can also differentiate into chondroblasts. The product of these cells produces a mass called a primary callus; this stabilizes the fracture site and
favors bone formation. Once completed, this process is called clinical union. Osteogenesis then dominates to transform the primary callus to a secondary callus. The final stage, remodelling of woven bone to lamellar bone is necessary to produce mature bone (9).

In non-unions, healing stops with no attempt to bridge the fracture with a callus; instead it is replaced with fibrous tissue. Certain factors can cause a fibrous non-union including: infection, avascular supply, shearing forces, loss of apposition and disease (such as tumors). Radiographic imaging shows bone ends become round and dense compared to fresh fractures that are clear-cut (17).

Fractures are usually reduced and fixed to enhance healing. Reduction is the procedure of restoring a fracture or dislocation back to its correct alignment. Fixation provides stability and maintains alignment of bone fragments during healing. It may be by a plaster of Paris cast, or internal fixation, where surgery is required to implant an internal fixator made of inert metal. It may also be external fixation where metal stabilizes the fracture at a distance from the injury (17,18).

For an organ that often heals itself, bone can have difficulty regenerating. For many fractures and bone defects, orthopedic intervention is not enough to initiate healing as it had resulted in a critical size defect (CSD). The concept of a CSD is based on the observation that defects of a certain size will not heal spontaneously in the lifetime of the animal (19,20). The gold standard treatment of CSD is autologous bone grafts (20), however, as with all treatments comes with disadvantage, which will be explained in 2.3.4.1. Considerable research focuses on four areas: non-unions, spinal fusion, calvarial and long bone CSDs (19). Problematic healing is currently being investigated, using scaffolds and mesenchymal stem cells for tissue engineering (19,20).

2.2.8 Cellular Communication
As before in 2.2.6, bone remodelling constantly alters bone structure throughout life. Other than the systemic factors that influence this, there are also local paracrine factors that allow cross talk between osteoblasts and osteoclasts (21). Osteocyte death
by senescence is sensed by osteoblasts that are under regulation of osteocyte-secreted
sclerostin (21,22). Direct cell-cell communication occurs by osteocyte-osteoblast gap
junctions that may sense this cell death. Osteoblasts attract osteoclast precursors to the
area and stimulate osteoclastogenesis (22). Adhesion molecules Ephs and Ephrins are
expressed on the osteoblast cell surface for direct cell-to-cell contact with osteoclasts
and may play a role in osteoclast precursor migration (21,22). Simultaneous
expression of differentiation factors such as RANKL by osteoblasts induces
osteoclastogenesis. Cell-cell communication between preosteoclasts cells results in
cell proliferation and fusion. Sema4D from osteoclasts inhibits bone formation by
osteoblasts (21). After an area is resorbed, osteoclasts die by apoptosis and osteoblasts
secrete new bone tissue (22). There is also cross talk between cells of the immune
system that also regulates bone remodelling (21).

2.3 Bone Grafting

2.3.1 Overview
Bone loss due to disease or trauma can lead to premature disability and lowered
quality of life (23). Bone grafts are widely used and help promote bone formation
and regeneration. They function partly as scaffolds for osteoconduction, matrices for
attachment and induce the proliferation of osteoblasts that require anchoring, and sites
of intrinsic bone formation through osteogenesis (24). There are four different types
of bone grafts: autografts, allografts, alloplasts and xenografts (23).

2.3.2 Indications and Contraindications
Indications for autologous bone grafting are reconstruction of skeletal defects that
includes: tumors, trauma, infections, augmentation for fracture healing, delayed-
union, mal-union, non-union, joint fusion, reconstruction, and prevention of
morbidity in patients with risk factors (such as diabetes and smoking). Contraindications include limiting supply due to previous surgery, an active infection, local tumor, and the presence of external fixators (24,25).
2.3.3 **Properties**

There are three biologically important properties that form the foundation for bone grafting: osteoconduction, osteoinduction and osteogenesis.

2.3.3.1 **Osteoconduction**

Osteoconduction, defined as the ability of the graft to support osteoblast formation of new bone along a biological or alloplastic framework, with a porous matrix to support migration, attachment and proliferation of cells (5,26). This property is shown by autografts, allografts and many bone substitutes (24,27).

2.3.3.2 **Osteoinduction**

Osteoinduction is the enhancement of bone formation by the stimulation of osteoprogenitor cells to differentiate to osteoblasts (24,27). And therefore stimulating osteogenesis (28). The gold standard autograft displays this attribute, but complications and lack of supply have lead to the research of many growth factors to mediate bone formation. An osteoconductive and osteoinductive graft not only acts as a scaffold, but also stimulates new osteoblast formation; this is desirable as it allows faster graft integration (24,27).

2.3.3.3 **Osteogenesis**

Osteogenesis is simply bone formation from osteoprogenitor cells where bone can be produced spontaneously or transplanted (28). Surviving cells in the graft can produce new bone. Because of limited supply of gold standard autografts, significant research in BTE is a promising field to provide bone grafts for defects. Osteogenesis occurs in autografts only (24,27).

2.3.3.4 **Other Properties**

Mechanical properties are necessary for weight bearing and are employed by autografts and allografts. Grafts should have other desirable properties such as being: biocompatible, integrating with neighboring tissue without eliciting an immune response; biodegradable, metabolised by the body to be replaced by host tissue and
not release toxic breakdown products; and bioactive, promoting osteointegration and stimulating bone ingrowth and differentiation (23).

2.3.4 Graft Structure and Uses
Grafts can be classified as cortical, cancellous, or corticocancellous depending on the structure present. Bone varies in structure depending on where it is harvested from, and thus, also varies in their ability to provide structural support and osteogenesis. Cortical bone grafts are used for stability and support and is replaced much slower than cancellous bone grafts. Cancellous bone grafts are more osteogenic, however it cannot provide efficient structural support, therefore bone grafts needs to be carefully selected for a specific function before harvesting (27). Mixed properties may be obtained from a single graft (24).

2.3.5 Types of Grafts
Currently, bone grafts to be used as substitutes come from many sources. These are autografts, allografts, alloplasts and xenografts (23).

2.3.5.1 Autograft
An autograft (also known as an autologous and autogenous graft) is when bone is moved from one site of the body to another within the same individual (24) and is the gold standard for bone grafting (29). As these grafts are from the same patient, it is non-immunogenic, and has the properties necessary for healing: osteogenic, osteoconductive and osteoinductive. However, they have several disadvantages such as limited supply, post-operative complications, anatomical incompatibilities and donor site morbidity, as the anterior iliac crest is the most common site for harvesting bone (23,25,30). The quality of these grafts can also be adversely affected by the patient’s comorbidities and age (25). All bone requires blood supply in the site of implantation. Additional blood supply may be needed; this is done by extraction of the periosteum and accompanying blood vessels. This graft is called a vital bone graft (27).
2.3.5.2 Allograft

An allograft is tissue transferred from one individual to another of the same species (24). It is usually taken from cadavers who have donated bone and is typically sourced from a bone bank (27). The structure of the allograft provides an osteoconductive scaffold for fibrovascular growth and attraction of cells from the host that eventually leads to bone development (29). To prevent disease and immunogenicity, donor screening and tissue processing is required. The graft can be freeze-dried, demineralized and irradiated, which is disadvantageous as the bone will lose osteogenic potential, osteoinductive properties and integrity (4,31,32). Chemosterilized, autolyzed, and antigen-extracted bone weakens strength and integrity, but leaves osteoinduction intact (4). Because of this processing, allografts are used mostly for filling skeletal defect cavities (4).

Advantages of this graft is that there is a large supply and no donor site morbidity (32). Failure of allografts is higher compared to autografts, which is due to immunogenic properties. The major histocompatibility complexes (MHC) I and II antigens on the graft are recognized by T-lymphocytes, resulting in immune rejection of the graft by cell-mediated toxicity, antibody-mediated toxicity, and antibody-dependent cell-mediated toxicity. To reduce immunogenicity, tissues can be modified as mentioned above and host can be matched to donor (4).

2.3.5.3 Alloplast

Alloplasts (or synthetic materials) are biocompatible, inorganic materials that are widely used due to osteoconduction, strength and incorporation into bone. These can be used as cancellous bone substitutes or fillers, where structural stability is not necessary. These materials are porous for cell and vessel invasion, and are eventually replaced by new bone (27,32). The advantage of these materials is that there is no risk of disease transmission and it can be mixed with autografts or allografts (32). Alloplasts can be bioinert, where there is no adherence to tissue in the implantation site. They can be bioactive, chemically reacting to surrounding tissue to form a strong bond. They can be bioresorbable, where the material contributes to the metabolic processes of the host to regenerate the tissue (33). Bone is a composite material, made of an organic and an inorganic component, providing flexibility and strength
respectively. Current work is looking into the development of new hybrid materials with combined properties of several materials, with focus on hydroxyapatite (HA) as the inorganic component (34,35).

2.3.5.3.1 Ceramics

Ceramic-based materials constitute a large proportion of materials for restoring bone defects and can be mixed with other graft material or used alone. They are very similar to the inorganic matrix of bone and are osteoconductive. They are not osteoinductive, as new bone is not stimulated and they are used best for filling contour defects (31). The ideal alloplasts are resorbable into the body and have similar strength to cancellous bone (29). Ceramic-based substitutes include, but are not limited to: calcium phosphate, calcium sulfate, and bioglass. These can be used alone or in combination. The pore size of ceramics should be similar to cancellous bone to allow fluid circulation and cell colonization (36).

HA is a crystalline structure of calcium phosphate with the chemical formula Ca_{10}(PO_{4})_{6}(OH_{2}) (29). Natural HA makes up the majority of the mineral phase of bone (37). Synthetic HA has a similar chemical makeup to human hard tissue allowing a favorable chemical and biological affinity to bone tissue (37,38). Due to these similar chemical properties with mineralized bone matrices, HA provides excellent osteoconductive, bioactive and biocompatible properties (29,36,38). Although hydroxyapatite has good cytocompatibility, disadvantages include low solubility in physiological pH and differing mechanical properties from bone (36). Despite these, HA is accepted as a widely used biomaterial for bone regeneration (37).

Nano-hydroxyapatite (nHA) is stable in normal temperatures and pH and is poorly soluble in water. Additionally, it has a high hardness, bioactivity, no cytotoxic properties and is biocompatible with skin, muscle and hard tissues. The application of nHA to bone during bone implantation results in rapid bone formation and a solid biological fixation to bone due to its osteoconductive and bioactive properties (33). nHA has a similar structure to the minerals found in bone and is thought to enhance the mechanical and osteoconductive properties of implants. Compared to micro-
hydroxyapatite (µHA), nHA has a high surface area to volume ratio that could control protein interactions (adsorption, configuration and bioactivity) and therefore enhance osteoblast adhesion (37).

Tri-calcium phosphate is also a biocompatible crystalline structure like HA with the formula Ca₃(PO₄)₂. Its structure is similar to the mineral phase of bone with higher degradation rates compared to HA, which occurs by dissolution and resorption by osteoclasts. Osteoconduction is improved by small particle size and an interconnected sponge-like matrix that allows fibrovascular invasion and bone production (29,36). Calcium phosphate cements are also available with the advantage of changing shape required for the bony defect. The disadvantages of cements include the requirement for close contact with bone for osteoconduction and a lack of a porous matrix for tissue invasion (36).

Bioactive glass is biocompatible, resorbable, osteoconductive and bonds to bone without fibrous tissue. It is made of silica, sodium oxide, calcium oxide and phosphate. The surface of bioactive glass is apatite and is replaced by bone – the bone bonding response (36). This is dependent on the silica content and results in a strong bond that later results in a strong integrative response to generate an osteoconductive location (29). Biocompatibility is shown by the absence of inflammatory infiltrate. The chemical reaction of bioactive glass is dependent on temperature, pH and the surface layer. Its porous network provides an osteoinductive scaffold and also helps resorption and bioactivity (36). Disadvantages of bioglass include little structural integrity (29).

Calcium sulfate functions as an osteoconductive bone filler that is completely resorbed, restoring anatomic features and structural properties (29). There is significant loss of weight bearing properties during degradation, hence should not be used for structural integrity (36).

### 2.3.5.3.2 Implantable Metals

The use of inert metals as pins, screws or plates are used for fracture stabilization. Stainless steel and titanium are most frequently used. Disadvantages of these include
stress shielding, inflammatory osteolysis, interference with radiological studies, and are permanent unless removed. Removal provides additional surgical related risks including infection and morbidity (39,40). While providing structural integrity, biodegradable metals degrade over time and thus leave no disadvantages associated with permanent inert metals. For example, magnesium alloys are biocompatible, non-toxic, and provides appropriate structural integrity (39,40). Disadvantages include unequal degradation rates to that of bone formation. This may be altered by combining metals with other materials (39).

2.3.5.3.3 Biodegradable Polymers
Implantable polymers, which can be natural or synthetic, have appropriate properties for cell attachment and growth. They are described as biocompatible, with some being biodegradable with modifiable degradation rates. The body resorbs degradable polymers, which is advantageous as healing can occur without leaving any material behind. The physical properties of polymers can be disadvantageous, such as limited mechanical properties, processability and variability between batches. They can be used alone or mixed with other materials in order to improve mechanical properties and processing (36).

2.3.5.4 Xenografts
Xenografts are grafts from another species, such as bovine. In order to reduce the risk of disease transmission it must be deproteinized, leaving only a calcified matrix of non-vital bone (12,27). Advantages and disadvantages of xenografts are similar to that to processed allografts (32).

2.3.6 Obtaining Grafts
Autografts are indicated for most applications with other types of bone grafts indicated if autograft supply is not enough or unattainable. Cancellous grafts can be obtained from superficial sites by surgery. These include: the greater trochanter, femoral condyle, medial malleolus, olecranon, distal radius, and the proximal metaphysis of the tibia. Larger cancellous and corticocancellous grafts can be contained from the anterior superior iliac crest and posterior iliac crest. Cancellous
grafts can also be obtained from the trabecular cavity during reaming procedures (24). Whole bone grafts can be obtained from the fibula (27).

2.4 Bone Tissue Engineering

2.4.1 Overview

Tissue Engineering is “an interdisciplinary field of research that applies the principles of engineering and life sciences towards development of biological substitutes to restore, maintain or improve tissue function” (41). The concept requires four key factors: a biocompatible scaffold similar to bone ECM, osteogenic cells to synthesize bone tissue, morphogenic signals to assist cells to differentiate to the desired phenotype and sufficient vascularization for nutrient and waste exchange (42).

2.4.2 Strategies

There have been different BTE methods that can be classified into two categories: cell or growth factor based strategies. Both provide an environment of bone regeneration by the introduction of osteogenic cells onto a 3D scaffold (41). However, cell-based strategy combines osteogenic cells with biomaterial scaffolds ex vivo, while relying on growth factors from the repair site to stimulate regeneration. Growth factor-based strategies rely on growth factors for an osteoinductive effect to recruit osteogenic cells from the local site (41,43). A cell-based strategy works independently of local osteogenic cells, and thus is an attractive approach for patients that have little to no osteogenic cells, including those with trauma, diabetes, and osteoporosis. Furthermore, recruited cells release growth factors at physiological doses allowing a more effective bone regeneration. In contrast to growth factor-based strategy, there is difficulty determining the optimal dose of growth factor for large animals, and delivery and release of the factor into the circulation (41). Supraphysiological doses are sometimes necessary for an osteoconductive effect (43).

2.4.3 Osteogenic Cells and Cells with Osteogenic Potential

A cell-based approach for bone regeneration is effective and assists in recruiting progenitor cells during the early stages of repair. The mechanisms that bone
regeneration occurs in BTE are: early release of osteogenic, vasculogenic molecules and growth factors, template formation for cell recruitment, and bone matrix synthesis and vascularization of the construct. Cell types come from many sources including mesenchymal, embryonic, induced-pluripotent, adipose-derived, and human exfoliated deciduous teeth (SHED) stem cells. For successful graft incorporation and healing the properties of the cell must include: isolation and expansion efficiency, expression and stability of bone markers and bone formation (42). Only osteogenic sarcoma cell line (SAOS-2) and mesenchymal stem cells (MSCs) will be discussed in this review as these are the pre-determined cells of choice.

2.4.3.1 Osteogenic Sarcoma Cell Line
The SAOS-2 cell line was isolated and characterized by J. Fogh and G. Trempe from an 11 year old Caucasian girl in 1973 (44). It was derived from a malignant bone tumor that shares several features with osteoblastic cells that makes them commonly used as osteoblastic models (45). Features which make SAOS-2 appropriate as an osteoblastic model include: generation of a mineralized ECM, a strong alkaline phosphatase (ALP) reaction, PTH and vitamin D reaction, and the presence of bone matrix proteins (46).

2.4.3.2 Mesenchymal Stem Cells
MSCs are multipotent stem cells that can differentiate into multiple lineages including osteoblast, chondroblast and adipocyte (47). Under appropriate stimuli (such as injury) MSCs can differentiate as required for tissue repair (48). MSCs have recognized potential for BTE as they are involved in natural bone development and have been widely used with many biodegradable scaffolds (42,49).

They come from many sources including: bone marrow, peripheral blood, umbilical blood, synovium, deciduous teeth, tooth pulp, amniotic fluid, adipose, brain, skin, heart, kidneys and liver (42). They are mostly isolated through a bone marrow aspirate procedure (6), are easily expanded in culture to give rise to distinct colonies (50), and are non-immunogenic (41,47). With a bone marrow aspirate, there are only a small number of MSCs, therefore cells must be expanded but this may affect differentiation capacity (49). Bone marrow aspirate concentrates are commonly
isolated from a patient’s iliac crest and are an excellent source of osteogenic stem cells and osteoconductive growth factors for bone regeneration. Aspirates specifically contain MSCs, endothelial progenitor cells (EPCs), HSCs, platelets, lymphocytes and granulocytes and all have a necessary role in bone regeneration (42). With the ability of high proliferative rates and resistance to freeze damage they can be expanded in vitro to acquire cell numbers of choice. MSCs express various markers that allow definition (42).

MSCs are described to be fibroblastoid, a spindle-shaped morphology (48,50,51), and have the ability to adhere to tissue culture plastic (42,50). Efficiency of colony formation and culture conditions vary between species, which depend on a number of factors such as fetal bovine serum (FBS) (50). MSCs also secrete cytokines and growth factors that influence differentiation and aging of other cells in the body. They can also invade and migrate through ECM to create important cell transpositions (48).

To enhance bone regeneration, MSCs are stimulated to differentiate down the osteogenic lineage. The 3D scaffold may be imbedded with osteogenic growth factors that also stimulate the migration and differentiation of progenitor cells. Differentiation to osteoblastic cells before implantation is shown to assist repair as the mature osteogenic cell population can immediately synthesize bone (42). MSCs do not spontaneously differentiate in culture. After expansion, differentiation requires appropriate culture conditions and/or induction agents such as fibroblastic growth factor (FGF-2), transforming growth factor beta (TGF-β), and bone morphogenetic protein two (BMP-2) (42,47).

There are several disadvantages using MSCs. Studies show that there is a maximum of 24-40 population doublings until senescence is reached. With increasing donor age and systemic disease, osteogenic differentiation in vitro and in vivo significantly decreases (42).

2.4.4 Three-Dimensional Scaffold
Scaffolds are a support, delivery vehicle, or matrix to facilitate migration, binding, or transport of cells and/or bioactive molecules used to replace, repair, or regenerate
tissue (5). The ECM is important to tissues as it regulates cellular activity. Bone cannot grow in a 3D manner in vitro; therefore a 3D structure that mimics the ECM supports growth of new bone tissue. This scaffold provides a temporary environment for cell attachment, migration, proliferation, differentiation and ECM deposition (6,12,26). As described before, factors that assist bone regeneration in tissue engineering include: osteoconductivity, a porous matrix for cell attachment, migration, proliferation and neovascularization; osteoinductivity, possession of proteins and growth factors for inducing surrounding MSCs to differentiate to the osteoblastic cell lineage; osteogenecity, osteoblasts present at the site of new bone formation produce osteoid; and osteointegration, mineralized tissue can bond to implant material (5,26).

When implanted it becomes vascularized and integrated into host bone. Scaffolds should be biocompatible, not evoking an immune response when implanted and invoke a healing or physiological inflammatory response (12). The scaffold should be able to transfer load to surrounding tissue, mechanically strengthen the implantation site after insertion and also resist physiological loading (6).

Adequate interconnected pores within a scaffold are required for osteogenesis and vascularization as this allows nutrient, metabolite and waste exchange, avoiding hypoxia and necrosis (52,53). An interconnected matrix promotes cell growth and distribution throughout the matrix (12). There have been various studies that research the optimal pore size of the scaffold material. A summary of pore sizes and their associated characteristics are found below in Table 2.1. There is an upper limit for increasing porosity as this results in reduced mechanical and compressive strength that is essential for weight-bearing bones (12,53,54). Increasing porosity with decreasing strength also increases the difficulty in scaffold production (53).

Table 2.1: Pore Sizes and Associated Characteristics. The pore size (µm) for scaffolds provides various factors for three-dimensional cell culture.
<table>
<thead>
<tr>
<th>Pore size (µm)</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 -150</td>
<td>Cell attachment</td>
<td>Murphy &amp; O’Brien 2010</td>
</tr>
<tr>
<td>100 – 150</td>
<td>Cell size, migration and fluid transport</td>
<td>Bose et al., 2012; Costa-Pinto et al., 2011; Murphy &amp; O’Brien, 2010</td>
</tr>
<tr>
<td>200 - 350</td>
<td>Optimal Osteogenesis</td>
<td>Bose et al., 2012</td>
</tr>
<tr>
<td>200 – 500</td>
<td>Vascular ingrowth</td>
<td>Meyer et al., 2010</td>
</tr>
<tr>
<td>300 – 800</td>
<td>Osteogenesis and vascularization</td>
<td>Murphy &amp; O’Brien 2010</td>
</tr>
</tbody>
</table>

Degradation should occur at a rate similar to the formation of new tissue without releasing toxic products and changing the pH of the local environment (5,26). Manipulating chemical and physical properties of the scaffold can alter degradation rate (55).

An approach for the fabrication of 3D scaffolds is using a naturally occurring substance such as natural polymers. They may have different functions in their original environment, but they are found to be biocompatible and have modifiable biodegradable rates. Further information about polymers was discussed in 2.3.5.3.3 (12). Chitosan will be discussed, as it is the predetermined scaffold material for this project.

### 2.4.4.1 Chitosan

Chitosan (CS) has shown excellent properties for being a suitable biomaterial for BTE. It is a linear polysaccharide that is obtained from deacetylated chitin (an aminopolysaccharide found in the exoskeletons of crustaceans) (36,56). The polymer chain is made of glucosamine and N-acetyl glucosamine with β -1,4 linkages (57). When N-acetyl glucosamine units are greater than 50%, the polymer is chitin. When
the number of N-glucosamine units is superior, it is CS (56). The degree of CS deacetylation ranges from 40-98% (57). Molecular weight is dependent on origin and preparation and ranges 5x10^4 and 2x10^6 Da. The degree of acetylation and polymerization determines the molecular weight of CS and thus, are important factors for its various uses (57). Solubility of chitosan is dependent on the free amino and N-acetyl groups that are soluble in an acidic pH (56,57).

CS has a highly cationic nature allowing its use in many biological applications as a biomaterial, giving it the properties of bioadhesion, increased absorption, transfection efficiency, anti-hypercholesterolemic, anti-microbial, anti-inflammatory and anti-tumor effects (56,57).

With bioadhesive properties, it can interact with anionic glycosaminoglycans (GAGs) and proteoglycans. Interactions with GAGs are important as cytokines and growth factor action can be altered. Antimicrobial properties are thought to be due to CS’s cationic nature disrupting cell wall synthesis, structure and cellular transport (56). CS has additional properties that make it an appropriate biomaterial for BTE: biodegradable, accelerated healing and biocompatible (56). Degradation of CS can occur by chitosanases, which are absent in mammals, so the majority of degradation occurs by lysozymes. Degradation, similar to CS’s other properties is also dependent on the degree of deacylation. Wound healing properties are due to macrophage activation and the production of cytokines. This accelerates healing while also protecting against infection (56). CS has excellent biocompatibility with tissue with no pathological inflammatory response (58).

CS can be molded into various structures depending on usage, including microspheres, paste, membranes, sponges, fibres, and porous scaffolds (12,56). CS is mechanically weak and lacks bioactivity (56) therefore load bearing applications are limited. These disadvantages can be overcome by combining CS with bioactive, mechanically strong materials such as hydroxyapatite (59).

2.4.4.1.1 Freezing and Lyophilization of Chitosan Scaffolds
CS scaffolds can be produced by freezing and subsequent lyophilization (freeze-drying). In a process called phase separation, the freezing of a CS solution causes the
formation of ice crystals, and the simultaneous precipitation of CS that surrounds them. Water diffuses to developing crystals and causes growth with respect to size. Freeze-drying the ice crystal will leave a space (pore) surrounded by precipitated CS.

The surrounding thermal gradient during the freezing process plays a major role on whether pores will form. A large thermal gradient gives rise to a heat transfer governed system and causes water diffusion and ice crystal formation in the direction of the gradient, resulting in layered sheets. A small thermal gradient gives rise to a mass governed system and causes slower diffusion of water molecules to developing ice crystals, forming spheres growing in all directions. These spheres are freeze-dried to leave a pore. Ice remolds with time, these ice crystals become elongated and anneal in the direction of the thermal gradient (60,61).

2.4.5 Morphogenic Signaling
BMPs are a group of growth factors that belong to the TGF-β superfamily (62). They have a strong effect on bone growth with important roles on early skeleton formation (63). BMP-2 can be used in various clinical applications including bone defects, non-unions, spinal fusion, osteoporosis and root canal surgery due to powerful osteoinductive properties (62,63). Implantation of BMP-2 results in MSC infiltration, cartilage formation, vascularization, bone formation and remodelling of tissue (64).

2.4.6 Bioreactors
The bioreactor is a dynamic system used for in vitro culture (41). The use of a bioreactor to cultivate 3D scaffolds is used to achieve homogenous bone development. Three factors allow this: improved cell seeding efficiency, increased mechanical stimulation of osteogenic cells, and removing the diffusion mediated limitation of nutrient and waste exchange (41,42,65). Environmental factors such as: temperature, pH, pressure, nutrient supply and waste removal, can be manipulated for optimal cell growth (42,65). This allows automated and standardized tissue manufacturing, reducing production costs, and lead the way for industrial-scale production of BTE (42). The bioreactor should be composed of biologically inert and non-corrosive material to prevent release of toxins. It is usually made from synthetic polymers that can withstand sterilization techniques (65).
There are several types of bioreactors with different flow patterns that improve cellular proliferation and differentiation (41,42). These include stirred flasks, rotating bioreactors and perfusion bioreactors. Discussion will focus on perfusion bioreactors, as it is the pre-determined bioreactor for this thesis (42).

Perfusion systems consists of a chamber that hosts the 3D scaffold, and a peristaltic roller pump to deliver culture medium, allowing laminar flow and mass transport of nutrients through the entirety of the scaffold (42,65). Fluid flow can be steady, oscillating or pulsatile with each significantly influencing osteogenic cells (42). Perfusion system studies show there is improved osteogenic cell distribution, density, proliferation, differentiation, and deposition of ECM through the entire scaffold in vitro (41,42), allowing improved bone growth in vivo compared to static culture systems. As bone tissue is mechanically sensitive, bioreactors can be designed to mechanically stimulate scaffolds. This is associated with increased ALP, mineralized matrix and osteogenic gene expression (42). It was found that oscillating flow was less stimulatory than pulsing or steady fluid flow (65). Grayson et al. demonstrated that a linear velocity of 400–800 mm/s was optimal for their system based on histological analyses, connexin expression, and protein contents (66). A flow rate of 0.01 to 3 mL/min has been shown to increase cell proliferation, mineralization and protein expression (67–69).

Uniform and efficient seeding of cells is necessary for successful bone grafts as this results in homogenous distribution of cells and increases viability (42,70). Efficient seeding limits biopsy size, extent of cell expansions and is directly associated with bone mineralization. Increased cell density is associated with increased gene expression, differentiation and mineralization. Manual, static seeding of cells onto the scaffold is most commonly used, but has low seeding efficiency and non-uniform distribution. High efficiency and uniformity can be obtained using bioreactors for dynamic seeding. Perfusion bioreactors have the highest seeding efficiency and uniformity; dilute cell suspension flows through the pores and allows cell seeding throughout the entire scaffold (42).
2.5 Summary

In conclusion to this literature review, bone is an incredible connective tissue with a high potential for regeneration. Despite this, the occurrence of critical sized defects (due to the nature of the size of the bony wound) causes limitations in healing with consequential soft tissue formation and therefore, reduction of functions of locomotion and protection. The healing and reparation of CSDs are attempted in a variety of ways, such as the gold standard autografts and allografts, xenografts, and synthetic materials, with each having their own advantages and disadvantages (primarily concerned with availability, disease transmission, graft rejection, healing potential and surgical morbidity). Thus, there is demand for bone substitutes superior in all these aspects. Bone tissue engineering can solve the low availability and surgical morbidity of superior autografts, by isolating the patient’s stem cells by a bone marrow aspirate, differentiating them to bone-producing osteoblasts and combining them with a biocompatible scaffold for support in 3D culture. This 3D environment simulates physiological conditions for osteoid production, while using a bioreactor provides additional physiological factors such as shear force, and a simulated blood circulation by the constant supply of nutrients and removal of waste. Although further work needs to be done, 3D culture of osteoblasts on a scaffold has a high promise of clinical involvement in the future.
3 Materials and Methods

3.1 Materials

Pre-made $\alpha$-minimal essential media ($\alpha$ MEM) and Dulbecco’s Modified eagle Medium (DMEM), Dulbecco’s Phosphate-Buffered Saline (DBPS), nano-hydroxyapatite (nHA), and glacial acetic acid (~99% purity) were purchased from Sigma. Powdered media ($\alpha$ MEM and DMEM) and LIVE/DEAD® assay was purchased from Invitrogen. Anti-Anti (antibiotics and antimicotic solution), Fetal bovine serum (FBS), Trypsin with ethylenediaminetetraacetic acid (EDTA) and phosphate buffered saline (PBS) were purchased from Gibco. 80% deacetylated Chitosan powder was purchased from Wako. MTS cell proliferation assay was purchased from Promega. Pharmed BPT and Tygon 3350 tubing were purchased from Saint Gobain. Micro-hydroxyapatite was purchased from Acros Organics, Triton-X was purchased from BDH Chemicals Ltd.

3.2 Methods
3.2.1 Chitosan Scaffold Manufacture and Analysis

3.2.1.1 Primary Chitosan Scaffold Manufacture

3.2.1.1.1 Chitosan Scaffold Manufacture

The first attempt of making scaffolds was as follows. 80% deacetylated chitosan (CS) powder was added to 1% (v/v) acetic acid (AA) to produce 0.5, 1.0, 1.5, 2.0 and 2.5% (w/v) solutions, as according to Table 3.1 below. The solutions were mixed on a plate shaker for 20 minutes or until dissolved so that there were no visible large particles of CS in solution. 5 mL of each solution was added to each well of a Corning® Costar® 6 well cell culture plate (Sigma-Aldrich, USA) and left in a cool, dry place overnight to allow air bubbles that were incorporated during mixing to escape. Samples were then frozen at -80 °C for at least 24 hours.

Table 3.1: Primary Chitosan Scaffold Protocol. Well number of 6 well culture dish labelled one to six (column 1). Concentration of CS as % w/v (column 2). CS added to solution in grams (column 3), and volume of 1% (v/v) AA in mL (column 4).

<table>
<thead>
<tr>
<th>Well Number</th>
<th>CS (% w/v)</th>
<th>CS (g)</th>
<th>1% (v/v) AA (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.025</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>0.050</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>0.075</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>0.100</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>0.125</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>3.0</td>
<td>0.150</td>
<td>5</td>
</tr>
</tbody>
</table>

3.2.1.1.2 Lyophilization/Freeze-Drying Method

Frozen CS solutions were freeze-dried in a FreeZone Triad Freeze Dry System 74000 Series (Labconco, Missouri, USA) as follows. The temperature of the freeze dryer shelf was reduced to at least -40 °C before samples were placed inside with
subsequent activation of the vacuum. After 1 hour the temperature was increased to 0 °C for the primary drying step. After 20 hours the temperature was increased to 20 °C to assist in the removal of residual water for secondary drying. 24 hours later the samples were collected. 3 mm and 8 mm biopsy punches (KRUUSE, Denmark) were used to create scaffolds that were used for analysis.

3.2.1.1.3 Scanning Electron Microscope
Preparation for Scanning Electron Microscopy (SEM) involved a scaffold that was cut transversely with a scalpel to reveal the interior. Both pieces were mounted on an aluminium stub using carbon tape as an adhesive. Samples were coated with approximately 15 nm of platinum palladium using an Emitech K575X Peltier-cooled high resolution sputter coater (EM Technologies Ltd, Kent, England) and viewed by a JEOL JSM-6700F Field Emission Scanning Electron Microscope (JEOL Ltd, Tokyo, Japan) at 5.0 kV and a LEI detector to acquire x25 and x100 images.

3.2.1.1.4 Micro-Computed Tomography
3 mm scaffolds were mounted on a sample holder of a Sky Scan 1172 high-resolution \( \mu \)CT scanner (Bruker, Kontich, Belgium). The scaffold was positioned so that radiation passed perpendicular to the samples largest width. Using micro-computed tomography software (Bruker), voltage was set to 30 kV and current to 175 \( \mu \)A. Camera settings were set to medium camera pixel. Approximately 1000 images of the scaffold in the form of transverse slices were taken.

3.2.1.2 Secondary Chitosan Scaffold Manufacture

3.2.1.2.1 Freezing Studies
To determine if freezing temperature and duration affected the scaffold architecture, 5 mL of 1% CS (w/v) solution was each placed in six 5 cm diameter petri dishes (BD Biosciences, Belgium) and left undisturbed overnight. 3 dishes were frozen at -80 °C for 24, 48 and 72 hours. The other 3 dishes were frozen at -22 °C for 24, 48 and 72 hours. Samples were then freeze-dried as described in 3.2.1.1.2. An 8 mm biopsy punch was used to create scaffolds samples for analysis.
3.2.1.2.2 Container Studies
Scaffolds were produced in containers of varying geometries to determine if this was a determinant of scaffold architecture. 1% (w/v) CS solution was used for the following experiments. Using 2 transfer pipettes (Biologix, USA) with an 8 mm diameter, 3 mL of solution was drawn up and the openings of the pipettes were then sealed with parafilm. Two 5 cm diameter petri dishes were filled with solution and sealed with a lid. Two 15 mL Falcon Tubes (Corning Life Sciences, USA) were filled completely with solution. One pipette, petri dish and Falcon tube was frozen at -22 °C and the other ones were frozen at -80 °C for 24 hours before being freeze dried according to 3.2.1.1.2. 3 mm scaffolds were punch biopsied for analysis.

3.2.1.2.3 Secondary Scaffold Scanning Electron Microscopy
Scaffolds were cut in half using a scalpel to produce two semi-circles in order to expose the interior surface and were mounted so that the interior was facing upwards. The other half was placed so that its most superior surface faced upward. Analysis was performed as according to 3.2.1.1.3.

3.2.1.3 Macroconcentrations of Chitosan Scaffolds
To improve the integrity of scaffolds, higher concentrations of CS were investigated. Macroconcentrations simply refers to larger concentrations of CS powder than previously used in 3.2.1.1.1. CS powder was added to 1% (v/v) AA to produce concentrations of 1.5, 2.0, 4.0, 8.0, 10.0 and 12.0% (w/v) as according to Table 3.2. The solutions that were highly viscous were manually mixed using a 1000 µL pipette tip to ensure the CS was dissolved. Samples were frozen at -22 °C (and from onwards, all scaffolds are frozen at this temperature) and freeze-dried as previously stated in 3.2.1.1.2.

Table 3.2: Macroconcentrations of Chitosan Scaffold Protocol. Well number of 6 well culture dish labelled one to six (column 1). Concentration of CS as % w/v (column 2), CS added to each well in grams (column 3). Amount of 1% AA (v/v) added in mL (column 4).
<table>
<thead>
<tr>
<th>Well Number</th>
<th>CS (% w/v)</th>
<th>CS (g)</th>
<th>1%AA (v/v) (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>0.075</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>0.100</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>0.200</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>8.0</td>
<td>0.400</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>0.500</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>12.0</td>
<td>0.600</td>
<td>5</td>
</tr>
</tbody>
</table>

3.2.1.4 Chitosan and Hydroxyapatite Scaffold Manufacture

3.2.1.4.1 Chitosan and Micro-Hydroxyapatite Scaffold Manufacture
To investigate the bioactivity and integrity of scaffolds, micro-hydroxyapatite (µHA) was added to 12 wells of 5 mL of 1% (w/v) CS solution as according to Table 3.3 (low concentrations) and 3.4 (macroconcentrations, defined simply as larger concentrations than used in Table 3.3) below. Dish was placed on a plate shaker for 1 hour or until dissolved and frozen at -22 °C for 24 hours before being freeze-dried.

Table 3.3: Chitosan Scaffold with Micro-Hydroxyapatite Protocol. Well number of 6 well culture dish labelled one to six (column 1). The volume of 1% (w/v) CS solution added in mL (column 2). µHA concentration showed as a % w/v (column 3), and µHA added in grams (column 4).
Well Number | 1% (w/v) CS (mL) | µHA (% w/v) | µHA (g)  
--- | --- | --- | ---  
1 | 5.0 | 0.00 | 0  
2 | 5.0 | 0.06 | 0.003  
3 | 5.0 | 0.10 | 0.005  
4 | 5.0 | 0.20 | 0.01  
5 | 5.0 | 0.40 | 0.02  
6 | 5.0 | 0.60 | 0.03  

Table 3.4: Chitosan Scaffold with Macroconcentrations of Micro-Hydroxyapatite. Well number of 6 well culture labelled one to six (column 1). Volume of 1% (w/v) CS added in mL (column 2). Concentrations of µHA shown as a % w/v (column 3). Amount of HA added in grams (column 4).  

| Well Number | 1% (w/v) CS (mL) | µHA (% w/v) | HA (g)  
--- | --- | --- | ---  
1 | 5.0 | 1.0 | 0.05  
2 | 5.0 | 2.0 | 0.10  
3 | 5.0 | 4.0 | 0.20  
4 | 5.0 | 10.0 | 0.50  
5 | 5.0 | 15.0 | 0.75  
6 | 5.0 | 20.0 | 1.00  

3.2.1.4.2 Chitosan and Nano-hydroxyapatite Scaffold Manufacture  
It was investigated if nano-hydroxyapatite (nHA) was superior than µHA with regards to a greater amount incorporated into the scaffold for bioactivity and structural integrity. 5mL of 1% (w/v) CS solution was placed into each well of a 6 well cell
culture plate. nHA was added to each well as according to Table 3.5. The dish was placed on a plate shaker for 1 hour or until contents were dissolved. Samples were frozen at -22 °C for at least 24 hours before being freeze dried as previously stated in 3.2.1.1.1 and underwent SEM analysis as in 3.2.1.1.2.

**Table 3.5: Chitosan and Nano-Hydroxyapatite Scaffold Protocol.** Well number of 6 well culture dish labelled one to six (column 1). Volume of 1% CS (w/v) solution in mL (column 2). Concentration showed as a percentage of the solution after subsequent nHA addition (column 3). Amount of nHA in grams added to the solution (column 4).

<table>
<thead>
<tr>
<th>Well Number</th>
<th>1% (w/v) CS (mL)</th>
<th>nHA (% w/v)</th>
<th>nHA (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.0</td>
<td>2.5</td>
<td>0.125</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>3.0</td>
<td>0.150</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>3.5</td>
<td>0.175</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>4.0</td>
<td>0.200</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>4.5</td>
<td>0.225</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>5.0</td>
<td>0.250</td>
</tr>
</tbody>
</table>

**3.2.1.4.3 8% (w/v) Chitosan scaffolds with Macroconcentrations of nHA**
8% CS (w/v) was chosen as the concentration as pores were well above 100 µm. therefore nHA could be added without having a significant effect on pore diameter. Macroconcentration simply refers to using larger amounts of nHA than used prior in 3.2.1.4.2. 2.4 g of CS powder was added to 30 mL of 1% (v/v) AA to produce an 8% (w/v) solution of CS. 5 mL of this solution was placed into each well of a 6 well dish. nHA was added to each well as according to Table 3.6. The solutions were highly viscous and therefore were manually mixed using a 1000 µL pipette tip to make sure the powders dissolved. Samples were frozen at -22 °C and freeze-dried as previously stated in 3.2.1.1.1.
Table 3.6: Chitosan Scaffold with Macroconcentrations of Nano-Hydroxyapatite Protocol. Well number of 6 well culture dish labelled one to six (column 1), volume of 8% CS (w/v) solution in mL (column 2), concentration of nHA shown in % w/v (column 3), amount of nHA added to each well in grams (column 4).

<table>
<thead>
<tr>
<th>Well Number</th>
<th>8% (w/v) CS (mL)</th>
<th>nHA (% w/v)</th>
<th>nHA (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.0</td>
<td>2.5</td>
<td>0.125</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>3.0</td>
<td>0.150</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>3.5</td>
<td>0.175</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>4.0</td>
<td>0.200</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>4.5</td>
<td>0.225</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>5.0</td>
<td>0.250</td>
</tr>
</tbody>
</table>

3.2.1.5 Scaffold Characterization

3.2.1.5.1 Scanning Electron Microscopy
SEM images were taken as in 3.2.1.1.3 of 3 areas from the central region of an 8% (w/v) CS and 5% (w/v) nHA scaffold. ImageJ software (National Institutes of Health, Maryland, USA) was used to determine the average pore diameters. For improved visualization of pores, images were first converted to black and white using the threshold function to convert pore walls black and pores white. The next function was to filter the image to minimize background noise, removing specks and objects. The scale was set to convert pixels to µm. From each sample, 30 pores were measured. Data was plotted on Prism 6 (GraphPad Software) to generate a graph and statistical results.

3.2.1.5.2 Micro-Computed Tomography of Scaffolds
CT images were taken as done previously in 3.2.1.1.4. Using Skyscan Software ‘CTan’ (Bruker), open and closed porosity was determined as a percentage using the method, ‘Advanced Porosity Analysis’ (Bruker microCT).
3.2.1.5.3 Scaffold Stabilisation and Rehydration Testing

From here onwards, scaffolds used for experimentation are 8% w/w CS and 5% nHA, as prepared in 3.2.1.4.3. When ‘scaffold’ is mentioned it will refer to the aforementioned material concentrations.

Freeze-dried scaffolds are precipitated polymers of chitosan acetate. If placed in aqueous conditions i.e. water, acetic acid would liberate hydrogen ions and hence redissolve the chitosan. Using a solvent (absolute ethanol) allows the removal to acetic acid without liberating hydrogen ions and hence stabilises the scaffold. 8 mm scaffolds were immersed in absolute ethanol for 1 hour and followed by immersion in 90, 80, 70 and 50% ethanol solutions for 30 minutes each. This was followed by immersion in distilled water for 30 minutes. Scaffolds were then placed into culture dishes with distilled water, frozen and freeze-dried again as in 3.2.1.1.1. SEM analysis was performed as in 3.2.1.1.2. Scaffolds that have been treated with alcohol will be referred to as ‘stabilised’.

3.2.1.5.4 Scaffold Degradation and pH

This method was used to determine if scaffolds degraded and affected the local pH in vitro. This was achieved by placing the scaffold in simulated physiological conditions to determine structural integrity over time, as the scaffold needs to remain stable to support the local environment for bone regeneration. Ideally, the degradation rate should match bone regeneration. To ensure a precise measurement of dry weight was made, the scaffolds were freeze-dried before and after degradation step. Scaffolds were weighed a 9 stabilized and freeze-dried scaffolds weighted and placed into individual wells of a 12 well culture plate. 2 mL of PBS was placed into each well. The plate was placed into an incubator at 37°C and 5% CO₂. At time 24, 48 and 168 hours, 3 scaffolds were removed, immersed in distilled water and frozen. An Orion 230A pH meter (Thermo Fisher Scientific, Auckland, New Zealand) measured the pH of each well at each of the time points when scaffolds were removed. When all scaffolds are frozen they all underwent freeze-drying as previously stated in 3.2.1.1.1. Scaffolds were weighed once again and data analyzed. The sensitivity of the weighing
instrument was to 3 decimal points. The measurement taken was when there was no fluctuance of the scale display, that is, when the reading was constant.

3.2.1.5.5 Scaffold Swelling Tests
To investigate the response of scaffolds in aqueous solution, 3 stabilized and freeze-dried scaffolds were placed in separate wells of a 6 well plate and immersed in 5 mL of PBS. Using forceps, scaffolds were immediately weighed (time = zero) after PBS immersion by placement onto a paper towel on a scale 6 times, ensuring the scale is zeroed before each measure. The scaffolds were placed in a 37°C, 5% CO₂ incubator. Scaffolds were weighed at 1, 3 and 7 days post immersion in PBS in the same manner as previously done at time = zero.

3.2.1.5.6 Scaffold Sterilisation
Two methods were attempted for scaffold sterilization. A freeze-dried and stabilised scaffold was placed into an autoclave at 121 °C for 20 minutes after 15 PPI was reached. Once autoclaved the scaffold underwent gross observation and SEM analysis as in 3.2.1.1.3.

The following protocol involves sterile conditions in a laminar flow hood. Stabilized and freeze-dried scaffolds were placed in 15 mL Falcon tubes with 10 mL of absolute ethanol. These are left to rest for at least 24 hours. The ethanol is removed and the scaffolds were transferred to a 15 mL Falcon tube with 10 mL of sterile distilled water. After 24 hours the scaffolds were removed and placed in a 6 well plate and immersed with 5 mL of growth media. All subsequent methods and protocols involved in using the scaffold were done under sterile conditions.

3.2.1.5.7 Fourier Transform Infrared Spectroscopy
FTIR was employed to collect infrared spectra to describe molecular vibrations. An 8% (w/v) CS and 5% (w/v) nHA scaffold was crushed into a fine powder using a mortar and pestle. 0.01 g of this powder was mixed with 0.1 g of potassium bromide (KBr) and further mixed using a mortar and pestle. The resulting mixture was pressed into discs and analyzed using a Spectrum RX1 (Perkin Elmer Instruments, USA) with
a 4 cm\(^{-1}\) resolution. Spectra were collected over the range of 400 – 4000 cm\(^{-1}\); with the background noise corrected by KBr. 16 scans were taken to give an average spectra.

**Energy Dispersive X-Ray Spectroscopy**

EDS was employed to determine the elemental composition of the scaffold. An 8% (w/v) CS and 5% (w/v) nHA scaffold was analyzed using a JEOL-2300 Energy Dispersive Analyzer and JEOL AnalysisStation software (JEOL, Germany). Accelerating voltage used a negative potential of 15 kV, and the sample was analyzed using ‘spot analysis’ for 2 minutes to collect elemental peaks.

**3.2.2 Cell Culture Investigations with Osteogenic Sarcoma Cell Line and Mesenchymal Stem Cells**

**3.2.2.1 Growth Media Production**

Under sterile conditions, a 500 mL bottle of ready to use Dulbecco’s Modified Eagle Media (DMEM) or Alpha-Minimum Essential Media (α-MEM) culture medium had 55 mL of media removed. 50 mL of heat inactivated FBS and 5 mL of Anti-Anti (10,000 units/10 mL penicillin, 10 µg/10 mL streptomycin, 25 µg/mL amphotericin B) were added to the media bottle. 40 mL aliquots were placed in 50 mL Falcon tubes (to prevent freezing-mediated bursting) and frozen at -22 °C for storage. This media solution of DMEM or α-MEM with 10% FBS and 1% Anti-Anti solution is referred to as growth media. Growth media using powder was made according to manufacturer’s instructions. A 1 L solution had 110 mL of media removed and 100 mL of FBS and 10 mL of Anti-Anti was added. The resultant solution was filter sterilized with a syringe (TERUMO®) and a 0.2 µm syringe filter (AVS Filter Technologies). This solution of DMEM or α-MEM with 10% FBS and 1% antibiotic/antimicotic solution is referred to as growth media.
3.2.2.2 SAOS-2 Cell Line Culture

3.2.2.2.1 SAOS-2 Cell Thawing
For SAOS-2 (American Type Culture Collection; Virginia, USA) cell culture, growth media will refer to α-MEM. Cryovials (Corning Life Sciences; USA) containing the frozen cell line were thawed rapidly in a 37 °C water bath. Under sterile conditions in a laminar flow hood the contents of the cryovial are added to a 15 mL Falcon tube containing 10 mL of growth media. The Falcon tube was then centrifuged for 3 minutes at 120 g. The supernatant was discarded and the cell pellet was resuspended in 1 mL of growth media. Cells were then plated in a 25 cm² cell culture flask and left in a 37 °C, 5% CO₂ incubator.

3.2.2.2.2 SAOS-2 Cell Culture and Passage
After the thawing process the cells were allowed to reach 70-80 % confluence, which was observed by phase contrast microscopy. Once the desired confluency was reached the cells were passaged as follows. Under sterile conditions in a laminar flow hood the growth media was removed from the cell flasks and washed twice with Dulbecco’s phosphate buffered saline (DPBS) to remove debris and unattached cells. 2 mL of trypsin/EDTA was added to remove adherent cells on the culture surface. The culture flask was placed in the incubator to optimize trypsin activity. After 5 minutes the flasks were tapped on a bench to assist the dissociation of cells. Using a phase-contrast microscope confirmed non-adherent cells in solution. In the hood, 6 mL of growth media was added to the flasks to neutralize the trypsin. The contents of the flask were added to a 15 mL Falcon tube that was centrifuged for 3 minutes at 120g. The supernatant was removed and the cell pellet was resuspended in 2 mL of growth media. 6 mL of growth media was added to two 25cm² cell culture flasks. The resuspended cells were added to the flasks drop wise. The amount was dependent on the desired rate of reaching 70-80% confluency. The flasks were then placed into the incubator. When cells reached passage 3, they were used in experiments or cryopreserved.
3.2.2.2.3 SAOS-2 Cryopreservation
A 20% dimethyl sulfoxide (DMSO) (EMD Chemicals, Pennsylvania) solution was made with growth media under sterile conditions and kept on ice. Cells were lifted using trypsin, centrifuged and resuspended in 2 mL of growth media. Cells were counted using a Countess Automated Cell Counter (Invitrogen). Cell suspensions were diluted to a cell density of 1x10^6 cells/mL or centrifuged, resuspended to a cell density of approximately 1x10^6 cells/mL. 500 µL of these cell suspensions were placed into a 1.2 mL cryovial and 500 µL of the prepared DMSO solution was added drop wise. Cryovials were sealed and placed in a Nalgene® cryofreezing container (Thermo Scientific, USA) with isopropanol to control freezing rates before being placed in an -80 °C freezer. After 3 days the cryovials are removed and stored as required at -80 °C for up to 6 months. Long-term storage greater than 6 months was done in liquid nitrogen.

3.2.2.2.4 SAOS-2 Cell Seeding
Three sterilized CS scaffolds were immersed in α-MEM media for 24 hours. Scaffolds were then removed from the media and individually placed into the wells of a 6 well plate. 3 glass coverslips were placed in other wells and acted as a control. Following cell passaging, 6000 cells were seeded onto each scaffold and coverslip in 100 µL of media, and placed in an incubator at 37 °C and 5% CO₂ for 60 minutes to allow cells to adhere, 5 mL of media was then added into each well. Cells were maintained for 24, 48 and 96 hours then underwent a LIVE/DEAD® assay.

3.2.2.2.5 LIVE/DEAD® Cell Viability/Cytotoxicity Assay
The numbers of live and dead cells were assessed using the aforementioned assay as follows. Under sterile conditions, media was removed from each of the wells and replaced with 5 mL of DPBS warmed to 37 °C. This is subsequently removed and 100 µL of assay solution (4 µM calcein AM and 4 µM of ethidium homodimer-1 in DPBS) was pipetted onto each scaffold and incubated for 30 minutes. A glass coverslip was added to the top of scaffolds before fluorescent visualization with confocal microscopy.
Visualization of fluorescence was done with a confocal laser-scanning microscope (Zeiss LSM 510 Confocal Microscope) and Zen 2009 software (Carl Zeiss MicroImaging GmbH, Jena, Germany). Living cells were identified due to metabolism of calcein AM to calcein (excitation 494 nm, emission 517 nm). Dead cells were identified due to incorporation of ethidium homodimer-1 into the nucleus as cell membranes were damaged (excitation 528 nm, emission 617 nm). Cell counts were performed in 3 randomly chosen fields per scaffold at x4 and x10 magnification. Viability of cells in each image was calculated as follows:

\[
\text{Cell Viability (\%) } = \frac{\text{Number of Live Cells}}{\text{Total Cell Number}} \times 100
\]

Average viability was calculated and presented with the average total cell number. This was to provide a comprehensive view of the cellular response to the scaffold material and prevents the misrepresentation of the scaffold as cytocompatible (for example, if 90% of the cells were viable but only 10 cells were present this would represent cytotoxicity). A one-way ANOVA with Bonferroni’s post hoc analysis was used to compare cell viability and total cell number for all of the scaffolds.

### 3.2.2.6 CellTiter 96® AQueous One Solution Cell Proliferation Assay

Also known as the MTS assay, this demonstrated that the scaffold is not cytotoxic and therefore allowed cells to proliferate. An 8% (w/v) CS and 5% (w/v) nHA scaffold, collagen scaffold and Triton-X were chosen for this assay. A modified International Organization for Standardization (ISO) to test for in vitro cytotoxicity was performed. The modification involved a larger weight of scaffold as done prior by Stace 2011. A 2 mg sterile CS/nHA scaffold was immersed in 2 mL of growth media, a sterile 2 mg collagen scaffold was immersed in 2 mL of growth media, and 2 mL of a 0.1% Triton-X solution was made with growth media. All 3 were placed in an incubator for 48 hours. These 3 solutions were known as extraction vehicles (elusion fluid) that could have taken up potentially cytotoxic elements from each scaffold and therefore affected proliferation of cells. Collagen was known not to be cytotoxic and was the negative control as there was no cytotoxic response, whereas Triton-X, a detergent, will kill all cells and therefore acted as the positive control, as there was a cytotoxic response. The CS/nHA scaffold was the experimental (test) vehicle.
3000 SAOS-2 cells were seeded onto 9 wells of a 96 well plate, 100 µL of chitosan, collagen and Triton-X vehicle were added to 3 wells each. Another 3 wells were pipetted with 100 µL of growth media made up the blanks. Cells were incubated for 0, 24, 48 and 72 hours. Time 0 hour cells immediately went through the MTS assay once seeded.

The MTS assay was done according to manufacturer’s instructions. 20 µL of MTS solution was added to each well and left to incubate for 2.5 hours. The absorbance value was read using a Synergy 2 Multi-Mode Microplate Reader (Biotek®) at 490 nm and data was recorded using Gen5 1.11 software. The software automatically produced data and provided means, standard error and coefficient variance. Data was graphed using Prism 6 (GraphPad Software).

3.2.3 Three Dimensional Perfusion Circuit

3.2.3.1 Perfusion Circuit Components
This project involved the use of a 3D perfusion circuit that was designed, fabricated and utilized by Stace 2011. It consists of a custom designed bioreactor, media reservoir, peristaltic pump and tubing. Images of components and assembled bioreactor are found below in Figure 3.1, with a conceptual diagram of the entire circuit shown in Figure 3.2.

The Major Scientific MFU-02 pump (Major Science, USA) consisted of 4 separate heads with surrounding rollers that rotate to compress the tubing in order to create peristaltic flow. The pump allowed control over flow rate and flow direction could be reversed if required. Flow rates were manually calculated, as rates provided by the manufacturer were erroneous. Flow rates were calculated by running the circuit for 1 minute, and measuring the volume of fluid that was pumped into a 50 mL Falcon Tube.

The silicon tubing used was Tygon 3350 as it was permeable for gas exchange, non-toxic and had a low protein affinity. Another type of tubing, Tygon Pharmed BPT was used to replace the Tygon 3350 that was compressed by the rollers of the pump.
Although Tygon Pharmed BPT had a low ability for gas exchange, it was durable and was resilient to damage by the roller heads.

The bioreactor was fabricated by Electromechanical Technology for Teaching and Research (EMTECH), University of Otago. It comprised of a chamber to house the scaffold and designed to allow laminar flow only. The growth chamber that housed the scaffold is 8 mm in diameter to house scaffolds of 8 mm for rodent critical size defects. The growth chamber was surrounded on both sides by a metal mesh with notches to allow air bubbles to accumulate and avoid the scaffold. The components of the bioreactor allowed an easy fit and seal from the outside environment.

The media reservoir was a modified Schott bottle with side arms topped with 0.22 micron filter membrane to allow gas exchange. The bottle had a modified lid with two entry points made of stainless steel tubes. One allowed for media to return drop wise into the reservoir, and the other draws media out into the circuit. All components of the perfusion circuit were easily sterilized by autoclave or UV radiation.
Figure 3.1: Perfusion Circuit Components. (A) bioreactor components, left to right: outer chamber, inner chamber, titanium core, inner chamber, outer chamber, (B) mesh that surrounds scaffold chamber with arrow pointing to notch to allow air bubbles to escape, (C) assembled bioreactor, (D) media reservoir side arm components, from left to right: cap for bottom side arm, plastic washer, rubber seals, 0.22 µm membrane filter, cap (E) media reservoir top cap components, steel tubes within (from left to right) rubber seal, cap insert and cap, (F) assembled media reservoir, (G) Tygon (top) and PharMed BPT tubing (bottom).

Figure 3.2: Conceptual Diagram of Perfusion Circuit for 3D Culture. (A) media reservoir, (B) Tygon tubing, (C) PharMed BPT tubing, (D) peristaltic pump, (E) Tygon tubing, (F) bioreactor, (G) incubator, (H) entry/exit point for tubing through incubator. Arrows indicates direction of media flow.

3.2.3.2 Three-Dimensional Culture of Osteogenic Cell Line

6000 SAOS-2 cells in 20 µL growth media were seeded onto a sterile scaffold and left in an incubator for 1 hour, then 5 mL of growth media was added and left for a further 24 hours and allowed cells to attach. All components were sterilized by autoclave, except the bioreactor, which was sterilized for 24 hours in UV light on each side of the components. The perfusion circuit was assembled in a laminar flow hood as follows.
45 mL of growth media (warmed to 37 °C) was added to the media reservoir and side arms were sealed. PharMed and Tygon tubing was then assembled to the circuit according to Figure 3.2, but omitted connection of the tubes to the bioreactor. Using sterile forceps, the scaffold was carefully placed into the titanium core and sealed by the inner and outer chambers to form the bioreactor. The tubing was connected to the bioreactor. It was ensured the seeded surface of the scaffold faced the incoming direction of media flow.

The perfusion system was then placed in the incubator and tubing was pushed through the outlet. The tubing was placed on a roller head of the pump. The pump was activated initially at 0.1 mL/min. Before media reached the bioreactor, the speed was reduced to 0.03 mL/min (lowest setting). The bioreactor was adjusted so that the notches in the metal meshes faced upwards and manually angled to allow air to escape through the notches. Once air bubbles were pumped out, the bioreactor was placed with notches facing upwards. The speed was then increased to 0.3 mL/min. In 24 hours, the speed was increased to 0.6 mL/min and in another 24 hours the speed was increased to 1 mL/min. The scaffold was incubated for a total of 14 days. At 7 days, the 45 mL of growth media was replaced and pH measured. pH was also measured at the completion of the experiment at 14 days.

6000 SAOS-2 cells in 20 µL were seeded onto 24 UV sterilized coverslips placed into wells of a 24 well plate. 1 mL of growth media was added to each well and the plate was placed in an incubator. Media was replaced every 3 days. These coverslips acted as the control experiment.

### 3.2.3.3 Scaffold and Coverslip Fixation

After 14 days the scaffold was removed from the bioreactor and immersed into 10 mL of 4% (v/v) paraformaldehyde solution. The scaffold remained in solution for at least 6 hours before being embedded in paraffin wax. The wax block is then cut longitudinally using a microtome to acquire 5 µm sections of scaffold and placed on slides. Decalcification prior to sectioning was required due to damaged samples. The slides were stained as follows in 3.2.3.4. An uncultured 8% (w/v) CS scaffold with no added nHA followed the scaffold preservation protocol as above. As there was no
nHA and did not go through cell culture it cannot be stained for mineralization or cells.

Glass coverslips seeded with SAOS-2 cells were fixed as follows. Media was removed from the wells of a 12 well plate and replaced with enough 4% paraformaldehyde to cover the glass slips. After 20 minutes of fixation in an incubator, the paraformaldehyde was removed and replaced with 70% ethanol to completely fill the well (prevent losses due to evaporation). The plate was then placed in a fridge (4 °C) until required for histological staining as in 3.2.3.4.

3.2.3.4 Histology Protocols
All staining was performed in the University of Otago Histology Unit. All materials were provided and protocols were provided and are as follows. Most staining solutions were aqueous and wax is immiscible with water. This wax must be dissolved with xylene and ethanol series and is described as follows. Slides were placed in xylene baths for 2 minutes each, 3 times. This was followed by 2 baths of absolute ethanol for 2 minutes each, 95% alcohol for 2 minutes, and 70% alcohol for 2 minutes and finally left in tap water prior to processing.

3.2.3.4.1 Haemotoxylin and Eosin (H&E)
Deparaffinised slides or coverslips were stained with Gill’s Haematoxylin for 4 minutes and rinsed well in running water baths. A slide was then dipped in acid alcohol for 4 seconds for differentiation, and washed in running water until sections turned ‘blue’. If the blue change did not occur, the acid alcohol and washing steps were repeated. Dehydration of slides followed using 70%, 95% and absolute alcohol. The slides were then stained with 0.5% alcoholic eosin Y for 1 minute. Dehydration followed once again using 70%, 90% and absolute alcohol. Slides were then cleared in xylene (1 minute, 4 times) and mounted using DPX (distrene 80, dibutyl phthalate, xylene, butylated hydroxytoluene) or Entellan mounting media. Imaging was performed using an Olympus AX70 Light Microscope.
3.2.3.4.1.1 Fluorescent Microscopy of Haematoxylin and Eosin Stained Scaffolds
Using an Olympus AX70 Light Microscope slides were visualized with fluorescent settings. The H&E slide was imaged at x20 magnification, using 496 nm excitation and 519 nm emission wavelength. Bandpass filter was 460 – 490 nm. This resulted in a ‘green’ emittance.

3.2.3.4.2 Van Gieson
Dewaxed slides or coverslips in water were stained with Gill’s Haematoxylin no. 2 for 4 minutes, rinsed, and ‘blued’ in Scott’s Tap Water for 1 minute. Sections were rinsed in running water and then stained with Van Gieson for 4 minutes. Sections were then dehydrated, cleared with xylene and mounted, and imaged as previously mentioned in 3.2.3.4.1.

3.2.3.4.3 Mason’s Trichrome
Dewaxed slides or coverslips in water were stained with Weigert’s Haematoxylin for 10 minutes and washed in tap water. This was followed by staining in acid fuchsin solution for 5 minutes and rinsed in distilled water. The sections were treated with 1% phosphomolybdic acid for 5 minutes and rinsed briefly. They were then stained with methyl blue solution for 2-5 minutes and rinsed again before treating with 1% acetic acid. Slides/coverslips were dehydrated with alcohol, cleared with xylene, mounted and imaged as previously mentioned in 3.2.3.4.1.

3.2.3.4.4 Alizarin Red
A 2% Alizarin Red solution was made up with 10 mL of distilled water and 0.2 g of Alizarin Red powder. The pH was adjusted to 4.2 using sodium hydroxide and hydrochloric acid. Dewaxed coverslips/slides in water were air dried and then stained with alizarin red for 5 minutes. Coverslips were then dipped in distilled water to remove excess stain. Slides/coverslips were dehydrated with alcohol, cleared with xylene, mounted and imaged as previously mentioned in 3.2.3.4.1.

3.2.3.4.5 Von Kossa
Dewaxed coverslip or slides, plus a human prostate control section were immersed in tap water. The prostate control section is known to have calcium deposits and will
show obvious black staining with Von Kossa. Sections were washed in distilled water and covered with 5% silver nitrate. The sections were either placed under a 60 watt bulb (4-5 inches away) for 30-60 minutes or UV light for 10-20 minutes. Following washing in several changes of distilled water, slides are treated with 5% thiosulfate for 2-3 minutes. Slides are then counter-stained with Van Gieson and dehydrated, cleared, mounted and imaged as in 3.2.3.4.1.

3.2.4 Bone Marrow Cell Retrieval

3.2.4.1 Animal Ethics
The Hercus Taeiri Resource Unit (University of Otago) was covered by their Animal Ethics Committee protocols for culls.

3.2.4.2 Rat Euthanasia
A male, adult Wistar rat was euthanized according to the University of Otago Animal Welfare Office CO₂ protocol. The rat was placed in a plastic chamber connected to a CO₂ source. CO₂ was let into the chamber until it was observed that the rat was still and not breathing. Confirmation of necropsy was by palpating for a pulse, observing respiration and testing for the pedal reflex.

3.2.4.3 Rat Femur Retrieval
The following was a modified protocol by Stace 2011. On autoclaved drapes, the rat was shaved around the thighs and lower back. Another set of drapes was sprayed with chlorhexidine gluconate (hibitane) disinfectant before the placement of the rat dorsal side up. The hip joint was first dislocated. This was done by placing pressure on the pelvis and lifting the femur superiorly until dislocation was felt. Using a number 22 scalpel on a number 4 scalpel handle, incisions were made from the lateral aspect of both knee joints to the vertebral column. Further incisions were made through the musculature of the leg until the femur was reached. Surgical retractors pulled the skin and muscle back for observation. Incisions were made to separate the surrounding muscles from the femur. The muscle tendons were removed from the femur using additional incisions made proximally and distally to reach the hip and knee joints respectively. A scalpel was used to cut at both joint capsules until the femur was free.
Any additional muscle and connective tissue was removed before placing the femurs into a 50 mL Falcon tube with 40 mL of hibitane.

### 3.2.4.4 Bone Marrow Retrieval

This protocol followed that by Stace 2011. In sterile conditions in a laminar flow hood, the femora were placed on paper towels. Using a number 22 scalpel blade and a number 4 scalpel handle, the femora were cut midway to expose the medullary cavity. 10 mL of DMEM was drawn into a syringe fitted with a 20 gauge needle and inserted into the medullary cavity, where growth media was flushed inside to expel the bone marrow. The contents were collected below in a plastic 94 mm dish. The contents of the dish were filtered through a 100 µm cell strainer on top of a 50 mL Falcon tube. 10 mL of media (containing marrow components) was plated into four 25 cm³ culture dish and placed into an incubator at 37 °C and 5% CO₂. Each femur’s contents were collected into separate plastic dishes.

### 3.2.5 Bone Marrow Cell Culture

This protocol also followed that by Stace 2011. After 24 hours for cell attachment, isolation of cells began. In sterile conditions within a laminar flow hood, the culture plates were rinsed with 2 mL of DPBS, three times, to remove debris and unattached cells. 6 mL of growth media was placed into flasks. The plates were placed back into an incubator for 3 more days. After 3 days the cells were passaged as done previously in 3.2.2.2.2.
4 Results

4.1 Scaffold Manufacture

4.1.1 Primary Scaffold Manufacture

4.1.1.1 Gross Appearance
Chitosan (CS) dissolved in acetic acid (AA) resulted in pale yellow solutions that increase in color intensity and viscosity with higher concentrations. Air bubbles get increasingly trapped with increasing viscosity. Freezing at -80 °C resulted in the appearance of central fractures with the periphery looking like cream-tinged ice. As depicted in Figure 4.1, freeze-drying results in approximately 1 cm high discs that have the presence of dull, yellow and glossy material with cracks (arrowhead), with the severity of cracks increasing as CS concentration increased. There are smaller cracks that originated from larger cracks. There were some peripheral regions (arrows) in the wells that were not fractured and were opaque, foamy and cream in color. It appeared that the peripheral, foamy regions emerged in random locations of the 6 well plates as the chitosan concentration increased. The peripheral region resulted in inadequate amounts for production of 8 mm diameter scaffolds, especially with increased chitosan concentration for strength requirements. A biopsy punch was used to retrieve samples from the center of the well and it compressed rather than cut, resulting in samples that looked like pointed ovals when looking from a lateral aspect.
Figure 4.1: Freeze-Dried Chitosan Scaffolds. Wells contain 5 mL of CS (w/v) solution that has been freeze-dried as stated previously. There are noticeable fractures (arrowhead) in the center of each scaffold with a white foamy periphery (arrow) of each well. CS concentrations: A) 0.5%, B) 1.0%, C) 1.5%, D) 2.0%, E) 2.5%, F) 3.0%.

4.1.1.2 Scanning Electron Microscopy
Scanning electron micrographs (SEM) of a 3 mm scaffold from all samples retrieved from the central area showed a smooth surface on the most superior layer, with the interior showing a highly layered architecture of chitosan sheets. The smooth layer is shown in figure 4.2 (A). 4.2 (B) demonstrated the layers of chitosan under the top surface after the top layer is removed by scalpel. The layers became more extensive with increasing chitosan concentration and did not appear to be interconnected.
Figure 4.2: Scanning Electron Microscopy Images of Freeze-Dried Chitosan Scaffolds Frozen at -22 °C. (A) Shows the transverse view of a 1% (w/v) CS scaffold showing the smooth surface (arrow) of the most superior layer. (B) Shows the transverse view of the interior of a scaffold made from 1% (w/v) CS, showing a layered architecture with no obvious orientation of layering. Images taken at x25 magnification.

4.1.1.3 Micro-Computed Tomography Analysis

Micro-computed tomographs (µCT) of a 3 mm 1% (w/v) scaffold from the central area provided approximately 500 images. Figure 4.3 shows a highly layered architecture in all CS concentrations with an obvious pointed oval shape of the scaffold due to biopsy compression as mentioned previously. Further analyses of these images were not undertaken due to the absence of a porous structure.
Figure 4.3: Micro-Computed Tomography Image of 1% (w/v) Chitosan Scaffold. A highly layered architecture is seen with layers becoming closer together at the sides due to compression by punch biopsy. Scale bar = 1.0 mm increments.

4.1.2 Secondary Scaffold Manufacture

4.1.2.1 Freezing Studies

4.1.2.1.1 Gross Appearance
Scaffolds produced by freezing in -22 °C were similar in appearance to the peripheral region of the primary scaffolds, with the entirety looking like cream, translucent ice. The scaffolds that were frozen at -80 °C showed the characteristic cracks as described before in 4.1.1.1 Freeze-drying resulted in the -22 °C samples looking opaque, cream in color and having a foam-like consistency as depicted in Figure 4.4 (A). There was increased roughness of the surface with increasing freeze times. The -80 °C samples had cracks that increased in intensity with increased freezing time as shown in Figure 4.4 (B).
Figure 4.4: Freeze-Dried Chitosan Scaffolds Frozen at Various Temperatures and Durations. (A) Shows three 1% (w/v) chitosan scaffolds frozen at -22 °C and (B) shows three 1% (w/v) chitosan scaffolds frozen at -80 °C. Scaffolds are frozen for 24, 48, and 72 hours and are displayed from left to right.

4.1.2.1.2 Scanning Electron Microscopy Analysis

SEM analysis of the scaffolds frozen at -22 °C showed a highly porous structure in all scaffolds as shown in Figure 4.5 (A). The transverse samples showed some distortion of the pore walls due to mechanical handling by forceps and cutting with a scalpel so that it does not give a clear outline of the pore. The -80 °C samples depicted in Figure 4.5 (B) show the characteristic layered architecture as previously described. With increasing freezing time there seemed to be a change in pore size. Porous structures looked more elongated in shape and the amount of layers appeared to increase.
Figure 4.5: Scanning Electron Microscopy Images of Chitosan Scaffolds Frozen at Various Temperatures and Durations. (A) three 1% (w/v) chitosan scaffolds frozen at -22 °C, sectioned to show a longitudinal view. (B) three 1% (w/v) chitosan scaffolds frozen at -80 °C and sectioned to show a transverse view. (i) scaffolds frozen for 24 hours, (ii) scaffolds frozen for 48 hours, and (iii) scaffolds frozen for 72 hours. Images taken at x100 magnification.
4.1.2.2 Container Studies

Adequate visualization of the frozen samples were unable to be obtained due to the opaque container walls obscuring the view. The gross appearance of freeze-dried samples frozen at -22 °C looked opaque, cream and foam-like, whereas samples frozen at -80 °C looked cracked both as previously stated in 4.1.2.1.1. (A) and (B) demonstrate SEM images of scaffolds produced in 15 mL Falcon tubes, with (A) showing a porous structure after being frozen at -22 °C, and (B) showing a layered structure after being frozen at -80 °C. (C) and (D) shows SEM images of scaffolds produced in large petri dishes, where (C) shows a porous structure after being frozen at -22 °C, and (D) shows a layered structure after being frozen at -80 °C. There seems to be no obvious orientation of all the porous structures. The sample in a transfer pipette proved to be unsuccessful as freeze-drying caused the frozen scaffold material to expel from the container when the vacuum pump was turned on, causing it to melt and change shape.
Figure 4.6: Scanning Electron Microscopy Images of 1% (w/v) Chitosan Scaffolds Prepared in Various Containers. (A) Shows a scaffold frozen at -22 °C in a 15 mL Falcon tube, (B) shows a scaffold frozen at -80 °C in a 15 mL Falcon tube, (C) shows a scaffold frozen at -22 °C in a petri dish, (D) shows a scaffold frozen at -80 °C in a petri dish. (i) images are shown at x25 magnification, (ii) images are shown at x100 magnification.

4.1.2.3 Macroconcentrations of Chitosan in Scaffolds

4.1.2.3.1 Gross Appearance

Addition of concentrations above 2% (w/v) CS to 1% (v/v) AA resulted in very viscous and pale yellow solutions, with no obvious difference between concentrations. The use of a 1000 µL pipette tip was sufficient to mix and break down clumps of chitosan without the introduction of significant amounts of air bubbles. Scaffolds from now onwards were prepared by freezing at -22 °C. These frozen scaffolds had translucent surfaces with an opaque, yellow-cream interior. Once freeze-dried the scaffolds looked cream colored, dense foam-like structures with a paler periphery. The remains of air bubbles on an 8% CS (w/v) scaffold are shown in Figure 4.7 and are shown as the small circles on the top surface. Biopsy sampling was done without difficulty as scaffolds retained their shape when pressure is applied.
Figure 4.7: Freeze-Dried 8% (w/v) Chitosan Scaffold. 5 mL of 8% CS (w/v) was freeze-dried in a well of a 6 well plate. There is a space in the bottom left of the scaffold to demonstrate biopsy sampling. Noticeable specks in the CS due to air bubbles (arrow).

4.1.2.3.2 Scanning Electron Microscopy Analysis
As shown in Figure 4.8, SEM analysis revealed a porous architecture with a trend of decreasing pore size as chitosan concentration increased. There was an obvious increase in the homogeneity of the pores at 4% (C) and 8% (w/v) CS (D), with less rectangular pore shapes and more ovoid and circular in nature. Increasing concentrations beyond 10% resulted in scaffolds of insufficient pore size for cell culture, which was judged by pore measurement and observation.
Figure 4.8: Scanning Electron Microscopy Images of Macroconcentrations of Chitosan Scaffolds. (A) 1.5%, (B) 2%, (C) 4%, (D) 8%, (E) 9%, (F) 10%, (G) 11% and (H) 12% (w/v) CS. Pores are measured as depicted by red text. Images are taken at x100 magnification.

4.1.2.4 Chitosan and Micro-Hydroxyapatite Scaffolds

4.1.2.4.1 Gross Appearance
CS solutions of 1% (w/v) that were described previously in 4.1.2.1.1 became increasingly white and opaque with the addition of micro-hydroxyapatite (µHA). Higher concentrations saturated the solution and were suspended in solution with some particles resting on the surface of the culture well. The freeze-dried scaffolds were opaque and cream in color, with foam-like consistency as previously found.

4.1.2.4.2 Scanning Electron Microscopy Analysis
As shown in Figure 4.9, SEM analysis of the internal structure of the scaffolds showed a highly porous architecture. As µHA concentration increased there was an observable decrease in pore size. There was also an increase of the walls collapsing into the pore. These collapsing walls looked like frills, looked frail and obscured the observers view into the pore.
Figure 4.9: Scanning Electron Microscopy Images of 1% (w/v) Chitosan Scaffolds with Various Micro-Hydroxyapatite Concentrations. (A) 0%. (B) 0.05%. (C) 0.1%. (D) 0.2%. (E) 0.4%. (F) 0.6% (w/v) µHA. Pores are measured as depicted by white text. Images taken at x100 magnification.
4.1.2.5 Chitosan and Macroconcentrations of Micro-Hydroxyapatite

4.1.2.5.1 Gross Appearance
The CS solutions increased in opaqueness with increasing µHA with a large layer of µHA that settled on the bottom of the culture wells, especially at 4% (w/v) and above. The appearance of the freeze-dried scaffolds showed the cream, opaque color gradually become chalk like in appearance with increasing µHA concentration. There was also a large increase in scaffold friability, as biopsy sampling was a great difficulty. The µHA samples at 4% and above crumbled to fine pieces.

4.1.2.5.2 Scanning Electron Microscopy Analysis
As depicted in Figure 4.10, SEM analysis shows an obvious porous architecture at 1% µHA and a much less obvious porous structure in 2% µHA. At 4% µHA there was the absence of pores and the presence of what looked like layers within layers. 15% and 20% µHA showed very simple layers that look like frills.
Figure 4.10: Scanning Electron Microscopy Images of 1% (w/v) Chitosan Scaffolds with Macroconcentrations of Micro-Hydroxyapatite. (A) 0%, (B) 1%, (C) 2%, (D) 4%, (E) 15%, and (F) 20% (w/v) µHA. Pores are measured as depicted by red text. Images taken at x100 magnification. Pore size distributions were unable to be measured for C, D, E, F as layers were present.
4.1.2.6 Chitosan and Nano-Hydroxyapatite Scaffolds

4.1.2.6.1 Gross Appearance

Mixing nano-hydroxyapatite (nHA) into a 1% (w/v) CS solution resulted in solutions that increase in opaqueness with increasing nHA. The opaqueness caused by using nHA was less than that caused by µHA. Once frozen, scaffolds looked translucent with a cream tinge. Once freeze-dried the scaffolds were cream colored, foam-like with a white periphery, and with no obvious difference between scaffolds (Figure 4.9). Using a skin biopsy punch decreased in difficulty with increasing nHA concentrations and the samples were easily cut out with their shape retained. A 1-2 mm layer of a white chalk-like material was formed on the base of the scaffolds with a small quantity remaining adhered to the culture plate as depicted in Figure 4.11 where biopsy samples have been removed. This lead to the thought that the solutions must be saturated of nHA and that the concentration is overestimated. The most inferior layer of nHA could be easily broken away while the uppermost layers remained attached to the scaffold.
Figure 4.11: Chitosan Scaffolds with Various Amounts of Nano-Hydroxyapatite. 5 mL of 1% CS (w/v) with various amounts of nHA was freeze-dried in each well of a 6 well plate. (A) 2.5%, (B) 3.0%, (C) 3.5%, (D) 4.0%, (E) 4.5%, (F) 5.0% nHA (w/v). Obvious punch biopsy retrieval shown is shown. There is a faint white substance on the well floor where biopsy samples are taken.

4.1.2.6.2 Scanning Electron Microscopy Analysis

Figure 4.12 shows a SEM analysis of a transversely cut scaffold illustrating a porous architecture. Pore openings were not obvious, with the walls collapsing inwards minimizing visualization. Cut pore walls look like frills. The 4.5% (w/v) nHA sample showed layers of CS rather than the required porous structure. However when nHA is increased to 5% (w/v) the porous structure remains. Pore size was difficult to quantify by measuring pore sizes on SEM images.
Figure 4.12: 1% Chitosan Scaffolds with Various Nano-Hydroxyapatite Concentrations. 5 mL of CS was freeze-dried in wells of a 6 well culture plate with various amounts of nHA. (A) 2.5%, (B) 3%, (C) 3.5%, (D) 4%, (E) 4.5% and (F) 5% nHA (w/v). Pore sizes are measured as depicted by red text. Images taken at x100 magnification.
4.1.2.7 8% (w/v) Chitosan and Macroconcentrations of Nano-Hydroxyapatite

4.1.2.7.1 Gross Appearance
The mixing of these materials together resulted in viscous, cream colored solutions that were paler in comparison to 4.1.2.3. The nHA and CS dissolved sufficiently with a 1000 µL pipette tip. Freezing resulted in a substance similar to 4.1.2.3 and had a white inferior layer. Freeze drying resulted in a dense foam-like structure with a chalky base layer, the most inferior part easily broke away with the uppermost material left incorporated into the scaffold.

4.1.2.7.2 Scanning Electron Microscopy Analysis
As shown in Figure 4.13, SEM analysis reveals a porous structure. Visualization into the pores is obscured in some samples as the pore walls collapse inwards. Figure 4.13 (A) below shows a rough surface with no porous or layered architecture. There was no observable trend in pore size with increasing nHA concentration.
Figure 4.13: Scanning Electron Microscopy Images of 8% (w/v) Chitosan Scaffolds with Various Nano-Hydroxyapatite Concentrations. 5mL of 8% (w/v) CS with various amounts of nHA was freeze-dried in a culture dish. (A) 2.5%, (B) 3.0%, (C) 3.5%, (D) 4.0%, (E) 4.5% and (F) 5.0% nHA (w/v). Pores are measured as depicted by red text. Images taken at x100 magnification.
Using ImageJ software, an SEM image was binarised and threshold adjusted so that pores were white and chitosan was black. A filter was applied to remove small pixels of white. 30 pores were then measured from each of the 3 samples. Each of these different images is shown below in Figure 4.14. The average pore diameter was 105.26 µm, 108.59 µm and 104.12 µm for each sample and is shown in Figure 4.15. There was no statistical difference between samples as analyzed by an ordinary 1-way ANOVA with a Tukey’s Multiple Comparisons Test (p <0.05). Table 4.1 shows the parameters of scaffold pore diameter.

![ImageJ Analysis of 8% (w/v) Chitosan and 5% (w/v) Nanohydroxyapatite Scaffolds.](image)

**Figure 4.14: ImageJ Analysis of 8% (w/v) Chitosan and 5% (w/v) Nanohydroxyapatite Scaffolds.** (A) shows untouched SEM image, (B) shows thresholded image in black and white, (C) shows filtered black and white image. Images taken at x100 magnification.
Figure 4.15: Graphical Representation of Average Pore Diameters of Three Scaffold Samples. Scaffold sections were filtered with ImageJ and had pores measured. There was no statistically significant difference between all samples. 30 pore diameter was measured for three samples (n = 30, data points expressed as mean with error bars representing ± SE).

Table 4.1: ImageJ Analysis of Scanning Electron Micrographs to Demonstrate Mathematical Parameters of Scaffold Pore Diameter.

<table>
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<th>Parameter</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
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4.1.3  8% (w/v) Chitosan and 5% (w/v) Nano-Hydroxyapatite Scaffold Characterization

4.1.3.1 Micro-Computed Tomography of Scaffolds
An 8% CS and 5% nHA (w/v) scaffold was chosen for µCT based on SEM results of pore diameter and the highest possible nHA concentration. The majority of pore sizes needed to be above 100 µm, to provide conditions compatible with cell migration. The highest concentration of nHA would provide optimal and additional bioactivity without loss of structural integrity. Open and closed porosity for 8% CS/5% nHA (w/v) scaffold are shown below in Table 4.2, with porosity results of a 5 and 10% CS with 5% nHA (w/v) scaffold shown for comparison.

Table 4.2: CTan Porosity Analysis of 5, 8 and 10% (w/v) Chitosan and 5% (w/v) Nano-Hydroxyapatite Scaffolds.

<table>
<thead>
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<th>Porosity Type</th>
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<th>10%CS/5% (w/v) nHA</th>
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<td>Closed porosity (%)</td>
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<td>79.0</td>
<td>75.2</td>
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</table>

4.1.3.2 Scaffold Rehydration and Stabilisation
During rehydration in serial ethanol dilution, it was observed that particles from the scaffold would break off and be suspended in solution, or sink to the bottom of a falcon tube. After freeze-drying there was a ‘web’ of fibrous material that resembled cotton wool and surrounded the scaffolds and walls of the petri dish. This web was easily removed. During distilled water immersion the scaffold did not dissolve and indicated that the AA had been rinsed away. SEM results (Figure 4.16) show no obvious change in pore size with ethanol washing and immersion in distilled water. The appearances of the pores are obscured with neighboring collapsing pore walls.
Figure 4.16: Scanning Electron Microscopy Images of Scaffolds Pre- and Post-Ethanol Series. (A) Shows a pre-ethanol series scaffold with appropriate pore sizes. (B) Shows a post-ethanol series scaffold with pore sizes still remaining appropriate. Frills of cut scaffold wall obscures view of several pores. Pores measured as depicted by white text. Images taken at x100 magnification.

4.1.3.3 Scaffold Sterilisation

Once autoclaving (121 °C for at least 20 minutes at 15 PPI) was completed the scaffold came out moist and a brown-orange color (Figure 4.17). SEM analysis (Figure 4.18) revealed appropriate sizes of porosity. Additional characterization of the pore size was not carried out due to physical changes.

Figure 4.17: Autoclaved Scaffold. Scaffold is orange in color with a white nano-hydroxyapatite surface (arrow).
4.1.3.4 Scaffold Degradation and pH Study

Nine pre-weighed scaffolds were placed in phosphate buffered saline (PBS) and left in an incubator over several time periods. It was observed that there were particles of scaffold settling in solution. At each time point (48, 96 and 168 hours), 3 scaffolds were weighed after being freeze-dried. The average mass values were plotted on Prism 6 (GraphPad Software) against its original weight (t = 0 hours). Graphical representation is shown in Figure 4.19.

Over a time period of 168 hours, there were no statistically significant differences between pre-degradation and post-degradation weights of all samples, as analyzed using a two-way repeated measure ANOVA with a Bonferonni’s post-hoc test, even though some scaffolds were observed to lose and even gain weight.

The pH of PBS was measured with a pH meter and shown as a graph in Figure 4.20. The pH of PBS was originally 7.4 and was observed to decrease at 24 hours and remained consistent until 168 hours. This drop in pH ranges from 6.98 – 7.02, There was a significant difference between 0 hours and 24, 48 and 168 hours (p <0.001), as analyzed using a 1-way ANOVA with Bonferonni’s post-hoc test. No statistically significant difference was found between other hours. These statistical results are shown in Table 4.3.
Figure 4.19: Graphical Representation of Scaffold Degradation During Incubation. Columns indicate mass of samples pre- and post-degradation that have underwent various degrading periods. No statistically significant difference was found between columns (pre- vs post-degradation) as analyzed using a two-way repeated measure ANOVA with a Bonferroni post-hoc test ($p > 0.05$). Columns represent mean values ($n = 3$; error bars represent ± SE of the mean).

Figure 4.20: Graphical Representation of pH Change During Scaffold Incubation. Represents change in pH over 168 hours. There was a significant difference in pH between 0 and 24, 48 and 168 hours ($p < 0.001$). As analyzed using a one-way ANOVA with a Bonferroni post-hoc test ($n = 3$, data points expressed as mean ± SE).
Table 4.3: Statistically Significant Differences in pH Across Incubation Periods of Scaffolds. (** = p < 0.001, n.s. = no statistically significant difference). Analysis performed using a one-way ANOVA with a Bonferroni post-hoc test (n = 3).

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4.1.3.5 Swelling Studies
Nine scaffolds were immersed in PBS and left in an incubator for up to 168 hours. At each time point, scaffolds were removed and immediately weighed. It was observed that scaffold mass had increased with time and is graphically displayed in Figure 4.21. Sample 1 showed a statistically significant difference (P < 0.01) between 0 hours and both 72 and 168 hours, and there was a significant difference (P < 0.05) between 0 and 24 hours. Sample 2 has a statistically significant difference between 0 and 168 hours (p < 0.01) and 72 hours (p < 0.05). And in sample 3, there was a statistically significant difference between 0 hours and 168 hours (p < 0.01), and 24 hours with 168 hours (P <0.05). Results from statistical analysis are shown in Table 4.4.
Figure 4.21: Graphical Representation of an Incubated Scaffold Swelling over 168 hours. Graph indicates change of mass of 3 scaffolds over time. As analyzed using a two-way ANOVA and Bonferroni post hoc (n = 6, data points expressed as mean ± SE).
Table 4.4: Statistically Significant Differences in Swelling of Incubated Scaffold. Table A) sample 1, table B) sample 2, table C) sample 3. These tables show if swelling was significantly different between incubation durations within each sample. As analyzed by two-way ANOVA with Bonferroni’s multiple comparison test \((n = 6)\) (* = \(p < 0.05\), ** = \(p < 0.01\), n.s. = no statistically significant difference found).

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4.1.3.6 Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectroscopy was used to characterize the scaffold composition. The infrared spectra in Figure 4.22 showed strong PO$_4^{3-}$ \((v_2)\) bands appearing at 1092 cm$^{-1}$ and at 1042 cm$^{-1}$. The PO$_4^{3-}$ \((v_1)\) band appeared at 962 cm$^{-1}$. Medium intensity CO$_3^{2-}$ bands were shown at 873 \((v_2)\) and 1421 \((v_2)\) and suggests that CO$_3^{2-}$ was incorporated in the HA lattice. There were typical chitosan absorption bands at 1654 cm$^{-1}$ and 1595 cm$^{-1}$ due to carbonyl (C=O) stretching and amine (N-H) deformation of CS molecules. The bands at 1319 cm$^{-1}$ and 1378 cm$^{-1}$ occurred due to C-CH$_3$ symmetric deformation.
Figure 4.22: Fourier Transform Infrared Spectroscopy of an 8% CS and 5% nHA scaffold. Peaks and bands labelled as shown. (A) 3423 cm\(^{-1}\) OH stretching, (B) 3100-3470 cm\(^{-1}\) broad OH stretching, (C) 2885-2990 cm\(^{-1}\) aliphatic C-H stretching, (D) 1651 cm\(^{-1}\) amide I, (E) 1422 and 1461 cm\(^{-1}\) CO\(^3\)-, (F) 1380 cm\(^{-1}\) C-O stretching, (G) 1252 cm\(^{-1}\) free primary amino group, (H) 1030-1033 cm\(^{-1}\) PO\(^4\)- stretching (I) 1030 cm\(^{-1}\) PO\(^4\)-.

4.1.3.7 Energy-Dispersive X-ray Spectroscopy

Energy dispersive X-ray Spectroscopy (EDS) was done to further characterize the scaffolds, specifically their elemental composition. Results (Figure 4.23) indicated large sodium and chloride peaks followed by large carbon and oxygen peaks. Smaller peaks of phosphate, potassium and calcium followed this. Carbon and oxygen made up the majority of the scaffold with 51.18% and 23.44% respectively. Chloride was 13.16% and sodium was 8.67%. Calcium was 2.1% with phosphate and potassium being both 1% or less. A nitrogen peak was absent.
Figure 4.23: Energy-Dispersive X-Ray Spectroscopy of an 8% CS and 5% nHA scaffold. Peaks are labelled as shown with a table showing element, keV necessary for detection, mass (%) and error (%). Peaks demonstrate the number of counts (X-rays that are received and processed by the detector) and keV demonstrates the energy level of the counts that correspond to an associated element.
4.2 Cell Culture Experiments on 8% (w/v) Chitosan and 5% (w/v) Nano-Hydroxyapatite Scaffolds

4.2.1 Cell Viability LIVE/DEAD® Assay

Growth media changed color from red to orange after 24 hours. There were no other observable differences between the 3 samples. Confocal microscopy revealed green ovoid shaped cells and irregular red cells. The fibroblastic morphology of the cell line was present at every time period, along with the presence of more ovoid cell shapes suggesting cells have not attached yet. Cell viability and total cell number was measured.

For SAOS-2 cells cultured on scaffolds, the average cell viability at 24 and 48 hours were similar at 73%, with cell viability at 96 hours increased to 87%. The average total cell number was 43, 53 and 64 at 24, 48 and 96 hours respectively. These values are presented in Figures 4.24 (A) and (B) respectively, with statistical analysis results displayed in Table 4.5. LIVE/DEAD® assay images of the scaffold are shown in Figure 4.25 and were analyzed to acquire viability and total cell number results. The viability of cells at 96 hours had a statistically significant difference compared to 48 and 24 hours. There was no statistically significant difference in viability between 48 and 24 hours.

For SAOS-2 cells cultured on coverslips, the average viability was 72, 81 and 97% for 24, 48 and 72 hours respectively. The average total cell number was 30, 33 and 58 for 24, 48 and 72 hours respectively. Figure 4.26 shows the viability and total cell number results from the coverslip experiment. Table 4.6 demonstrates the statistically significant differences of viability and total cell number on a coverslip and only demonstrated a significant difference of viability between 96 and 24 hours.

Figure 4.27 shows viability and total cell number comparison of scaffold versus coverslip. There were no statistically significant differences between scaffold and coverslip experiments between viability and total cell number for all time periods.
Figure 4.24: Graphical Representation of LIVE/DEAD® Assay of SAOS-2 Seeded 8% CS and 5% nHA scaffold. (A) The average SAOS-2 viability (as a % of total cells, ± SE) over increasing culture time. (B) The average total cell number (±SE) over increasing culture time (n=3). Blue = viability, red = total cell number.
Table 4.5: Statistically Significant Differences of Viability and Total Cell Number Between Culture Periods Using a LIVE/DEAD® Assay on an 8% CS and 5% nHA scaffold. (* = p < 0.05, n.s. = no statistically significant difference) as analyzed using a one-way repeated measures ANOVA with a Bonferroni post-hoc test (blue = viability, red = total cell number) (n=3).

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Figure 4.25: Confocal Micrographs of SAOS-2 Seeded Scaffolds. Green = live cell (calcein AM), red = dead cell (ethidium homodimer-1). (A) and (B) 24 hours. (C) and (D) 48 hours. (E) and (F) 96 hours. (A), (C), (E) represents x10 magnification, (B), (D), (F) represents x5 magnification.
Figure 4.26: Graphical Representation of LIVE/DEAD® Assay Using SAOS-2 Seeded Coverslips.

(A) average SAOS-2 viability over increasing culture time (as a % of total cells, ± SE). (B) average total cell number over increasing culture time (±SE). Blue = viability, red = total cell number. (n=3).
Table 4.6: Statistically Significant Differences of Viability and Total Cell Number Between Culture Periods Using LIVE/DEAD® Assay on Coverslips. * = p <0.05, n.s. = no statistically significant difference, as analyzed using a one-way repeated measures ANOVA with a Bonferroni post-hoc test (blue = viability, red = total cell number) (n=3).

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Figure 4.27: Graphical Representation of LIVE/DEAD® Comparing Scaffold and Coverslip Viability and Total Cell Number. (A) The average SAOS-2 viability (as a % of total cells, ± SE). (B) The average total cell number (±SE). n.s. = no statistically significant difference found between test and control groups, as analyzed using a one-way repeated measures ANOVA with a Bonferroni post-hoc test (orange = coverslip, green = scaffold) (n=3).

4.2.2 CellTiter 96® AQOne Solution Cell Proliferation Assay
Scaffolds of CS/nHA and collagen were immersed in growth media to allow potential cytotoxins to leach out into the media in order to create a scaffold extraction vehicle. Vehicle is defined as a substance (growth media) to facilitate the use of another material (potential cytotoxins). These ‘vehicles’, along with Triton-X solution
(0.01%) in growth media were used as media to grow cells. The MTS assay was a colorimetric assay to assess cell viability. It involved the tetrazolium dye MTT that is reduced to formazan by cellular enzymes, which reflected the amount of viable cells.

It was observed that the yellow MTS gets increasingly purple as time progressed, especially in the chitosan and collagen wells. There was little change in colour of the yellow MTS of Triton-X vehicles. As shown by Figure 4.28, the absorbance reading of the Triton-X vehicle fluctuated around zero, and illustrates that there is very little to no enzymatic conversion of MTS to formazan and hence, no viable cells. The absorbances of both chitosan and collagen increased with time, illustrating that cells are proliferating and metabolizing MTS to formazan, with the highest absorbance values at 72 hours indicating higher amounts of formazan. This could indicate larger amounts of cells metabolizing MTS or cells have longer period to convert large amounts of MTS. There was no statistically significant difference between the chitosan and collagen extraction vehicles, as collagen is considered to be non-toxic it is implied that chitosan has no negative affect on cell proliferation. There was a statistically significant difference between Triton-X and chitosan absorbances at 0 hours (p <0.05) that indicates absorbance readings of formazan, media buffer, or differences in cell count.
Figure 4.28: Graphical Representation of Vehicle Absorbances Using MTS Assay. Line graph shows absorbance reading of CS, collagen and Triton-X vehicles over 72 hours. Absorbance = mean ± SE. There were statistically significant differences between Triton-X and both Collagen and Chitosan at 24, 48 and 72 hours (p < 0.001). There was a statistically significant difference between Chitosan and Triton-X at 0 hours (P < 0.05). There was no statistically significant difference between Collagen and Chitosan at all time points (n=3).

4.2.3 Three Dimensional SAOS-2 Cell Culture

4.2.3.1 Histology of SAOS-2

Figure 4.29 shows light microscopy images of haematoxylin and eosin (H&E) stained test and control scaffolds, and an image of a seeded coverslip that were all cultured for a time period of 14 days. The porous nature was evident by the sectioning of the scaffold, with ‘clumps’ of CS forming thick and thin walls. In both test and control images chitosan was shown to stain pink by eosin. In the test scaffold (A) and (B) there were light-pink stained structures found throughout the CS thought to be nHA (arrowhead), which indicates that nHA is acidic or positively charged allowing staining by eosin. Haematoxylin stained structures were found throughout the scaffold and were stained purple (arrows). These structures varied in shape and size and location. They concentrated in the top quarter of the scaffold and were dispersed throughout the entire scaffold but were less concentrated or found as singular entities. Image F demonstrated H&E staining of a coverslip, with purple nuclei and pink cytoplasm. Figure 4.30 shows a light micrograph of an H&E stained scaffold using fluorescent filters. The fluorescent yellow showed CS matrix and the green showed
cell-like structures. Yellow appeared due to auto-fluorescent eosin. Green is due to cellular structures.

Figure 4.29: Haematoxylin and Eosin Stained Scaffold and Coverslip containing SAOS-2. (A) Test x10 objective, (B) test x20 objective with cellular structures (arrow) and hydroxyapatite (arrowhead), (C) control x10 objective, (D) control x20 objective, (E) glass coverslip x10 objective, (F) glass coverslip x20 objective.
Figure 4.30: Fluorescent Microscopy of Haematoxylin and Eosin Stained Scaffold containing SAOS-2. Yellow fluorescence indicates CS and green indicates cells. Image taken at x20 magnification.

Figure 4.31 shows light microscopy images of Masson’s Trichrome (stains collagen blue, cell nuclei blue-black, and cytoplasm orange-red) stained test and control scaffolds, and an image of a seeded coverslip that were all cultured for a time period of 14 days. The CS matrix was stained a dark purple/red in Images A-D and was darker in Images A and B. Image A and B revealed pink and blue structures that corresponds to nHA (arrowhead) a cellular structure (arrow) respectively, that was similar to that found in H&E staining. Image E and F demonstrated staining of a coverslip, with nuclei stained black, blue or purple, and cytoplasm staining purple/pink.
Figure 4.31: Masson’s Trichrome Stained Scaffold and Coverslip containing SAOS-2. (A) Test scaffold x10 objective, (B) test x20 objective, with a cellular structure stained blue (arrow) and hydroxyapatite stained pink (arrowhead), (C) control x10 objective, (D) control x20 objective, (E) glass coverslip x10 objective, (F) glass coverslip x20 objective.
Figure 4.32 shows light microscopy images of Alizarin Red stained test and control scaffolds, and an image of a seeded coverslip that were all been cultured for a time period of 14 days. Image A–D revealed a lightly red stained CS matrix, with A and B staining slightly darker. Image B revealed a cell structure similar to that found in H&E staining (arrow). Images E and F revealed clusters of dark red staining, with cell cytoplasm stained less intensely. There was no staining evident in the spaces between cells.
Figure 4.32: Alizarin Red Stained Scaffold and Coverslip containing SAOS-2. (A) Test x10 objective, (B) test x20 objective with cell cluster (arrow), (C) control x10 objective, (D) control x20 objective, (E) glass coverslip x10 objective, (F) glass coverslip x20 objective
Figure 4.33 shows light microscopy images of Von Kossa stained test and control scaffolds, and an image of a seeded coverslip were all been cultured for a time period of 14 days. Image A and B revealed a red stained CS matrix due to Van Gieson counter staining and black specks throughout the matrix that corresponds to Von Kossa staining of calcium deposits. Image B showed a structure similar to the cells found in H&E staining and has black specks within (arrow). In Images C and D there were no black stained structures, as there are no cells or nHA. Images E and F revealed specks of black found in the cell cytoplasm. Image G showed areas of black staining in the prostate section and is found throughout the section (arrows).
Figure 4.33: Von Kossa Stained Scaffold, Coverslip containing SAOS-2 and Prostate Control Section. (A) Test x10 objective, (B) test x20 objective with an arrow pointing to a cell cluster containing black stained structures, (C) control x10 objective, (D) control x20 objective, (E) glass coverslip x10 objective, (F) glass coverslip x20 objective, (G) control human prostate x4 objective with black stained regions (arrows).

Figure 4.34 shows light microscopy images of Van Gieson stained test and control scaffolds, and an image of a seeded coverslip that were all been cultured for a time period of 14 days. Van Gieson stained for blue/black nuclei, red collagen and yellow muscle. Images A-D revealed that CS is stained red. Image B revealed a structure (arrow) that was stained red but not as intensely as CS. This structure had a similar appearance to the cells found in H&E staining. In Image A, lightly stained regions were found through the CS matrix (arrow) and corresponded to nHA. These regions were not found in Images C and D. Image E and F showed that both the nucleus and cytoplasm are stained red, with the nuclear contents staining darker red or black. This stain is similar to 4.33 as Von Kossa used Van Gieson as the counter stain.
Figure 4.34: Van Gieson Stained Scaffold and Coverslip containing SAOS-2. (A) Test x10 objective with arrow pointing to nHA, (B) test x20 objective with arrow pointing to cell cluster, (C) control x10 objective, (D) control x20 objective, (E) glass coverslip control x10 objective, (F) glass coverslip x20 objective.
4.2.4 Bone Marrow Cell Culture
Observation by phase contrast microscopy 24 hours after plating marrow showed a heterogeneous population of cells, where certain populations were elongated and spindle shaped, while the other was polygonal or flat shaped with little to no processes. Following protocol, cells were passaged at day 3 even though it was not 70-80% confluent but was approximately 40-50%. This resulted in the appearance of the spindle and polygonal cell populations that attach but had a reduced ability to proliferate. The second and third passage took a large amount of time to become confluent so passage was not done at 70-80% confluency, but was approximately 20-30%. These passaged cells were found individually or in small clusters of 2-3 cells and did not proliferate. Figure 4.35 demonstrates a spindle and flat cell.

Figure 4.35: Phase Contrast Micrographs of Bone Marrow Cell Populations. (A) spindle cell and (B) flat cell. Images are taken at x10 objective
5 Discussion

5.1 Scaffold Manufacture

The aim of this thesis was to fabricate a chitosan (CS) and hydroxyapatite (HA) scaffold, with pore diameters greater than 100 µm and porosity similar to bone. These requirements allow cell attachment, migration and fluid transport. Additional characteristics of the scaffold were bioactivity to promote boney integration and ingrowth; and biocompatibility so there is no immune response or cytotoxicity. When this scaffold was 3D cultured in a bioreactor (providing shear stress and a circulation) a further aim was to produce a 3D sample of osteoid tissue.

5.1.1 Primary Scaffold Manufacture

The inaugural development of a scaffold was unsuccessful as indicated by data and gross observation. Scaffolds were mechanically weak and the majority was comprised of a layered structure and therefore is incompatible with cell culture.

Our findings in Figure 4.5 demonstrated that freezing scaffolds at -80 °C resulted in a layered architecture following freeze-drying. This means that the scaffolds will not be able to support cell growth, as there is the absence of a porous architecture. In a study by Cooney et al., the freezing of CS solutions below -50 °C resulted in a layered architecture and illustrated that mass transfer (concentration gradients) dominates the system. As the author’s scaffold was frozen at -80 °C, it was clear that the scaffolds were governed by mass transfer during freezing, and when combined with SEM analysis, the layered architecture is evident (60).

Initially only scanning electron micrography (SEM) was performed but this only provided a limited view of the entire scaffold, which may have missed structural
differences that appeared in other locations. To garner a greater appreciation, several SEM images of different locations (from superior to inferior and medial to lateral) should be taken. For improved visualization we used micro-computed tomography (µCT) to analyze the entire structure of one biopsy sample, giving approximately 1000 image slices that confirmed layers throughout. These images could be analyzed with software to calculate pore size and porosity. However with µCT, processed images can give inaccurate estimations, as thresholding can reduce or increase pore size, and modify pore walls that could be analyzed as non-pore. As this was only a small 3 mm biopsy sample from the larger sample made in the wells, there could very well be different architectures throughout the samples in the wells. µCT imaging, although provided a comprehensive view of one small sample, is limited by the fact it cannot image larger samples and is time consuming, taking several hours for one 3 mm sample at a medium camera resolution.

Results indicated by Figure 4.1 that increasing the CS concentration caused the severity of cracks or fissures and the layered architecture to become more extensive, and that there was an increasing amount of air bubbles becoming trapped in solution. This demonstrated that increasing the concentration of solute altered the physical properties during scaffold development. Work by MacKenzie demonstrated that solutes retained water during the freeze-drying process so when secondary drying commences, these solutes shrank and generated stresses causing cracks (76). Our findings suggested that increasing solutes resulted in a larger amount of water retention, and therefore produced larger stresses and cracks. Another reason for the appearance of fissures may be due to the freezing process. Water froze from the outside in, expanding upward in its confined container. As water in the central portion of the well freezes it will expand and generate stresses on the peripheral ice, causing it to crack and allow its expansion. Alternatively, it may be possible that the cracks are formed by large ice crystals that extend across the well, so when lyophilized, it leaves a sharp groove surrounded by CS.

Increasing the CS concentration (up to 3.5% (w/v)) resulted in an increasingly viscous gel that retained air bubbles due to mixing. After resting overnight these bubbles rose to the surface and disappeared with bubbles in the least viscous gels disappearing first. This was important, as air bubbles that remained in the frozen scaffold would
introduce inappropriately sized pores during the freeze-drying process. Additional studies involving the introduction of bubbles into solution may be an alternative method of introducing pores. This may be beneficial for highly viscous solutions that will not let bubbles escape.

The fissures and the peripheral porous region appeared at random locations and were of varying sizes. There must have been heterogeneous ice nucleation conditions within and around each sample. Possible reasons for this would have to alter the thermal gradient (ΔT) and hence heat or mass transfer (60). Plastic is a poor conductor of heat so the periphery of each sample is in contact with a well wall and will have a slower freezing rate compared to the material in the center. This occurrence was observed by Stace 2011, who froze scaffolds at -80 °C in plastic wells which resulted in porous peripheral regions and layered central regions (39). However this would not explain why these peripheral regions appear randomly. Alternative explanations may involve what is in contact with the well plates: air, freezer wall, and other frozen objects that can increase or decrease ΔT. Future studies should look into creating homogenous freezing conditions for samples in order to generate homogenous scaffolds, such as freezing in liquid to create the same temperature surrounding the CS gels.

Cooney et al. explained the presence of a smooth surface on top of every sample, which is shown by Figure 4.2 (A, arrow). A solution exposed to air in the freezer formed a ‘skin’ at the initial air-liquid interface that caused significant resistance to heat transfer. This resulted in an even ice crystal formation at the beginning of the freezing process (60). This skin also appeared between the scaffold-well interfaces. This layer of CS was non-porous and must be removed prior to cell culture, as it is a barrier for cellular migration. Additional work should look into the interfaces between the CS gel and the surrounding wells and air so that this skin does not form and is porous all the way through. This will have to investigate the various conductances of materials.

A study by Madihally froze scaffolds at -78 °C and this resulted in the formation of pores whereas the author’s did not. In contrast, the methods showed that scaffolds
were produced at 1 and 2% (w/v) CS with 1.2% (v/v) AA inside pre-cooled, flat bottomed glass tubes with a diameter of 1.5 cm and 1.2 mm wall thickness. These tubes of 3-5 mL of solution were frozen in liquid at specified temperatures. This study illustrated that smaller diameter scaffolds in high conductance materials (compared to plastic that the author used) that is in close contact with surrounding temperature can still result in the formation of pores that averaged to be 140 µm for 1% and 90 µm for 2% (w/v) CS. This supports the idea that the conductance of materials can have significant effects on the rate of freezing and pore formation.

5.1.2 Secondary Scaffold Manufacture
In order to generate a porous scaffold, freezing conditions (such as temperature and duration), and container shape were altered. The development of a porous scaffold was successful as indicated by data, however, scaffolds were still mechanically weak and lacked bioactivity.

5.1.2.1 Freezing Studies
Findings as shown by Figure 4.4 indicated that freezing scaffolds at -22 °C resulted in a porous architecture and that -80 °C resulted in a layered architecture. Increasing the freezing time appeared to elongate pore diameter and increase the extensity of fissures and layers.

Freezing scaffolds at -22 °C in concordance to a mass transfer governed system (temperature greater than -50 °C) resulted in spherical ice crystal growth and thus, once freeze-dried we were left with pores (60). This means that freezing at higher temperatures and/or providing a small thermal gradient caused pore formation. Several studies indicated that freezing at -20 °C resulted in a porous architecture (59,71–73). Between these studies and the author’s, similarities in scaffold preparation to post-freeze-drying include CS concentrations, AA concentrations, and temperatures of freezing. Differences include additives (calcium nitrate, ammonium phosphate) to produce HA in situ, and treatment by NaOH post-freeze drying to neutralize the AA. From this evidence we can suggest that temperature is the main determinant in whether layers or pores form.
Increasing freezing times appeared to elongate pore diameters as shown by Figure 4.5. This indicated that pore dimensions are dependent on freezing time and results in the formation of pores that are not a sphere-like shape. In a study by Cooney et al., freezing beyond a certain time point resulted in pores that became longer in diameter that also became less interconnected. Pawelec, Husmannm Best & Cameron suggested that a longer freezing duration resulted in the preferential growth of large ice crystals due to the constant remodelling and annealing of ice (61). A possible explanation for elongated pores may be due to the thermal gradient. Instead of ice forming in the direction of the gradient ice may remodel in this direction resulting in elongated pores. Pores should be measured at more freezing points to determine if there is a point at which pores begin changing dimensions, and whether freezing should be halted and the scaffold be put through immediate freeze-drying. Additional study should look into the geometry of the pores and the influence of this on live cells. It was found by Bidan et al. that cross shaped pores resulted in twice the amount of ECM deposition compared to square shaped pores (74). A study by Ripamonti illustrated the effects of highly crystalline biomimetic matrices on bone formation. It was demonstrated that a specific geometry induces the formation of bone by the anchorage of endogenous BMP and other growth factors (75). Looking into the effects of ovoid pores and other geometries on cells should be investigated as these can optimize osteoid production.

At 72 hours of freezing time, the author’s scaffold showed elongated pores when frozen at -22 °C, whereas Cooney et al.’s scaffold had a layered architecture at -20 °C. Both samples were of similar volumes but different shapes. The author’s scaffold was cylindrical; Cooney’s was a rectangular prism and therefore, had a greater surface area for heat transfer. Cooney’s scaffolds were also made in metal molds adding to the propensity of a higher heat conductance. The greater surface area and metal molds may explain the reasoning as to why layers were present when Cooney’s scaffold was frozen at -20 °C. This further supports the notion that small thermal gradients or slow heat transfer results in pores (60).
5.1.2.2 Container Studies

Figure 4.6 indicated that the container the CS solution was frozen and freeze-dried in had no influence on pore formation. Different containers frozen at -22 °C and -80 °C showed the characteristic porous and layered architecture respectively. The sample frozen in a transfer pipette was expelled during the freeze-drying process and could not be used for analysis.

In contrast to the author’s study, a study by Madihally and Matthew demonstrated the production of radially oriented pores that were larger and elongated towards the center, with smaller, interconnected pores in the periphery when scaffolds were produced in cylindrical containers. Scaffolds that were produced in a shallow square dish resulted in uniform and perpendicular pores to the container dimensions (72). This demonstrates that the thermal gradients acting on the container play a role in pore shape. The three-dimensional nature of the thermal gradients must be taken into account, as heat is lost from the center to the periphery, and therefore the scaffold will not be homogenous throughout. Similar to the peripheral/central structural differences in the cylindrical container by Madihally and Matthew, freezing scaffolds (as previously discussed) at -80 °C produced a peripheral porous material and a layered center. It is possible that pores formed initially and elongated substantially to become layers when exposed to strong thermal gradients or for long periods of time.

The limitation exists in the author’s study, that SEM imaging only takes a single view at one location and therefore, will not be able to detect patterns in pore architecture. The author suggests that SEM analysis be performed from the periphery to the center of an entire freeze-dried sample in order to see if there is a pattern in pore formation.

5.1.3 Chitosan and Hydroxyapatite Scaffold Manufacture

5.1.3.1 Micro-Hydroxyapatite

Micro-hydroxyapatite (μHA) was added to produce a composite scaffold with CS that is bioactive and has enhanced structural integrity. Figure 4.9 indicated that the addition of μHA resulted in reductions in pore diameter, the development of layers and reduced structural integrity. At minute amounts, μHA saturated the solution and excess settled on the well floor. Very little HA dissolved with the excess floating in
solution or settling, so once freeze-dried the little that have dissolved precipitates with the floaters and settlers drying out and dispersed among the chitosan. At very large amounts, the freeze-dried scaffold was friable and collapsed with agitation.

The addition of increasing amounts of µHA resulted in reductions of pore size. This means that there was a limit on the amount of µHA you can add before pore sizes were inappropriate. However, we were not able to reach this limit as the structure instead became layered and weak. Comparable to Cooney et al., the addition of solutes may have altered thermal conductivity and viscosity resulting in smaller pores. A possible explanation of these layers may be, once again, the altered thermal gradient, that converted pores to a layered structure.

As bone is comprised mostly of minerals (~70%), it was thought that higher concentrations of HA could be used. However, 4% µHA and above when combined with 1% (w/v) CS resulted in friability of the scaffold as demonstrated in Figure 4.10, where the absence of pores was due to a broken sample. MacKenzie demonstrated that the solute matrix collapses during freeze-drying due to water vapor damaging the structural integrity. This indicated that there are no interconnections between layers or pores in the scaffold and therefore is incompatible with tissue engineering (76). However, bone is also comprised of an organic component (30%). Therefore, a higher amount of CS may be necessary to form the solute matrix to support µHA. Other studies involving collagen have used up to 60% collagen and 40% (w/v) HA, 30% collagen and 70% (w/v) HA, and 5% collagen and 70% (w/v) HA and exhibited no friability (77,78). This indicated that collagen might be of superior structural integrity to CS. Future work may involve the addition of collagen to chitosan and hydroxyapatite scaffold to improve the inherent weak strength.

5.1.3.2 Nano-Hydroxyapatite

Results from Figure 4.12 indicated that the produced composite scaffolds (1% CS with varying nHA) were porous. These scaffolds had a higher structural integrity than previously made scaffolds made with µHA.
Compared to µHA, nano-hydroxyapatite (nHA) particles are of the nano scale and
have a greater surface area to volume ratio (79). Pore measurements ranged 46.0 –
223.2 µm, it was expected that the addition of nHA would reduce pore diameter, but
this was not obvious with SEM analysis. This means several things: further nHA can
be added without the reduction of pore diameter, additional imaging studies are
needed, or nHA is insoluble with very little dissolution of ions into acidic solution
(compared to dissolved chitosan that when increased, pore diameter would reduce).
There was a faint white surface on the base of the well under where a sample has been
taken (Figure 4.11) and a small layer of nHA on the base of every scaffold. This
indicated that calcium and phosphate has saturated the solution with the excess nHA
settling to the bottom of the well. Other studies have shown greater amounts of added
HA, a 2% (w/v) CS and 12% (w/v) nHA scaffold was prepared in situ by Kong. However, pore sizes were 20 – 60 µm ranges and deemed unsuitable for cell culture
(59). Several studies show that nHA added to CS does not influence the porous
structure or significantly affect porosity. Instead, only the microscopic morphology of
the CS surface was different, where nHA particles were “scattered like islands”
homogenously throughout (71,79). This means that further nHA can be added without
changing the structure. However, the settling of nHA at the bottom of the well
indicates rapid saturation of the solution by calcium and phosphate ions and/or that
nHA is not very soluble. This layer of nHA would be wasteful.

Biopsy sampling was easily made with no friability exhibited and is demonstrated in
Figure 4.11. Previous studies have shown that addition of nHA to scaffolds resulted in
an increased compressive modulus (79). This further supports the use of HA as part of
the composite scaffold. Combined with this improved structural integrity, nHA is
similar in size to HA found in bone and shows significant increases in protein
adsorption and cell adhesion. Therefore, nHA is a suitable ceramic for the production
of CS composites.

Future work should look into introducing nHA into solution that allows significantly
greater amounts to dissolve and integrate with CS. This may involve in situ
precipitation, increasing temperature, or increasing the available surface area of HA.
Once a suitable scaffold has been finalized, mechanics testing should be investigated
to compare compressive and tensile strength to that of bone.
5.1.4 Macroconcentrations of Chitosan

Our findings in Figure 4.8 suggest that increasing CS concentration to 10% (w/v) and above results in pore diameters beginning to fall below 100 µm. Increasing CS concentration produces dense, foam-like scaffolds with higher structural integrity to what was made previously.

This indicated that the methods used to freeze and freeze-dry scaffolds allowed the use of higher concentrations of CS. The majority of current literature used chitosan of concentrations 1, 2, 3 g/100 mL or percent (w/v) as further increases in CS results in reductions in pore size. (56,59,60,72). However, a study by Jana et al. developed scaffolds up to 12% (w/v) CS. The differences between this method and the authors were centrifuging prior to freezing to remove air bubbles, freezing the solution in 10 mL syringes at -20 °C for 24 hours, and lyophilization at -89 °C until dried. This resulted in highly interconnected porous scaffolds with pore size ranging from 100 – 500 µm, porosity of 86.1% at 12% (w/v) CS and high structural integrity (73). However this scaffold lacked bioactivity and needed the addition of HA. Jana et al. emphasized the duration of CS dissolving in weak acid prior to freezing (similar to the author’s, which was at least 24 hours) as protonation of the amine groups of CS was difficult to occur completely. This allowed protonation to increase with time and resulted in rigid CS chain conformation and viscosity of solution, and hence high structural integrity of the scaffold. Cooney et al. showed that increasing CS concentrations did not significantly affect pore structure even though the scaffold was denser. This suggested that ice crystal growth was not affected by CS concentration at -20 °C. However, this observation occurred at 1, 2, and 3% (w/v) CS hence there might be significant changes at higher concentrations (60). The clinical implications of being able to use high concentrations of CS may allow the development of a high-strength bone graft substitute that may be able bear weight or maintain its integrity in vivo and therefore expanding the use of CS as a bone tissue engineering material.

Additional studies around manipulation of CS concentration, freezing temperature and time, and lyophilization conditions might help determine the optimum conditions for appropriate pore size and porosity. It is recommended that repeating a 12% (w/v)
CS scaffold using the method developed by Jana et al. with nHA for increased bioactivity and to determine nHA effects on pore size.

5.1.5 8% (w/v) Chitosan Scaffolds with Macroconcentrations of nHA

Now that scaffolds with appropriate pore size and structural integrity have been produced, bioactivity can be increased by the addition of nHA. Composite scaffolds were made based on the 8% (w/v) CS scaffold as pore sizes were well above 100 µm. So when nHA was added pore sizes could reduce yet still be above 100 µm. SEM imaging revealed that all nHA concentrations resulted in pore sizes that appeared to be above 100 µm.

SEM data as shown in Figure 4.13 indicated that the addition of nHA (2.5 – 5% (w/v)) to 8% (w/v) CS solution resulted in pores that ranged from 22.7 – 414.3 µm in diameter. This means that the added nHA appeared to have no influence on pore diameter, supporting the above notion in 5.1.3.2.

This may be due to the limited dissolution of calcium and phosphate ions in acetic acid, so nHA remains immiscible and suspended in solution. In comparison to dissolved chitosan in acetic acid, increasing concentration resulted in reduced pore diameters. During preparation of the scaffold in the mixing step, nHA was suspended in solution causing it to become pale or it would settle to the bottom, indicating nHA is not very soluble. After freeze-drying a layer of white substance accumulated at the base of each well and suggests further settling of HA during the freezing process. This was a 1-2 mm thick and further indicates that nHA does not dissolve fully.

Additional study can investigate the actual weight of nHA in the scaffold (not including the white layer base) and whether nHA dispersion is homogenous throughout. Modifications to the protocol must be made to ensure homogenous dispersion by altering the viscosity of CS to keep nHA suspended or increasing solubility.
5.1.5.1 Pore Diameter Analysis By Scanning Electron Microscopy of 8% (w/v) Chitosan and 5% (w/v) Nano-Hydroxyapatite Scaffold

As shown in Figure 4.15 and Table 4.1, it was found that the average pore diameters were above 100 µm in all 3 samples, with 95% confidence intervals as follow: 97.13 – 113.4 µm, 99.29 – 117.9 µm, and 97.33 – 110.9 µm for samples 1, 2 and 3 respectively. The smallest pore diameter measured was 55.35 µm and the largest was 176.4 µm. There was no statistically significant difference found between pore diameters in the 3 SEM image samples. This indicated that the average pore diameter is suitable for tissue culture in 3D, however, even though the average diameter is suitable for cell culture, some pores are well below the appropriate diameter and therefore will not be suitable for cells.

Figure 4.15 indicated homogeneity between samples and therefore throughout the scaffold, as pore diameters were not statistically significantly different from one another. However, bias ensued due to the selection of SEM images, where clear pore outlines were necessary for accurate measurement. Other images taken for pore analysis had obscured pores due to unsuitable sectioning or walls collapsing inward. These unsuitable images may represent variation in pore sizes that deviated from those measured. As measurements were taken from one plane through a pore, it is unknown whether the measurement was taken at its largest or smallest axis and therefore will provide an inaccurate measurement of the largest pore diameter. There was also selection bias of pores for measuring. While care was taken to measure all available pores, very small ‘pores’ were not measured, as they may be artifacts from image processing. By comparing the original and processed image, it is evident that converting a 3D image to black and white binary produces false pores. So selection criteria involved restricting pores less than 50 µm to decrease this bias.

The use of automated imaging software for pore size was not pursued as analysis resulted in incorrect data, as pore diameter measured was an average of 10 µm that clearly does not correlate to the large pore sizes found on SEM and µCT imaging. As pore sizes are varied and are not all above 100 µm, this may negatively impair cell growth. Although the average is acceptable, there are several pores that will not be involved in cellular proliferation and migration. Therefore this would result in non-homogenous cell growth and ECM (osteoid) production throughout the scaffold.
However, as the scaffold degrades in vivo the smaller pores will coalesce to form larger pores that will be optimal for cell migration, fluid transport, neovascularization and osteogenesis.

μCT imaging may be improved if recorded using high camera pixel settings to improve the definition of pore walls. Freezing of scaffolds may provide another way of processing for image analysis, to improve pore wall strength during sectioning. Depending on availability, it is recommended to use the widely used mercury intrusion porosimetry as a way to calculate pore size and porosity (59,73). Modification of the scaffold may need attending to in order to increase the size of all pore diameter to be above 100 µm.

5.1.5.2 Porosity Analysis By Micro-Computed Tomography of 5, 8 and 10% (w/v) Chitosan and 5% (w/v) Nanohydroxyapatite Scaffold

SkyScan software analyzed the interconnectivity between pores and was 69.6% in 5%CS/5%nHA (w/v), 79.0% in 8%CS/5%nHA (w/v) and 75.2% in 10%CS/5%nHA (w/v) as shown in Table 4.2. The trend in Table 4.2 showed an increase in porosity from 5 to 8% then reduced when increased to 10%. These results suggested that the porosity of both 8 and 10% (w/v) CS was appropriate for tissue engineering. As cancellous bone had a porosity 70% or greater, allowing adequate nutrient and waste exchange we deemed any porosity comparable to this suitable (80). A possible explanation for why 5% (w/v) CS had a low porosity may be freezing times. Although all scaffolds were frozen for at least 24 hours, freezing times may vary for each batch (frozen longer than 24 hours). As explained before, lengthened freezing time may cause greater interconnectivity but beyond this interconnectivity will decrease as ice crystals anneal (61). Work by Jana et al. opposed the porosity trend demonstrated by the author’s study. The study showed high porosities for all samples that decreased as CS concentration increased. This suggested that the temperature of -20 °C and duration of freezing of 24 hours was ideal to provide highly interconnected pores (73).

Strengths of the porosity analysis by μCT included the analysis of an entire sample as μCT provided approximately 1000 images. However this sample was only 3 mm and
came from one region of the prepared sample in a well plate. This may have porosities different from other locations in the well. Limitations included the processing of the images to calculate porosity. Processing involved conversion to binary and involved altering pore wall boundaries slightly to remove artifacts. This may have provided inaccurate porosity measurements, as some continuous walls may break and be detected as interconnected. The limitation exists that only 1 sample of each CS concentration was analyzed, therefore we could not do any statistical analyses. Additional study can investigate what freezing temperatures and times result in optimal porosity. As stated in 5.1.5.1, mercury porosimetry may be of use to calculate porosity.

5.1.6 Scaffold Characterization

5.1.6.1 Scaffold Rehydration and Stabilization

After freeze-drying we were left with precipitated polymers of chitosan acetate that when introduced to aqueous media, will liberate acetic acid and re-dissolve the scaffold. Ethanol wash series removed acetic acid without impairing structural integrity or pore structure. Initially a few particles were visibly floating in solution. The final wash in distilled water resulted in an intact scaffold with no dissolving. SEM analysis as shown by Figure 4.16 revealed no observable differences in structure and indicates an ethanol wash series is suitable to remove aqueous acetic acid.

There was a variety of ways to remove acid, including: sodium hydroxide, sodium sulfate, tripolyphosphate and the attempted ethanol series. These deprotonate the amine group on chitosan and promote cross-linking (12). Studies have demonstrated that hydration with NaOH causes some shrinkage and distortion due to alkalinity induced changes to CS crystallinity, and that partial reversal of this change was observed at pH 7 solutions. Using ethanol series caused no significant volume or shape changes, and allows complete hydration. This could provide another way of sterilization using 70% alcohol, and equilibrated using media prior to cell culture experiments (72). Care must be taken not to use concentrated aqueous NaOH treatment and/or alcoholic NaOH with exposure to oxygen as this promoted further deacetylation (57) and cytotoxicity to cells. However, many studies used mild NaOH
as a neutralizer and do not report any structural irregularities (59,71,73). Therefore this indicated that mild NaOH or an ethanol series are appropriate for removing acid.

5.1.6.2 Autoclaved Scaffolds

As shown by Figure 4.17, orange colored scaffolds came out of the autoclave with the nHA still attached to the inferior layer. Autoclaving did not appear to have significant effects on structure with SEM analysis revealing pore sizes to be greater than 100 µm. This means that autoclaved scaffolds may be potential candidates for tissue engineering.

A study by Ji & Shi showed that autoclaving was an efficient method of sterilizing and caused thermal cross-links that increased the compressive modulus of a CS scaffold. A viability and proliferation assay demonstrated that autoclaving resulted in a biocompatibility scaffold with no formation of toxic products. Additionally, pores were homogenously distributed (81). Although the author decided not to use an autoclaved scaffold, the scaffold had potential for tissue engineering as sterilization was efficient and cross-links improved its integrity. Additional study should test cytocompatibility of an autoclaved scaffold with nHA to determine if cell viability or proliferation could be improved and whether it is superior to a non-autoclaved scaffold.

5.1.6.3 Scaffold Degradation and pH Study

Figure 4.19 indicated there was no statistically significant weight change over the incubation period of up to 7 days but there was a significant change in pH over this time period as shown by Figure 4.20.

These results (Figure 4.19) supported that scaffolds do not degrade significantly in vitro or at simulated physiological conditions, and will retain structural integrity at the implantation site. Therefore physiological temperature and buffered solutions did not affect degradation. However, this was not a true representation of a physiological system, as the scaffold will be exposed to mechanical forces of perfusion and cellular waste products that may degrade it. Realistically, scaffolds would be implanted for longer than 7 days and therefore should be in conditions similar to in vivo.
Additionally, mammals do not possess chitosanases and therefore lysozyme is the primary enzyme to degrade the scaffold (82). It may be appropriate to place scaffolds in buffer containing physiological amounts of lysozyme and look into developing predictable degradation rates.

Results (Figure 4.20) from pH measurements indicated a statistically significant difference (Table 4.3) in pH (7.42 lowers to 6.98-7.02) between time 0 and 24, 48 and 168 time points. This implied that hydrogen ions were leached from the scaffold or the buffer reacted with the scaffold to produce acid. A possible reason for the drop in pH was residual acetic acid that was not washed away with the ethanol series. A similar observation was found by She, Zhang, Jin, Feng, & Xu, pH dropped from 7.4 to 6.92 in 1 hour, and then to 6.53 in 24 hours. Differences in the experiment involve silk fibroin instead of HA, and a longer degradation time (8 weeks). Silk fibroin is a widely used fibrous protein in biomedical applications due to its biocompatibility and mechanical properties and is similar to CS hydrophilicity and therefore degradation. Hence it is thought not to be the cause of the pH drop, but the AA (83).

Limitations of this experiment included the use of static culture for scaffolds and small volumes of PBS. In a physiological system, this local acidity would be transported away from the site of production and have minimal effect of the local environment. To mimic this, PBS should be replaced frequently. The small volume of PBS used may have exaggerated the drop in pH as hydronium ions could exhaust the buffer. Larger volumes of PBS may be able to discriminate how likely the acidity is. Acidity must be controlled, a study by Han et al. suggested that acidic environments inhibit osteoblasts and stimulate osteoclasts leading to an osteoporotic environment (84). However, osteoclast function may improve graft incorporation by tunneling into the scaffold and promote neovascularization (9).

Additional study of degradation and pH needs to look into introducing shear stress and media similar to blood to determine if the scaffold can withstand mechanical forces, and whether blood components negatively impair integrity. The use of a bioreactor would provide shear stress however; this was not possible as there were time constrictions combined with the availability of only one custom made perfusion type bioreactor.
5.1.6.4 Swelling Studies

There was a significant difference (Table 4.4) found in weight between 0 and 168 hours in all samples and indicates that scaffolds are swelling and retaining fluid with time (described graphically by Figure 4.21). Swelling behaviour refers to water entering the material, combining with the hydrophilic groups and therefore, is influenced by amount of hydrophilic groups and intermolecular forces (56).

According to Zo et al., swelling kinetics was an appropriate indicator of large pores that are interconnected, representing good nutrient transport. Their results demonstrated a swelling of 94% of its original weight in 120 seconds (85). Jana et al described a scaffold that had a swelling ratio of approximately 30% in 2 weeks. In contrast, the author’s experiment resulted in a mean swelling of 10% over 7 days. This vast difference may be accounted for by the method of scaffold manufacture. The scaffolds manufactured by Zo et al. were 1% (w/v) CS with 1% (w/v) HA, Jana et al. used an 8% (w/v) CS scaffold, whereas the author used 8% (w/v) CS with 5% (w/v) HA. A more concentrated solute should have attracted more water, but less CS may have resulted in thinner pore walls, that can distend and retain more water. Alternatively, the author’s scaffold could have had small pores that were not interconnected. But this was not the case; as SEM and µCT imaging have shown appropriate pore sizes and adequate porosity similar to trabecular bone. It is hypothesized that the mechanical integrity of the author’s CS/nHA scaffold was superior leading to less swelling and deformation.

Additional studies need to look further into the effects of swelling of a scaffold once implanted. This may be beneficial as this ensures surface area is in contact between the scaffold and the implantation site, or harmful if the swollen scaffold increases the local pressure and inhibiting blood flow. It may be necessary to keep the scaffold in fluid prior to seeding with cells to make sure it reaches its maximum swelling. A longer time period for static culture may be required to see if the scaffolds swell further in 1 week.
5.1.6.5 Fourier Transform Infrared Spectroscopy

The Fourier transform infrared (FTIR) spectra of an 8% (w/v) CS and 5% (w/v) nHA scaffold suggested that nHA is incorporated into the structure, due to the presence of $\text{PO}_4^{3-}$ and $\text{CO}_3^{2-}$ absorption band, which infers carbonated HA (37,59). A summary of FTIR peaks and bands can be found below in Table 5.1.

Table 5.1: Summary of Fourier Transform Infrared Spectroscopy Peaks and Bands with Associated Characteristics. Contains typical peaks and bands for chitosan and hydroxyapatite spectroscopy.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak/Band (cm$^{-1}$)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan</td>
<td>1252</td>
<td>NH$_2$ of glucosamine</td>
</tr>
<tr>
<td></td>
<td>1031, 1074, 1155</td>
<td>Pyranose $\nu_3$ stretching</td>
</tr>
<tr>
<td></td>
<td>1606</td>
<td>Amide II</td>
</tr>
<tr>
<td></td>
<td>1650</td>
<td>Amide I</td>
</tr>
<tr>
<td></td>
<td>3420</td>
<td>OH/N-H stretching</td>
</tr>
<tr>
<td>Nano-Hydroxyapatite</td>
<td>962</td>
<td>$\nu_1$ (PO$_4^{3-}$) stretching</td>
</tr>
<tr>
<td></td>
<td>1050-1089</td>
<td>$\nu_3$ (PO$_4^{3-}$) stretching</td>
</tr>
<tr>
<td></td>
<td>1461</td>
<td>$v$ (CO$_3^{2-}$)</td>
</tr>
<tr>
<td></td>
<td>3571</td>
<td>$v$ (OH)</td>
</tr>
</tbody>
</table>

As depicted by FTIR spectra results (Figure 4.22), absorption bands of chitosan peaked around 3430 cm$^{-1}$ due to the stretching vibration of OH and N-H groups (86). The presence of amide I and II peaks (1650, and 1606 cm$^{-1}$ respectively) indicated that the CS used is not fully deacylated (87).
The spectra of nHA showed characteristic peaks $v_3$ ($\text{PO}_4^{3-}$) at 1050-1089 cm$^{-1}$, $v_1$ ($\text{PO}_4^{3-}$) at 962 cm$^{-1}$, $v(\text{CO}_3^{2-})$ at 1459 cm$^{-1}$. A small band was observed at 3571 cm$^{-1}$ that corresponded to $v(\text{OH})$ (86) The band at 1461 cm$^{-1}$ corresponded to $\text{CO}_3^{2-}$ and indicated that these groups have incorporated into the HA crystal structure and replaced $\text{PO}_4^{3-}$ (88).

Additional study should repeat FTIR with a CS only scaffold and a nHA only scaffold. This will allow us to compare the composite scaffold with these and allow us to determine if there is a shift in the peaks, and whether these peaks were a result of formed chemical bond between CS and nHA, or if there is no shift, then the composite is only a mixture. Using FTIR on scaffolds with lower and higher amounts of CS and nHA compared to the composite can determine if some of the CS or nHA peaks are small or absent.

5.1.6.6 Energy Dispersive X-Ray Spectroscopy
As shown in Figure 4.23, the elemental composition of the scaffolds determined by energy dispersive X-ray spectroscopy (EDS) indicated large peaks of sodium and chloride, carbon and oxygen, with smaller peaks for phosphorus, potassium, and calcium. There was the absence of a nitrogen peak. The anomaly for large sodium and chloride could be explained as the scaffolds were kept in storage using saline buffer that contains physiological quantities of these ions, therefore once dehydrated prior to analysis these ions were left in the structure and analyzed with EDS. The presence of various ions may be beneficial for bone tissue engineering. Doreya, Amany, & Sara stated that substitution of silicon and additional carbonate in place of phosphorous improves solubility, and bioactivity can be increased as shown by in vitro and in vivo studies (89). Sang et al. demonstrates that sodium substitution into HA resulted in enhanced osteoconduction (90). Cho, Yoo, Chung, & Rhee show that chloride substitution improved bioactivity and osteoconduction (91).
Additional studies involving ion substitution may be beneficial for these scaffolds, especially determining a way to dissolve more HA into solution. It must be looked into if the residual ions from the buffer can be washed away with fluid or if it indeed has been permanently incorporated into the structure. If ions are washed away, static cell culture on scaffolds that have these additional ions may improve bioactivity until placed in the bioreactor, until perfusion washes the ions away.

It was found that the mole ratio of calcium to phosphorus was 1.92, 1.43 and 1.045 in 3 random regions of the scaffold, suggesting different phases of precipitated calcium phosphate throughout the scaffold. For calcium hydroxyapatite, this ratio is 1.67. These ratios determined what type of calcium phosphate is analyzed (at a specific location by EDS) when combined with molecular formulae. For example, tricalcium phosphate was 1.5, tetracalcium phosphate was 2.0, and octacalcium phosphate was 1.33 (92). This ratio could indicate inferior mechanical properties and other calcium phosphate phases if the ratio was not 1.67 (93). The author’s results suggested that there was variation in calcium phosphate throughout the scaffold, however this was similar to ratios found in bone that range between 1.37 – 1.87 (94) and may be due to the variation of minerals. This variation may be due to heterogenous conditions through the scaffold (concentration, temperature, pH). The presence of trace elements, sodium and chloride may be the variation that caused the differences in calcium phosphate ratios. Nitrogen should have been present as CS was a polysaccharide with an amine group on each monosaccharide unit. The absence of a nitrogen peak may be explained due to low concentrations of CS used or the approach of how EDS was used. It is suggested to trial X-ray diffraction to determine the solid phases within the CS scaffold.

5.2 Cell Culture Experiments

5.2.1 Cell Viability LIVE/DEAD® Assay

As CS was a biocompatible polymer that has increased bioactivity and osteoconduction from nHA, it was expected that the cell line would exhibit excellent viability (89). However, data shown in Figure 4.24 (A) indicated non-optimal viability at 24 and 48 hours (73%) with good viability (87%) at 96 hours. In
comparison to the viability on coverslips, there was no statistically significant difference between them.

As shown in Table 4.5 (blue color), there was a significant difference of viability between 96 hours and both 24 and 48 hours of culture. This indicated that cells were increasing in viability over the course of cell culture. A study by Zo et al demonstrated that a CS and nHA composite scaffold showed excellent viability, with statistically significant differences between scaffold and static culture (85). Another study demonstrated viability of 95% using a 2% (w/v) CS scaffold (95). Reasoning behind the low viability at 24 and 48 hours may be due to poor cell culture techniques prior to seeding, and therefore resulting in higher amounts of dead cells. The increase in viability at 96 hours was likely due to cell proliferation of healthy cells in higher quality conditions and removal of dead cells with media replacement.

As shown by Figure 4.24 (B), total cell number increased with time however this was not statistically significant (Table 4.5, red color) and may be due to low sample sizes (n = 3). Viability and total cell number combined gives a complete view of the cellular response to the scaffold. If for example, viability were 90% this would demonstrate excellent viability. However, if combined with a total cell number of 10 this shows there were very few cells present and represents cytotoxicity (96). Therefore with high viability and total cell number this indicated the scaffold was biocompatible.

The scaffold and coverslip experiments (Figure 4.27) demonstrated no significant differences between viability and total cell number. The coverslip acted as the control and should have demonstrated high viability of cells. This indicated that there was a flaw in cell culture techniques as it was the same in both experimental and control groups. Future work to improve cell viability should look into frequent media changing to remove debris that could be detected as dead by the assay. Cells must also be passaged at 70-80% confluence to ensure fast growth of cells and hence high viability. Additionally, as there was no statistically significant difference in viability between scaffolds and coverslips, this indicates two things. Firstly, the scaffold was non-toxic as it is similar to the glass coverslip and secondly, there was no additional benefit from growing cells on scaffolds.
5.2.2 CellTiter 96® AQueous One Solution Cell Proliferation Assay

Figure 4.28 demonstrated similar absorbances of CS/nHA and collagen, with both increasing with culture time. Triton-X absorbance was very close to zero at all time points. This demonstrated that cells were proliferating in both CS and collagen vehicles and that no proliferation was occurring in Triton-X vehicles.

Collagen is a widely used biomaterial with biocompatibility properties and is suitable for a cell proliferation assay negative control (97), as it will not cause a cytotoxic response. Therefore, as CS and collagen’s absorbances were very similar throughout the culture period (with no statistically significant differences) it is suggested that the scaffold was biocompatible and non-cytotoxic. Triton-X is a detergent and disrupts any membranous structure, preventing proliferation. Hence it was appropriate as a positive control as it will cause a cytotoxic response. The statistically significant differences in absorbance found between CS and collagen, and Triton-X supported that CS and collagen are non-cytotoxic. A statistically significant difference was found between CS and Triton-X absorbance at 0 hours. This may be due to the leeching of CS or nHA into solution, or the slight metabolizing of MTT and therefore altered absorbance values.

The author’s experiment followed a study by Stace and showed very similar results, where CS and collagen showed no toxicity and Triton-X was toxic (39). This further supported that the CS/nHA scaffold was non-toxic and is appropriate for cell culture. Additionally, the data indicated that any steps in the production of the scaffold to post sterilizing supported the suitability for cell culture as no known toxic factors have been introduced, or have been removed during scaffold preparation.

5.2.3 Perfusion Circuit Three-Dimensional Cell Culture of SAOS-2

A limitation of this study was the availability of only one bioreactor. This combined with time constraints limits multiple uses and the inability for statistical analyses. Because of this, 3D culture is compared to 2D static culture on coverslips. Future research should involve the production of several bioreactors. This will allow various factors to be manipulated such as shear stress, seeding density and growth mediums.
5.2.3.1 Histology Protocol

Our work indicated that 3D cell culture of SAOS-2 cells seeded onto a scaffold for 14 days in a bioreactor resulted in the detection of clusters of cells that have proliferated from the surface of seeding to slightly deeper into the scaffold. There were some cells present throughout the scaffold and existed as singular entities. The cells were prominently detected by H&E staining and less by other stains.

As shown by Figure 4.29, the H&E stained individual structures purple throughout the pink CS matrix. As haematoxylin stains acidic structures purple it indicated that nuclei, ribosomes and/or rough endoplasmic reticulum have been stained. This indicated that cells were living prior to fixation as degradation of DNA and nuclei occurred prior to cell death (98). Alternatively, the cytoplasm may not be easily seen due to the nature of sectioning, or the nucleus:cytoplasm ratio may be large just as seen in lymphocytes. Future study should use a portion of the scaffold immediately after bioreactor cell culture for LIVE/DEAD® viability assay to determine if cells are indeed living or dead and will provide insight into how far cells have penetrated the scaffold.

Fluorescent microscopy (Figure 4.30) revealed the yellow auto-fluorescence of eosin stained matrix and green structures that looked like cells (525 nm excitation and 545 nm emission). This indicated that the matrix (which consists of CS and nHA) readily took up eosin as a stain. Similar to Baker et al, the cationic chitosan took up anionic eosin well due to opposite charges, and auto-fluoresced (99). The green structure did not auto-fluoresce like CS, thus it did not take up eosin. This further supported that the green structures were synonymous with the haematoxylin stained purple structures in H&E staining. As eosin has stained the CS and nHA matrix pink (as shown by Figure 4.29 H&E staining) these were the only structures that auto-fluoresce with light microscopy. Additionally, these green structures were not confined to the matrix whereas CS/nHA are. To additionally confirm cells were viable, the scaffold must be analyzed using a LIVE/DEAD® assay immediately after the intended culture period as mentioned above.
A study by Wang et al demonstrated the biocompatibility of $2 \times 10^6$ MSCs seeded onto a polyamide/nHA scaffold using phase-contrast microscopy. This *in vitro* study observed at 3 days that large amounts of cells migrated and proliferated into the pores with no sign of toxicity. Compared to the author’s study, which seeded cells for 24 hours before placement into a bioreactor, this was static culture in a well plate for up to 7 days (100). It may be beneficial for future studies to employ static culture on a scaffold for a longer duration prior to bioreactor usage, to allow cell attachment and proliferation throughout the scaffold before exposure to shear stress, which may have negatively affected proliferation and ECM production in the author’s work. Work by Seol et al. showed that seeding $1 \times 10^7$ MSC cells/g of CS scaffold (3% (w/v) CS, with similar pore diameters to the author) resulted in attachment, and rapid proliferation of cells in 28 days of static culture (101). This further supported the use of static culture of scaffolds prior to bioreactor implementation. Both Wang and Seol seeded MSC cells at a high density compared to the author, 1000-10,000 times larger in comparison to the author’s 6000 seeded cells per scaffold. Work by Holy, Shoichet and Davies suggested that the initial seeding density (ranging from $0.5 - 10 \times 10^6$ cells/cm$^3$) did not significantly affect the formation of tissue, but lower densities delayed the production of mineralization (102). The very low magnitude of seeding by the author may have negatively impaired cell penetration and proliferation, or 14 days culture simply was not enough time for cells to organize and form tissue. It is recommended to seed cells at a $10^6$ or similar magnitude. Future study should investigate the optimal seeding density onto CS/nHA scaffolds for the generation of osteoid tissue, using a cell proliferation assay and histology for analysis.

Another factor involved in cell proliferation in 3D culture is shear stress. Shear stress was introduced as 1 mL/min perfusion through the system. This showed that this rate resulted in viable cells. Studies showed that a 1 mL/min, continuous flow (non-pulsatile) enhanced proliferation, calcium deposition and expression of bone marker proteins (67). Similarly, another study reported that flow rates of 0.3, 1 and 3 mL/min resulted in increased proliferation, alkaline phosphatase and bone marker expression, and mineralization (68). In light of these studies, where this increased proliferation was not observed and indicated that the author’s fluid flow may need to be altered as the bioreactor used was the first of its kind. In contrast, another study showed that 1 mL/min resulted in cell death and low viability and recommended lower flows of
0.01, 0.1 and 0.2 mL/min as they resulted in higher viability, proliferation and expression of DNA and RNA (69). Although 1 mL/min was manually measured this may be completely different to the forces that the cells are exposed to, as there are cross sectional areas that differ in size throughout the circuit. Future study should start at very low fluid flows until tissue has been generated. Shear stress can then be investigated at varying rates to determine the optimal flow that enhances cell proliferation.

Sectioning of the scaffold additionally revealed the porous architecture with a heterogeneous matrix due to fabrication of scaffold by both CS and nHA. Various lightly stained portions within the CS matrix were found in H&E, Masson’s Trichrome (Figure 4.31), Alizarin Red (Figure 4.32), Von Kossa (Figure 4.33) and Van Gieson (Figure 4.34) stained scaffolds. These light areas are indicative of nHA clumps that have dispersed throughout. Although none of these stained specifically for nHA (except for alizarin red), we can speculate this is the case, as there was no light stained areas found in the control scaffold. Alizarin red intensely stained a coverslip of SAOS-2 cells, whereas the staining was not as intense in the CS/nHA scaffold. A possible reason for this reduction in staining intensity of mineralization on a scaffold section was likely to be the decalcification step during microtome sectioning, which was required due to destruction of the sections by the microtome.

The porous architecture of the scaffold was evident by histological staining and microtome sectioning. This further supports SEM and µCT analysis that have determined this also. In all histological images of the scaffold, there were noticeable thick and thin walls in the CS matrix with no noticeable pattern. This means that the process of scaffold generation produced irregular walls. Compared to a scaffold by Stace, who used 1% (w/v) CS and 0.25% (w/v) nHA to prepare the composite, there were only thin walls with no clumping (39). This suggested that the nature of these clumps was due to high concentrations of CS/nHA that may not have been mixed sufficiently with AA. Additional study needs to look into a way for the homogenous distribution of CS and nHA. This may involve looking into the mixing of solutes and freezing conditions as mentioned above in 5.2.1.
An unexpected finding occurred during 3D culture. At 7 and 14 days of culture, pH was measured after replacing the media. The media increased from 7.4 to 7.62 on both occasions. This indicated that acidity was lost or alkalinity was added to the media during cell culture. A study demonstrated that DNA and collagen synthesis increase, but a reduction in ALP expression at a slightly alkaline pH (103,104). This may negatively impair the mineralization of the osteoid tissue. Kaysinger demonstrated that a pH of 7.0 – 7.6 was a suitable range for osteoblast support (104). As the pH is just above this range there should be no significant effect on cells. The cause of this pH change could be reduced cellular metabolism or diffusion of gases through the tubing. As cells were present, this eliminated metabolism as a factor, although they may have lowered metabolism, as they were not proliferating. The tubing, which passed out of the incubator to the peristaltic pump, was in contact with a very low atmospheric CO₂. As this tubing allowed gas exchange CO₂ will be lost and therefore a loss of acidity. Future use of the bioreactor should limit the length of tubing outside of the incubator, a change of non-permeable tubing for outside the incubator and increasing the length of tubing inside the incubator for CO₂ equilibration.

5.2.4 Bone Marrow Culture

The results of bone marrow culture showed a heterogenous cells population of spindle and flat cells (Figure 4.35). It appeared that passaging cells reduced their ability to proliferate and/or become senescent with an increase in the ratio of flat:spindle cells. Further investigation of these cells for differentiation down the osteoblastic lineage and subsequent 3D culture was unable to be attempted due to insufficient cells.

The cell culture of bone marrow resulted in a heterogeneous cell population that was evident prior to the first passage. This indicated that bone marrow contained several phenotypes or morphologies of cells. A study by Neuhuber et al. demonstrated 6 morphologically distinct cell sub-populations after the culture of Fischer rat marrow. 3 subpopulations showed elongated, ‘spindle’ shapes while the other 3 had ‘flat’, polygonal morphologies with little or no processes. The author’s study showed both these populations in vitro. Alternatively, the culture could be contaminated with non-stem cell populations from marrow. However, mesenchymal stem cells (MSCs) are
differentiated by other cells by their ability to adhere to tissue culture plastic, and therefore non-MSCs should be removed by media replacement (42). Implications of this heterogeneity are the ability of these cells to proliferate and differentiate. Spindle cells had faster population doublings compared to the flat cells, and flat cells had a reduced ability to differentiate down the chondrogenic and adipogenic lineages (105). During cell expansion, it appeared that passaging prior to 70-80% confluence reduced the ability for cell proliferation and caused a relative decrease of spindle cells or increase in flat cells. This showed two things; firstly, detachment by trypsin may have negatively influenced cell division if done prior to, or during, early log phase. And secondly, spindle cells are lost, transformed to flat cells, or the flat cells are preferentially grown during a passage. A study by Bonab et al. showed that cells begin senescence and lose stem cell characteristics the moment in vitro culture began (106). As a result, cells must be used as soon as possible for experiments and expanded as little as possible. As an adult Wistar rat was used for its bone marrow it may have adverse of stem cell culture as mentioned above. A clinical consequence may be the use of elderly mesenchymal stem cells that have reduced proliferation and therefore a higher chance of cell culture failure. Therefore, implementing these stem cell technologies may require the harvesting of cells at a young age that are cryopreserved for later use in life.

Also shown by Neuhuber, it was found that the ratio of spindle to flat cells decreased over time and suggested that spindle cells are ‘immature’ and flat cells are ‘mature’ stem cells (105). This supports the author’s study, as spindle cells decreased with passaging, or time in general. It is possible that the spindle cells ‘differentiate’ into flat cells with time when stimulated by in vitro experimentation. Both spindle and flat cells could be seen individually or in clusters and showed that their proliferative potential still exists. A consequence of more flat cells was the reduced ability to differentiate down adipogenic and chondrogenic lineages. However, this did not apply to the field of bone engineering. Therefore bone marrow cells require less stringent protocols for cell culture.

Future studies should look into the optimization of stem cell proliferation. A modification to the protocol by Stace will be to not passage cells after 3 days of marrow isolation and instead should let cells become confluent to 70-80% and then
passaged 3 additional times. Additionally, following a protocol established in the Dias Lab, cells can be grown in plastic dishes after isolation, this will allow easy access for a cloning cylinder that will be placed on top of a proliferating spindle shaped colony, which can undergo subsequent detachment with trypsin. The cells can then be aspirated and resuspended to grow pure spindle-shaped cells. Confirmation of stem cells should use flow cytometry, using MSC markers such as CD44, CD1460 and STRO-1.

6 Conclusions and Future Directions

6.1 Scaffold Production
It was found that the development of pores was temperature dependent, -80 °C will not result in a porous structure, but form layers. 22 °C was found to be appropriate for pore development. Additionally, freezing conditions such as duration can influence the porous geometry. The use of hydroxyapatite to form a composite scaffold with chitosan exhibited favor to that of the nano scale, as a larger amount could be used without causing the scaffold to become friable or form layers. In contrast to the majority of literature, 8% (w/v) chitosan was used to produce a scaffold with adequate pore diameters, porosity and structural integrity. The structural parameters were maintained with the addition of 5% (w/v) nano-hydroxyapatite with average pore sizes above 100 µm and a porosity of 79%.

6.2 Scaffold Characterization

Scaffolds that were rehydrated by ethanol series did not show any significant structural changes and could be immersed in aqueous media without dissolving. Autoclaving demonstrated an effective way of sterilization with no change in the porous architecture other than a physical change in color. Scaffolds did not degrade significantly in vitro but the local pH surrounding scaffolds reduced from 7.4 to 6.98. It was shown that scaffolds swelled approximately 10% after immersion in aqueous solution. Fourier transform infrared spectroscopy showed that hydroxyapatite was incorporated into the chitosan scaffold. Energy Dispersive Spectroscopy demonstrated the presence of chitosan and hydroxyapatite, with the presence of high chloride and sodium peaks due to storage of scaffolds in saline.

6.3 Cell Culture Experiments

The LIVE/DEAD® assay showed that Osteogenic Sarcoma cells (SAOS-2) were viable with no significant difference compared to 2D culture on glass coverslips. A CellTiter 96® MTS proliferation demonstrated that cells proliferated when exposed to scaffold vehicles. Both assays demonstrated that the scaffold was biocompatible and did not contain any cytotoxins. 3D culture of SAOS-2 cells on a scaffold for 14 days at a flow rate of 1 mL/min resulted in the appearance of cells within the scaffold. These cells were concentrated at the cell seeded end with few dispersed deeper
through the scaffold. Histology showed the presence of cells and additionally demonstrated the porous nature and the incorporation of hydroxyapatite in the scaffold. Isolation of mesenchymal stem cells proved initially successful with the presence of a heterogenous cell population of spindle and flat cells. The proliferative capacity of these cells reduced with passaging and resulted in senescence.

6.4 Future Directions

There are several areas highlighted by this research for which further investigation should be sought after. Firstly, cells were found on the scaffold after 3D culture, once mesenchymal stem cell proliferation, differentiation, seeding and extracellular matrix production have been optimized the study should proceed into animal trials. Using Lewis rats, we can isolate stem cells and generate a cell-scaffold construct that has been grown in the bioreactor. As Lewis rats are genetically identical, the scaffold can be implanted into 8 mm critical sized calvaria defects without rejection. Here we can compare the scaffold against rats that have calvaria autografts to determine efficacy of bone healing by these synthetic grafts and whether this has been stimulated in critical defects.

Secondly, the use of high concentrations of chitosan to produce a scaffold demonstrates higher structural integrity. The scaffolds should be mechanically tested to measure tensile and compressive strength and/or a nano-indenter to test hardness. Therefore, we can compare and contrast between current bone graft substitutes. As chitosan can be molded into various shapes, this may lead onto the future use of chitosan scaffolds to produce grafts for bones involved with locomotion and weight bearing.
References


5. Sultana N. Biodegradable Polymer-Based Scaffolds for Bone Tissue Engineering. Springer; 2013.


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Appendices

Appendix A – Pore Measurement Supplementary Data

Table A1: Mean pore diameter of 3 SEM images of a 8%CS/5%nHA (w/v) scaffold.

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