Engineering 3-D constructs of human bone matrix in a mechanically-active environment

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A thesis submitted for the degree of
Doctor of Clinical Dentistry (Orthodontics)

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Date: 19th August 2016
“Do not assume anything and find out for yourself by doing the experiment”.

Professor M.C. Meikle’s counsel upon my commencement of this research project, paraphrasing John Hunter in his letter to Edward Jenner

“I think your solution is just, but why think? Why not try the experiment?”

August 2, 1775.
Acknowledgements

I would like to thank Professor Murray Meikle who gave me the opportunity to do a project that was both challenging and rewarding. He supervised me from Cambridge, UK but he never felt too far away with prompt e-mail responses. I will always remember his advice, which was a constant reminder throughout this research: “Do not assume anything and find out for yourself by doing the experiment”.

Doctor Trudy Milne, my NZ – based supervisor, is the most dedicated and supportive supervisor one could possibly wish for. Trudy taught me how to think scientifically, as well as practical laboratory skills like tissue culturing techniques. Trudy planted in my heart a strong interest in research, for many years to come. Thank you, Trudy for all your support.

Professor Mauro Farella inspired and encouraged me to undertake this research project with Professor Meikle. His ongoing support for my constant pursuit for more knowledge and inspired me to ask questions about all aspects of research and orthodontics in general. He loves every student and wants the best for all of us. His encouragement for me to go onto a PhD may come true!

I am grateful to Professor Cannon for his guidance, encouragement, editorial support, attention to detail and his dedication. Your help has been invaluable.

I don't think this research would have been possible without the technical support of FlexCell® engineers, who answered countless questions. Thank you Aisley Amegashie and the team.

Thank you to Andrew McNaughton from University of Otago Microscopy, with his expert guidance on confocal microscopy.
An enormous thank you is also dedicated to Penny and Carol from the ADA library in Australia. I think I have been the most challenging post-graduate student they have ever had, constantly asking for original publications and rare textbooks that have been scanned and e-mailed to me over the past three years, all with a smile.

My sincere gratitude goes to the NZ Dental Association Research Foundation for their financial support.

Last but not least, the past three years have been most challenging for my husband Max, who endured the sacrifice of our family life being put on hold for three years, while always encouraging me to pursue my career as an orthodontist. He supported me through this research both emotionally and with advice, and came to visit me many times in Dunedin over the last three years. Thank you for being there for me.
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<th>Description</th>
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<tbody>
<tr>
<td>2-D</td>
<td>Two Dimensional</td>
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<tr>
<td>3-D</td>
<td>Three Dimensional</td>
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<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
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<tr>
<td>ARF</td>
<td>Apposition-Resorption-Formation</td>
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<tr>
<td>B2M</td>
<td>Beta-2-Microglobulin</td>
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<tr>
<td>BLGLAP</td>
<td>Bone Gamma-Carboxyglutamate Protein</td>
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<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>CaAM/EthH</td>
<td>Calcein Acetoxymethyl /Ethidium Homo-Dimer-1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary Deoxyribonucleic Acid</td>
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<tr>
<td>DBM</td>
<td>Demineralised Bone Matrix</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>FDA/PI</td>
<td>Fluorescein Diacetate/Propidium Iodide</td>
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<tr>
<td>GAG</td>
<td>Glicosaminoglycans</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>HA</td>
<td>Hyaluronan</td>
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<tr>
<td>HAP</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HFO</td>
<td>Human Femoral Osteoblasts</td>
</tr>
<tr>
<td>HCO</td>
<td>Human Calvarial</td>
</tr>
<tr>
<td>kPa</td>
<td>Kilo Pascals</td>
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<tr>
<td>MPa</td>
<td>Mega Pascals</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
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<tr>
<td>μL</td>
<td>Microliter</td>
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<tr>
<td>mL</td>
<td>Milliliter</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>OCN</td>
<td>Osteocalcin</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PGA</td>
<td>Polyglycolic-Acid</td>
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<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
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<tr>
<td>PEGDA</td>
<td>Polyethylene Glycol Diacetate</td>
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<tr>
<td>PEO</td>
<td>Polyethylene Oxide</td>
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<tr>
<td>PLA</td>
<td>Polylactic-Acid</td>
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<tr>
<td>PLGA</td>
<td>Polylactide-Co-Glycolide Acid</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RT-qPCR</td>
<td>Reverse Transcription Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor- β</td>
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<tr>
<td>w/v</td>
<td>Weight/Volume</td>
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The thesis is divided into six chapters:

**Chapter 1** provides an introduction and summary of the aims of the project. The limitations of currently available bone scaffolds are reviewed.

**Chapter 2** reviews what is currently known about human calvarial and femoral osteoblasts and the process by which they mineralise their extracellular matrix when cultured *in vitro* as a monolayer (2-D). Human calvarial osteoblasts (HCOs) and human femoral osteoblasts (HFOs) were cultured in monolayers in this chapter providing an insight into the proliferation and mineralisation characteristics of these cells prior to utilising 3-D hydrogels.

**Chapter 3** reviews the literature with regard to hydrogels as a 3-D culture system. The benefits of using a semi-synthetic hyaluronan-gelatin-PEGDA cross-linked hydrogel are introduced. Experiments carried out in this chapter focus on validating assays to determine the proliferation and mineralisation rates for HCOs and HFOs grown in a hydrogel. The effects of increased cross-linking and stiffness of the hydrogel culture medium on cell morphology and proliferation were examined. Based on these findings, a suitable hydrogel model was chosen for use under mechanical compressive strain in the next chapter.

**Chapter 4** reviews the importance of a mechanical load for bone formation. The experiments undertaken in this chapter look at the suitability of the model to determine the effect of compressive intermittent strain on the expression of osteogenic genes.

**Chapter 5** is a general discussion overviewing the results. Recommendations are made to aid the future development of assays suitable for 3-D hydrogels and how to improve the hydrogel substrate for optimum osteoblast growth under mechanical load.

**Chapter 6** is an appendix consisting of protocols that were specifically developed for culturing osteoblasts in hydrogels.
Abstract

**Background:** A biocompatible, osteoconductive and osteoinductive framework for ingrowth of host cells that can mimic the remodelling behaviour of normal bone matrix would be of significant benefit to the tissue engineering field. A solution would be to create a mineralised matrix, an engineered ‘off the shelf’ bone replacement material, containing the multitude of growth factors that would facilitate its integration into bone and induce bone regeneration. Mechanical loading is an important determinant of bone mass and architecture, and has been shown to promote osteogenic differentiation *in vitro*. A suitable scaffold for culturing osteoblasts must therefore have the strength to withstand mechanical stimulation over extended periods of times. The present study explores the suitability of semi-synthetic hyaluronan-gelatin PEGDA cross-linked hydrogel for culturing of calvarial and femoral osteoblasts. This semi-synthetic material has a close resemblance to the extracellular matrix, and offers the opportunity to optimise its bio-mechanical characteristics through modification of hydrogel cross-linking.

**Aims:** 1) To develop a 3-D hydrogel cell-culture model for engineering artificial mineralised bone matrix *in vitro*; 2) Validate assays for the measurement of osteoblast proliferation and hydroxyapatite deposition; 3) Examine suitability of the model for the study of mRNA expression under mechanical strain.

**Methods:** Human foetal calvarial osteoblasts (HCO) and femoral osteoblasts (HFO) were cultured in thiol-modified hyaluronan-gelatin-PEGDA cross-linked hydrogel. Two degrees of cross-linking were used and cell proliferation and hydroxyapatite deposition was quantified over a 21-day culture period. Confocal microscopy was also used to assess the penetration of the hydrogel by the assay dyes. An enhanced cross-linked hydrogel was used for mechanical strain experiments. Quantitative polymerase chain reaction (qPCR) was used to measure mRNA expression of alkaline phosphatase (ALP), osteocalcin (OC) and bone morphogenetic protein 2 (BMP2) genes.
**Results:** Cell proliferation and hydroxyapatite deposition increased for each cell line over 21 days. The highly cross-linked hydrogel was found to be a better scaffold for osteoblast attachment. Confocal microscopy showed assay stains had limited hydrogel penetration. A cyclic compressive mechanical load did not affect ALP expression in either the HCO (p=0.243) or the HFO (p=0.132) but it caused a 53.9-fold increase in BMP2 in the HFO group (p=0.0043). No OC mRNA was detected in either group.

**Conclusion:** Thiol-modified hyaluronan-gelatin-PEGDA cross-linked hydrogel is a suitable scaffold for the study of osteoblast proliferation and hydroxyapatite deposition *in vitro*. Further experiments are required to assess the differences in the responses of calvarial and femoral osteoblasts to mechanical strain.
Chapter 1
The need for an ideal bone graft material.

1.1 Introduction and aims

The need for bone tissue engineering arises from a limited ability of native bone to regenerate a defect beyond a certain size. To date no ideal replacement graft has been developed. Successful engineering of a bone matrix with osteoinductive properties has the potential to alleviate, what some have called, a world crisis in bone tissue transplant shortage (Drury and Mooney, 2003; Stevens, 2008). Creating a suitable bone substitute has become one of the top priorities in the tissue engineering field (Hacker and Mikos, 2006).

An autograft, or bone from the same individual from a different donor site, is the biologic gold standard against which the biocompatibility of all new graft materials is compared. However, an autograft is associated with the high biologic cost of morbidity related to a second operation, the loss of tissue at a donor site, and a limited quantity of available tissue. The term allograft describes the use of tissue harvested from a different individual of the same species, while the term xenograft describes graft material, which is harvested from a different species. A number of synthetic materials have also been trialled as substrates for bone grafting but this has been met with limited success as they lack the properties of an ideal graft (Cypher and Grossman, 1996). These properties include osteointegration, which is the integration of the transplanted graft with no biological consequences; osteogenesis, or the formation of new bone by the live cells contained within the graft; osteoconduction, which is the ability of the graft to act as a framework enabling ingrowth of new bone on the graft surface; and osteoinduction, the most challenging property to achieve, which involves induction of surrounding mesenchymal stem cells to differentiate into bone forming osteoblasts. The evidence suggests that bone morphogenetic proteins play a significant role in the property of osteoinduction (See Section 1.2).

The field of tissue engineering would benefit from the creating of a
biocompatible, osteoconductive and osteoinductive framework for ingrowth of host cells that can mimic the remodelling behaviour of normal bone matrix. A possible solution would be to create a mineralised matrix containing the multitude of osteogenic and other growth factors that would induce surrounding bone to regenerate itself (Hauschka et al., 1986; Urist and Strates, 2009; Wozney et al., 1990). In other words, a bioengineered ‘off the shelf’ bone replacement material (Meikle, 2007).

Bone growth in vivo is appositional occurring through the addition of new layers to previously formed mineralised layers. The new layers require a scaffold within which osteoblasts can deposit a mineralised matrix, growth factors that promote osteoblast proliferation and mineral deposition, and a mechanically active environment. In vitro, appositional growth could potentially be reproduced by culturing osteoblasts in successive layers of a thin hydrogel scaffold (3-D environment with growth factors) and under intermittent compressive mechanical load.

Hydrogels are a class of polymers that can absorb large amounts of water without dissolving due to physical or chemical cross-linkage of the various hydrophilic polymer chains, from which they are composed (Lee and Mooney, 2001; Tibbitt and Anseth, 2009). They mimic closely the extracellular environment that cells experience in vivo, and can bind nutrients and growth factors. Hydrogels have also been reported to have beneficial effects on cell adhesion, morphology and maintenance of cell phenotype (Hoffman, 1993; Wang et al., 1990; Cukierman et al., 2001). Hence, hydrogels are good candidates to be used as a thin 3-D scaffold in an appositional bone-growth model. Hydrogels are discussed in detail in Chapter 3.

In this project, osteoblast/hydrogel constructs were cultured in a mechanically active environment as mechanical loading has been shown to be an important determinant of bone mass, bone architecture and osteogenic differentiation in vitro (Jagodzinski et al., 2004, Jagodzinski et al., 2008; Sittichokechaiwut et al., 2010; Li et al., 2013).

The primary aim of the present study was therefore to develop a 3-D hydrogel
cell-culture model and to assess whether human calvarial and femoral osteoblasts cultured in a hyaluronan-gelatin hydrogel scaffold, are able to proliferate and deposit a mineralised matrix, with a view to the development of a multi-layered model in the future.

In addition, this study included validating assays for the measurement of osteoblast proliferation and hydroxyapatite deposition in hydrogels and the examination of the model for its suitability for the study of mRNA expression under mechanical strain.
1.2 Characteristics of an ideal bone graft

An ideal bone graft would biologically mimic an autologous bone graft. It would contain components and characteristics that are necessary for bone repair and would release growth factors for osteoinduction, vascularisation and the recruitment of cells with the potential for osteogenesis (Cancedda et al., 2007). It would also act as a scaffold for deposition of a mineralised matrix by the osteoblasts (Liu et al., 2006).

Osteoinduction is the ability of the graft to induce surrounding osteoprogenitor mesenchymal stem cells to differentiate into osteoblasts, a process mediated by the release of bone morphogenetic proteins or BMPs (Greenwald et al., 2001). The importance of osteoinduction was first illustrated by the work of Urist and co-workers, who observed that when a devitalised bone is transplanted despite the absence of living cells, it is still able to act as an osteoinductive scaffold (Urist et al., 1965). The gradual and synergistic resorption of the graft by host multinucleate osteoclasts causes a release of osteogenic growth factors (BMPs), stored inside the scaffold, inducing the surrounding mesenchymal stem cells to proliferate and differentiate into osteoblasts.

An ideal graft material would also mimic the coupled remodelling kinetics of bone, known as the apposition-resorption-formation (ARF) sequence (Frost, 1969). When implanted, the original graft scaffold would be resorbed by host osteoclasts releasing BMPs, while simultaneously being replaced by new bone of host origin in accordance with the normal remodelling sequence.
1.3 Bone Morphogenetic Proteins (BMPs) and the process of osteoinduction

BMPs comprise an extensive group of signalling molecules that belong to the Transforming Growth Factor-β (TGF-β) superfamily of proteins, with over 20 members identified (Bragdon et al., 2011). BMPs 2, 4, 6, 7 and 9 are recognized as the main osteoinductive signals (Urist et al., 1998; Wang et al., 1990; Cheng et al., 2003).

Urist in 1965 discovered that demineralised bone extract could induce bone formation in extra-skeletal sites by a process he termed autoinduction. BMPs, tightly bound to the matrix of demineralised bone were responsible for osteoinductive or autoinductive activity, a process which induces undifferentiated mesenchymal cells to become osteoprogenitor cells and form new bone (Urist et al., 1965, Urist and Iwata 1973). Osteoinduction was observed in a series of over 70 experiments, in which sectioned cylinders of devitalised and hydrochloric acid-demineralised bone from animals and humans were transplanted into the muscles of various animals.

Figure 1. Process of transformation of implanted cylinders of decalcified bone matrix into new bone.

The end volume is about 75% of the volume of the original implant of dead, decalcified matrix. Adapted- Urist et al., 1965.

BMPs are an integral component of bone regeneration, yet finding a suitable carrier system for BMPs which mimics remodelling of bone is still a challenge (Lee et al., 2007, Liu et al., 2013). The extracted BMPs are hydrophobic, but as the proteins are purified, they become hydrophilic and are able to diffuse and induce osteogenesis. In tissue engineering, BMPs are attached to carriers to prevent early diffusion and to create a controlled release to the site (Burg et al.,
2000). If the carriers do not mimic remodelling of bone, issues arise with non-synchronized and uncontrolled release of the growth factors, uncoupled resorption of the implanted carrier and interference with healing (Seeherman and Wozney, 2005). Additionally, BMP-2- or BMP-7-loaded scaffolds have shown a range of complications including ectopic bone formation leading to neurological impairment and even increased malignancy risk with higher doses of BMP-2 (Carragee et al., 2011; Wong et al., 2008).
1.4 Allograft and xenograft

Allografts (grafts from the same species) or xenografts (grafts from different species) are commonly used in clinical practice, but have significant limitations. Such grafts are stripped of their cellular content with enzymes and detergents, which potentially results in weakening of the extracellular matrix. The repair capability of an allograft is less predictable than that of an autograft (same person) and the financial cost is high (Stock and Vacanti, 2001; Saito and Takaoka, 2003). Finally, allografts and xenografts carry the risk of viral or bacterial transmission and an immunological host response with inflammation and rapid resorption of the graft.

The discovery that BMPs are responsible for osteoinductive properties of devitalized and demineralised bone (Urist et al., 1965) led to commercial products that use powdered demineralised bone matrix (DBM) from animal and human bone. The process of demineralisation removes live progenitor cells and cell surface antigens, yet preserves the organic component of the bone matrix, which contains collagens and other proteins accounting for the osteoinductivity of DBM (Liu et al., 2006; Bartold et al., 2006). Commercial DBM products have shown mixed osteoinduction success with some animal models showing no osteoinduction, but instead encasement in a fibrous connective tissue (Garraway et al., 1998). The original demineralisation process introduced by Urist in 1965 utilised hydrochloric acid. Modern processing methods, however, vary widely and these may impact the osteoinductive properties of the graft (Russell and Block 1999).

DBMs have been widely used in dental bone augmentation, spinal fusion and multiple other applications. DBM bone powder has been incorporated into collagen sponges and hydrogels. The innovation has been implemented as a solution to the poor handling qualities of DBM powdered bone fragments during its clinical application at bleeding operative sites (Garraway et al., 1998).
1.5 Engineered scaffolds

Finding suitable carriers for BMPs has led to a rapid growth in engineered scaffolds. The main scaffolds that have been investigated as BMP carriers include natural polymers (collagen, hyaluronan, fibrin, chitosan, silk, alginate and agarose), inorganic materials (calcium phosphate cements and ceramics), synthetic polymers (polyglycolic-acid – PGA; polylactic-acid – PLA; and polylactide-co-glycolide – PLGA) and combinations of these materials (Seeherman and Wozney, 2005). Each have advantages and disadvantages and have shown mixed success (Meikle, 2007). Current research is focused on engineering a scaffold with biomimetic properties that would mimic the role of the extracellular matrix (ECM) in cellular adhesion, migration and proliferation (Elisseeff et al., 2005).

1.5.1 Natural polymers as carrier scaffolds

Naturally derived polymers most closely simulate the native cellular environment, and their biocompatibility and bioresorbability are the main advantages. However, naturally derived gel scaffolds isolated from biological tissues have a disadvantage of batch-to-batch variability. Other disadvantages include poor mechanical properties, contamination in large scale production, immunogenicity and disease transmission (Seeherman and Wozney, 2005; Yang et al., 2001).

The most common natural polymer used as a carrier scaffold is a reconstituted type-1 collagen gel derived from rat tails by solubilisation in acetic acid (Achilli and Mantovani, 2010). Collagen type-1 is the most abundant protein naturally found in ECM and it plays an important role in providing structural integrity and the expression of the osteoblastic phenotype in vivo (Stock and Vacanti, 2001; Masi et al., 1992). In vitro studies have shown successful culture of human osteoblasts using collagen hydrogel scaffolds (Ignatius et al., 2005). Studies investigating the implantation of type-1 collagen scaffolds in animal models have shown the importance of type-1 collagen in maintenance of the osteoblast phenotype resulting in the synthesis of bone matrix and ultimately in new
formation at graft sites (Xiao et al., 2003). Unfortunately, the process of collagen solubilisation changes its structure and alters the mechanical properties of the collagen matrix. Instead of covalent cross-linking of the larger fibers, the individual collagen fibers cross-link with each other and form larger fibers that are connected by weak hydrogen bonds, rendering this model unsuitable for mechanical loading (Pedersen and Swartz, 2005). It has also been shown that collagen gels prepared in this way are dimensionally instable and may shrink by up to 85% of their height during cellular reorganisation (Guidry and Grinnell, 1985). Finally, potential antigenicity, batch-to-batch variability and disease transmission have also been reported. This type of scaffold can still be used for short-term studies or in combination with another more rigid scaffold.

The need for the modification of the mechanical properties of the scaffold has led to the development of scaffolds, which include a thiol-modified hyaluronan-gelatin cross-linked by polyethylene glycol diacetate (PEGDA). It is this semisynthetic hydrogel, which is used by the present author for the present study (See Chapter 3).

1.5.2 Synthetic polymers

Synthetic resorbable polymer scaffolds have been widely used to overcome the aforementioned limitations of natural polymers. The FDA approved polymers polyglycolic-acid (PGA), polylactic-acid (PLA) and polylactide-co-glycolide (PLGA) differ in their properties, especially in their degradation times. PLGA, a co-polymer of PLA and PGA, is one of the most commonly used synthetic materials for preparing fibrous scaffolds. However, these scaffolds also have limitations.

Synthetic polymers with their biocompatible and biodegradable attributes have a good safety record in the form of surgical sutures and have also been used as delivery vehicles for BMPs with variable success (Saito and Takaoka, 2003; Schwartz et al., 1995). The main advantages of synthetic polymers is that they carry a low risk of disease transmission, display a range of degradation times, provide a versatile substrate with a range of physical properties and can easily be used in manufacturing processes due to a well characterised and reproducible
chemistry. However, these scaffolds may undergo unpredictable degradation, resulting in increased cytokine production stimulating inflammatory bone resorption. Their hydrophobic nature makes BMPs and other factor incorporation and cell attachment challenging (Yang et al., 2001; Bartold et al., 2006). PGA for example, loses its mechanical strength rapidly over a period of two weeks and becomes absorbed too fast for most anchorage-dependent applications. Additionally, all polyesters release acidic degradation products, which may affect biocompatibility. Finally, the polyesters do not have a chemically reactive side chain to enable cross-linking or modification by the attachment of other molecules (Yang et al., 2001). The latest research is focused on improving cellular attachment by surface treatments. Fibre electro-spinning to mimic the ECM and creating hybrid scaffolds is also evolving (Zhao et al., 2016).

Numerous animal experiments that investigated resorbable materials such as PLA and PGA acids and polycaprolactone (with or without tri-calcium phosphate), reported mainly fibrous tissue formation with a few microscopic islands of bone (Liu and Ma, 2004; Lam et al., 2008). These synthetic polymer materials act primarily as tissue spacers and can provoke a foreign body reactions in the host (Meikle et al., 1993; Coombes and Meikle, 1994). In such instances, macrophages and multinucleate giant cells which are rich sources of cytokines, migrate to the site, and stimulate interleukin-1β (IL-1β), IL-6 and tumour necrosis factor (TNF) production (Bonfield et al., 1992; Cardona et al., 1992, Anderson et al., 2008). Interleukins and TNF play important roles in immunoregulation and are also potent stimulators of bone resorption (Gowen et al., 1983a; Bertolini et al., 1986), which is a significant disadvantage when attempting to regenerate bone.
1.5.3 Bio-ceramics

The main bio-ceramic inorganic scaffolds are the calcium phosphate group, which include hydroxyapatite and tri-calcium phosphate (Yang et al., 2001; Liu et al., 2013). These are brittle, biocompatible, and readily available materials. To improve the mechanical properties of bio-ceramics, hybrid scaffolds have been designed, incorporating ceramics with polymers (Wei and Ma, 2004). Bio-ceramic materials have osteoconductive surface characteristics and can bind to bone (Bartold et al., 2006). However, being non-osteoinductive they cannot induce osteogenesis on their own unless BMPs are added (Burg et al., 2000). Hydroxyapatite (HAP) materials are non-resorbable, which prevents ingrowth of new bone (Liu et al., 2013). The porosity characteristics of HAP have also been improved, due to the lack of interconnectivity between pores in the older models (Burg et al., 2000). Tri-calcium phosphates have the limitation of rapid degradation with little bone formation (Bartold et al., 2006). Despite lack of osteoinductivity, much of current tissue engineering research is conducted in this field, with the aim of developing a bio-ceramic hybrid composite, matching the complex structure, resorbability and mechanical properties of bone (Olszta et al., 2007).

1.6 Summary

This chapter introduced the aims of the project, described the properties of an ideal bone graft, and the extent to which its characteristics have been achieved in engineered bone scaffolds. Numerous scaffold materials have been created with limited success. While an alternative to autogenous and allogeneic bone has been the goal of bone and biomaterials research for many years, a suitable bone matrix that contains osteoinductive properties similar to those found in natural bone has not been found.
1.7 References


Chapter 2

Calvarial and femoral osteoblasts and their mineralisation in a monolayer cell culture

2.1 Introduction

Prior to commencing work in 3-D culture, it was necessary to understand the mineralisation process of calvarial and femoral osteoblasts in a conventional monolayer culture. Although these osteoblasts have common features, there are some distinct differences, which will be discussed.

2.1.1 Differences in calvarial and femoral bone

Calvarial and femoral bones differ in their embryological origin, response to mechanical strain, mineralisation and resorption patterns, and osteogenic potential.

Different sites within the skeletal system differ in their embryological origin, mode of osteogenesis and function. Mammalian bones have distinct embryological origin (paraxial and lateral plate mesoderm and neural crest) and undergo two different modes of development (intra-membranous and endochondral) (Chung et al., 2004). The classic work on quail and chick embryos by Le Lievre, Le Douarin and co-workers has led to the discovery that the lower jaw and bones of the skull vault are derived from the neural crest cells which migrate to the head region (Le Lievre and Le Douarin, 1975, Couly et al., 1993). Differentiation and migration of neural crest cells relies on complex signaling pathways (Mishina and Snider, 2014). The posterior part of the skull, starting at the posterior sella turcica of the sphenoid complex and the rest of the skeleton are of mesoderm origin. The bones of the craniofacial skeleton, apart from the posterior cranial base develop by intramembranous ossification, which is a process of direct ossification without a cartilaginous precursor. The rest of the skeleton develops by endochondral ossification from cartilage anlagen, which
later becomes mineralised (Couly et al., 1993, Chung et al., 2004, Karaplis, 2008). Although the bone may be classified as cartilaginous, its subsequent growth occurs by an intramembranous process where the periosteum-derived cells convert to osteoblasts, which lay down successive layers of bone and participate in growth and remodelling (Meikle, 2002).

Calvarial and femoral bones have potentially different ways of regulating mineralisation. Mineralisation characteristics of calvarial and femoral bones were studied from samples of 300 human foetuses between 6-8 to 40-41 weeks (Dziedzic-Goclawska et al., 1988). Matrix deposition, maturation and mineralisation rate was gradual and slower in parietal than in the femoral bones. The authors concluded that the differences in kinetics of mineralisation processes between the weight bearing bones and bones that are not exposed to strong external forces are most probably genetically conditioned.

Some studies using cell culture show that femoral cells demonstrate a different pattern of mineralisation to calvarial osteoblasts. In a rat model, femoral osteoblasts showed a more dispersed mineralisation pattern in comparison to calvarial osteoblasts which showed a more well-defined pattern (Declercq et al., 2004). Variability in the observations could be explained by variations in the cell isolation techniques, the culture environments, the source of the cells and the age of the donor. It is also important to emphasise that caution is required in the interpretation of these studies as many have been carried out using cultured cell monolayers – an approach that does not adequately replicate the in vivo environment.

There are also differences in resorptive mechanisms between calvarial and long bones, specifically between the proteolytic enzymes used by the osteoclasts to degrade the cross-linked collagenous bone matrix. All osteoclasts produce cysteine proteinases as their main class of proteolytic enzymes (cathepsin K is the most important). Calvarial osteoclasts differ in that they also produce matrix metalloproteinases (MMPs), principally collagenase (Hill et al., 1994). A clinically relevant example of functionally different site-specific osteoclasts and their
enzymes is pycnodysostosis, which is a mutation in the cathepsin K gene, where the flat bones of the head are affected differently from the long bones (Everts et al., 1999, Everts et al., 2006). Further research is required in this field to determine which other proteolytic enzymes participate in the degradation of bone (Everts et al., 2006).

The osteogenic potential and regenerative capacity is thought to be higher in neural crest-derived bones compared to mesoderm-derived bones, which is the reason why some studies state their preference for neural crest-derived graft material (Koole, 1994; Quarto et al., 2010). In a retrospective study, iliac crest and mandibular symphysis grafts were compared in 50 patients, with follow up time between 3 to 10 years. Based on assessment of radiographic films, the authors concluded that the mandibular symphysis grafts performed better with significantly less resorption of the grafts. This study had many limitations, namely its retrospective nature, a heterogenous study sample, a vague definition of a successful graft and the absence of blinding during radiographic assessment (Koole et al., 1989). The conclusions were however supported by later animal studies which stated that the implantation of long bone grafts in calvarial defects and vice versa should be considered with great care (Van den Bos et al., 2008). Additionally, Kasperk (1995) compared mandibular and iliac crest-derived human osteoblast cells within individual patients. Although the sample size was small (N=4), this allowed for direct comparison of the two cell lines within individual patients, while limiting the confounding variable of individual variation. These samples showed that mandibular, neural crest-derived osteoblasts proliferated faster and showed comparatively higher levels of insulin–like growth factor II (IGF-II), higher basic fibroblastic growth factor (bFGF) and less alkaline phosphatase Other studies showed a difference in BMP expression patterns between calvarial and long bones (Suttapreyasri et al., 2006). Finally, calvarial and long bones demonstrate different responses to dynamic mechanical strain and this is further explored in Chapter 4.

In summary, differences in embryological origin of calvarial and long bones manifest in different bone development processes that are mediated by different
regulatory molecules. These differences have been suggested to account for the superiority of neural crest-derived bone in grafting procedures (Hall, 1999).

2.1.2 Bone mineralisation

Bone mineralisation is the process by which hydroxyapatite crystals are laid down within the collagenous organic matrix. This process is tightly regulated and is specific to osteoblasts (Aubin et al., 1998, Ducy et al., 2000). It begins with osteoblasts secreting a collagenous non-mineralised extracellular matrix called osteoid. This organic component of the extracellular matrix mainly consists of type-1 triple helix collagen, which gives bone its resistance to tensile forces. This is followed by formation of hydroxyapatite crystals within matrix vesicles and their secretion onto the extracellular matrix. These hydroxyapatite crystals make up the inorganic component of bone and provide structure and resistance to compressive load. They make up approximately two thirds of the total matrix (Aronow et al., 1990; Owen et al., 1990; Lian and Stein, 1992).

2.1.3 Phases of the mineralisation process

Mineralisation of the extracellular matrix can be divided into three phases; osteoblast proliferation, extracellular matrix development and maturation, and mineral deposition onto the matrix (Owen et al., 1990) (Figure 2). The initial peak in osteoblast proliferation is marked by increased histone gene expression (H4), a marker of DNA replication. The synthesis of type-1 collagen is followed by maturation and decline in proliferation. Early mineralisation of the matrix is marked by an increase in alkaline phosphatase (ALP). With the onset of hydroxyapatite accumulation, osteocalcin (calcium binding protein) increases, which is a marker of the mature osteoblast (Hauschka and Wians, 1989).
Figure 2. Relationship between proliferation, extracellular matrix development (ECM) and mineralisation.

Three periods are shown: 1: proliferation; 2: extracellular matrix development and maturation and 3: mineralisation. Each stage is connected to the next with a feedback loop and the expression of phenotypic markers H4 (histone), Col-1 (collagen-1), ALP (alkaline phosphatase), OP (osteopontin), OC (osteocalcin), HA (hydroxyapatite) are specific to each time period. Adapted – Lian and Stein, 1992.

The onset of mineralisation is marked by an increase in the concentration of calcium and phosphate inside matrix vesicles, which bud from the main hypertrophic cell membrane. Alkaline phosphatase at the vesicle membrane acts to produce a local increase of phosphate. Once the increase in calcium and phosphate exceeds the solubility point, deposition of hydroxyapatite 

$$\text{Ca}_5(\text{PO}_4)_3(\text{OH})$$

occurs within the matrix vesicles (Anderson, 1995; Orimo, 2010).

As the process continues, hydroxyapatite crystals penetrate the matrix vesicle membrane and become exposed to extracellular fluid. Calcium and phosphate concentration outside the vesicle must be sufficiently high to support continuous formation of new hydroxyapatite crystals. This results in a deposition of mineral-
forming clusters, which permeate the collagenous matrix, filling the spaces between collagen fibrils (Orimo, 2010). This is depicted in Figure 3 below.

**Figure 3. The process of mineralisation initiated in the matrix vesicle.**

Mineralisation will not commence without inorganic phosphate inside the matrix vesicle. Vesicles contain enzymes, one of which is ALP, which makes inorganic phosphate (Pi) available for mineralisation. Once hydroxyapatite crystals are formed from calcium and inorganic phosphate (Pi), they penetrate the membrane and become deposited between collagen fibrils. Adapted-Orimo, 2010.

In summary, mineralisation is a synchronised and regulated process in which osteoblasts produce the extracellular matrix and deposit hydroxyapatite crystals onto this extracellular matrix; different protein markers can be observed at different stages of the process (Lian and Stein, 1992; Owen et al., 1990; Stein et al., 1990).
2.1.4 Role of alkaline phosphatase in bone mineralisation

Osteoblasts are rich in alkaline phosphatase (ALP), which is a membrane-bound enzyme that hydrolyses monophosphate esters of inorganic pyrophosphate to generate the inorganic phosphate, which is essential for the mineralisation process. ALP is an early differentiation marker expressed by pre-osteoblasts and early osteoblasts and is used for phenotype identification and evaluation of osteogenesis (Anderson, 1995; Orimo, 2010). The late phenotypic protein is osteocalcin (OCN), which is expressed by mature osteoblasts and odontoblasts (Ducy et al., 2000).

The hypothesis that ALP has an important function in skeletal mineralisation was originally proposed in 1923 by Robert Robison. He saw that in young rats and rabbits there was considerable phosphatase activity within ossifying bone and cartilage. Later research in the 1960s by H.C. Anderson discovered that the earliest site of hydroxyapatite crystal deposition occurred in small membrane-bound matrix vesicles, which were rich in ALP. The name alkaline was given to the enzyme due to its optimum pH of 7. Experimental inhibition of ALP prevented calcification of isolated matrix vesicles. The most convincing evidence for the role of alkaline phosphatase in mineralisation stems from the genetic condition hypophosphatasia, in which the ALP gene mutation alters its catalytic activity resulting in the failure of calcification in cartilage, bone and dentine (Whyte, 1994; Anderson, 1995).

2.1.5 In vitro mineralisation by osteoblasts in a monolayer culture

Osteoblasts are mononuclear cells that differentiate from pluripotent mesenchymal stem cells through a cascade of progenitor stages to form mature matrix-secreting osteoblasts (Wlodarski, 1990).

The investigations carried out on monolayer cell cultures in the 1980s demonstrated that when osteoblasts are maintained in culture for extended periods in the presence of ascorbic acid, β-glycerophosphate and dexamethasone (triple supplement), extracellular matrix mineralisation is observed in the form
of ‘nodules’ (Bellows et al., 1986). Microscopically and histologically, these nodules resemble woven bone with an aggregation of cells embedded in a fibrous collagen network (Aronow et al., 1990).

Ascorbic acid is an essential ingredient for osteoblastic differentiation in cell culture and is necessary for deposition of a dense collagenous extracellular matrix (Bellows et al., 1986) and L-ascorbic acid 2-phosphate has been shown to be superior to ascorbic acid for in vitro culturing. L-ascorbic acid 2-phosphate is a long-acting vitamin-C derivative with a half-life of 7 days in culture conditions (Declercq et al., 2004). It stimulates the onset of extracellular matrix collagen synthesis, its deposition and maturation and the formation of nodules. It further supports cell growth and alkaline phosphatase activity (Takamizawa et al., 2004).

Mineralisation of nodules does not occur without a source of phosphate. For initiation of mineralisation, β-glycerophosphate (β-GP) is added as a substrate for alkaline phosphatase to generate inorganic phosphate. Removal of either the β-GP from cultures or the inhibition of ALP stops the initiation of mineralisation (Bellows et al., 1991). It has been reported that if β-GP is added in high concentrations, dystrophic calcification occurs. A concentration of 10 mM β-glycerophosphate is commonly used (Chung et al., 1992).

Addition of dexamethasone stimulates the number of nodules formed (Bellows et al., 1986). However, it has been reported that the degree of susceptibility to glucocorticoid treatment is dependent on the developmental stage of the cells and is also species-dependent (Declercq et al., 2004). For example, dexamethasone can be added to undifferentiated cells to induce differentiation into the osteogenic lineage (Ter Brugge and Jansen, 2002). In the presence of β-glycerophosphate, it has also been shown to increase mineralisation by increasing ALP activity (Cheng et al., 1994).

The formation and mineralisation of nodules involves aggregation of confluent cells in a swirled pattern, followed by retraction of cells into dense, multi-layered
structures. There is variation in the time taken for mineralised nodules to become visible. On average, osteoblast nodules will show extensive mineralisation by day 28. This is dependent upon the source of the osteoblasts, as osteoblasts isolated from different donors show marked variation in proliferation and osteogenic potential (Ter Brugge and Jansen, 2002; Aronow et al., 1990). Nodule formation is also dependent upon the initial cell plating density and only once the cells are confluent, and ALP is up regulated, can the mineralisation process commence (Aronow et al., 1990; Bellows et al., 1986;).

2.1.6 Summary

This section has outlined the differences between femoral and calvarial osteoblasts, which likely stem from their different embryologic origins. Hence studies of osteoblasts should include both types of cell. The major common limitation of most studies is that they were conducted using monolayer cultures on plastic surfaces, which are not representative of the 3-dimensional \textit{in vivo} environment. Hence is the need for 3-D culture systems, which will be discussed further in Chapter 3.
2.2 Hypothesis

HFOs and HCOs will have similar rates of proliferation and mineralisation over 21 days when cultured as a monolayer.

2.3 Aims

1. To compare the relative proliferation rates for HCOs and HFOs cultured in a mineralisation medium containing triple supplement.

2. To compare differences in hydroxyapatite formation between HCOs and HFOs cultured in a mineralisation medium containing triple supplement.

2.4 Materials and Methods

2.2.1 Cell culture

A commercial osteoblast cell line, which had been previously phenotyped was purchased. The human foetal femoral osteoblasts (HFOs) – of mesoderm origin (#4610) and human calvarial osteoblasts (HCOs)– of neural crest origin, (#4600 lot 3439) were purchased from ScienCell Research Laboratories, cryopreserved at P1 at >5 x10⁵ cells / ml per vial. The osteoblasts originated from foetuses (20-22 weeks old) and had a doubling time of 25 hours.

The use of a commercial osteoblast cell line provides homogeneity and ensures the maintenance of the osteoblastic phenotype over a number of generations. Continuous passaging and cultivation, however, can still result in a drift towards an undifferentiated phenotype. Therefore, it is recommended that cells of a low passage number are employed for any subsequent experiments (Casser-Bette et al., 1990; Brugge and Jansen, 2002). The ScienCell product sheet stated that up to ten passages were possible. Cells at passage 3-5 were used for all experiments.

Upon arrival on dry ice, the HCOs and HFOs were immediately transferred to liquid nitrogen until required. Following this, the cells were thawed and sub-
cultured on poly-l-lysine coated T-75 flasks for good attachment. Osteoblast media and osteoblast growth supplement (ObGS, ScienCell) were used to multiply the cells, as recommended by the supplier. Before reaching confluence, cells were trypsinised and transferred to a new T-75 flask until sufficient cell numbers were obtained. Following cell expansion, stocks of cells were frozen to ensure a source of low passage number cells for future experiments.

The cells were detached from the flask with trypsin, transferred to a 15 mL centrifuge tube and centrifuged at 220 xg at room temperature for 5 min. The cells were resuspended in 2 mL of medium before counting using a haemocytometer. When stained with Trypan Blue, live cells under the microscopy appear transparent. Dead cells taking up the stain and appear blue. The cells were centrifuged again at 220xg and the supernatant was removed. The cell pellet was resuspended at approximately 3.5 x 10^5 cells per mL of freezing medium consisting of 10% Dimethyl Sulphoxide (Cat No D-4540; Sigma Aldrich) and 90% FBS (refer to Appendix 6.3). Aliquots of 1 mL were transferred to cryovials and placed in a cooling chamber ("Mr Frosty™" Nalgen Labware, Rochester, USA) at -80°C which allows the cells to freeze at a rate of -1°C/min . After 24 hrs the frozen cells were transferred to liquid N₂ for long-term storage.

Osteoblasts were routinely expanded in Osteoblast growth medium, which contained Dulbecco’s Modified Eagle’s Medium (DMEM) with GlutaMAX™, antibiotic-antimycotic reagent, gentamycin and 10% foetal bovine serum (FBS).

When required, triple supplement was added to the growth medium to start the mineralisation process. Triple supplement includes 0.2 mM L-ascorbic acid 2-phosphate (Asc-2-P), 0.1 µM Dexamethasone and 10 mM β-glycerophosphate (β-GP) (See Appendices 6.5 and 6.6). Triple supplement stock solution was made and frozen in vials. Each vial was thawed prior to a medium change and fresh triple supplement reagents were added to the D-MEM with GlutaMax™ media at each medium change, every 2 days.
The addition of triple supplement to induce mineralisation and nodule formation is a well-established protocol. The present author undertook an independent verification of the process to ensure that both the HCO and HFO lines used in the study were capable of forming nodules. Cells were cultured in monolayer in either the presence or absence of triple supplement. These preliminary investigations allowed for the development of assay protocols that formed the basis of our later work with hydrogels.

For all experiments one each of the frozen HFO and HCO vials were thawed and immediately transferred to a T-75 flask, containing osteoblast growth medium and cultured until the cells were 80% confluent. The osteoblasts were then trypsinised, counted and seeded at 20,000 cells/cm² - 35,000 cells/cm² and cultured on a variety of plate sizes, including 6-well 24-well and 48-well with and without triple supplement in parallel for 21 days at 37°C in 5% CO₂/95% air.

2.2.2 Microscopic observation of nodule formation

The osteoblasts were observed under a light microscope (MRC/B146; Olympus, Hampton, Tokyo) at each media change over 21 days. Cellular arrangement, morphology, retraction and nodule formation were observed and photographed using a Canon G10 camera and Remote Capture-DC software.

2.2.3 Alizarin Red staining of calcium deposits

The ability to organise their extracellular matrix into mineralised nodules is a characteristic of functional osteoblasts. The mineralising potential of HFO and HCO cells grown in a monolayer, was examined over a 21-day period using an Alizarin Red-based mineralisation assay.

Alizarin Red stain is used to demonstrate calcium salts. However, alizarin is not specific for calcium salts and can also react with other metals, as it binds to cations. Binding also depends on the pH of the dye solution as the hydroxyl groups of alizarin need to be able to dissociate for salt formation to take place (Puchtler et al., 1968). A pH of 4.2 of the staining solution was confirmed with a pH meter prior to staining.
Cell culture plates were removed from the incubator, the media removed and the cells rinsed with sterile PBS. The Alizarin Red stain was applied to provide adequate coverage of the cultured cell monolayer. The cells in each well were fixed with 10% formalin for 1 min and stained with 1% Alizarin Red (pH 4.2) for 10 min at room temperature. The cells were washed four times with a wash buffer (PBS containing 0.05% Tween 20). The intensity of the Alizarin red stain was analysed qualitatively using light microscopy (Appendix 6.8).

2.2.4 Colourimetric detection of alkaline phosphatase activity

Alkaline phosphatase (ALP) is required for the initial step of the mineralisation process and is an early differentiation marker expressed by pre-osteoblasts and early osteoblasts (Anderson, 1995; Orimo, 2010). The ALP assay stains the outside secretory membranes of osteoblasts with naphthol phosphate and the diazonium dye fast blue to quantify alkaline phosphatase activity (Scherff and Groot, 1990).

Cell culture plates were removed from the incubator, the media removed and the cells rinsed with sterile PBS. Each well containing a monolayer of osteoblasts was fixed with 10% formalin for 1 min and washed with wash buffer (PBS containing 0.05% Tween 20) for 1 min. Longer fixation could lead to irreversible inactivation of ALP. To make the ALP substrate, 1 BCIP/NBT tablet (SigmaFast™ BCIP-NBT; Sigma Aldrich) was dissolved in 10 mL de-ionized H2O at room temperature and 500 µl was added per well and incubated in the dark at room temperature for 5-10 mins. The intensity of the stain was checked every 2-3 min. The substrate was removed to stop the reaction and the cells washed with wash buffer for 2 min and carefully aspirated. The stained cells were viewed under light microscopy (Appendix 6.7).

2.2.5 OsteoImage™ fluorescence assay for hydroxyapatite

A fluorescent assay for hydroxyapatite was used to provide a relative quantification of hydroxyapatite mineral formation and to trial the assay prior to its application to the 3D hydrogel culture studies. The OsteoImage™ (Lonza,
USA), a fluorescent staining reagent was used which binds to the hydroxyapatite crystals of the mineralised nodules deposited by osteoblasts (Wang et al., 2006).

HCOs and HFOs were seeded at 50,000/cm² in 48-well plates. Separate plates were used for three time points (7, 14 and 21 days). Six samples from each group were cultured in 300 µl mineralisation medium, which was changed every 2 days. The plates were incubated at 37°C in 5% CO₂/95% air.

HCO and HFO cells were stained using the OsteoImage™ assay according to the manufacturer’s instructions at 7, 14 and 21 days. After removal of the culture medium, each sample was rinsed with PBS (400 µL), fixed with 10% Formalin (100 µL) for 1 min and rinsed with OsteoImage™ wash buffer (400 µL). OsteoImage™ staining reagent (200 µL) was placed into each sample well and incubated at room temperature for 30 min in the dark. Each well was washed three times with wash buffer (400 µL) so as not to disturb the sample. After the final wash, the resulting level of fluorescence was measured in a plate reader (Synergy 2, Biotek, USA) at 485/528 nm excitation/emission wavelengths (Appendix 6.10).

2.2.6 PrestoBlue™ proliferation assay

The aim was to compare the proliferation rates of HFO and HCO cells in the presence of triple supplement over 21 days using PrestoBlue™, a resazurin-based fluorescent assay. Reduction of resazurin forms a fluorescent pink compound, which quantitatively reflects metabolic cell activity and proliferation (Lall et al., 2013).

The HCOs and HFOs were plated at 50,000 cells/cm² for the PrestoBlue assay in four 48-well plates (for each of the four time points). The cells were cultured in 300 µl mineralisation medium and incubated at 37°C with 5% CO₂/95% air. The medium was changed every 2 days. Samples of medium were cultured in parallel to account for any background fluorescence. At days 1, 7, 14 and 21 the samples were stained with PrestoBlue™ at a 1:10 ratio of culture medium according to manufacturer’s instructions and incubated for 1 h at 37°C in the dark. The
resulting level of fluorescence was measured using a Synergy™ 2 multi-mode microplate reader at 540 nm excitation/ 610 nm emission (Biotek, USA) (Appendix 6.9).

2.2.7 Data analysis

GraphPad Prism Version 6.00 software for Mac OS X (La Jolla California, USA, www.graphpad.com) was used for data analysis and presentation.

2.5 Results

2.5.1 Osteoblasts and triple supplement

The aim of this series of investigations was to compare proliferation and mineralisation characteristics of HCOs and HFOs cultured with or without the addition of osteogenic triple supplement. Cultured cells were stained for calcium-rich areas using Alizarin Red and with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) for the colourmetric detection of alkaline phosphatase.

Within the limitations of a subjective colourimetric assessment, there was an observed colour difference when the HCOs and HFOs cultured with or without triple supplement. Staining was more intense for the triple supplement group. This finding is consistent with those reported in the literature and is the result of β-glycerophosphate in the triple supplement acting as an external source of phosphate required for onset of mineralisation (Bellows et al., 1986; Ecarot-Charrier et al., 1988). However, it was also noted that HCO cells consistently stained less intensely with Alizarin Red at days 7 and 14 compared with the HFO cells. However, at day 21 there was minimal difference between HCOs and HFOs (Figure 4).

Alkaline phosphatase activity was confirmed at 21 days with the conversion of the BCIP to a dehydroindigo dimer product with the release of hydrogen ions causing the reduction of NBT to the insoluble NBT diformazan. Both HFOs and
HCOs in the presence of triple supplement showed similar AP activity at 21 days (Figure 5). Staining was more intense for the triple supplement group (Figure 5).

Figure 4. Effect of triple supplement on the formation of calcium-rich deposits.

The intensity of Alizarin Red calcium-rich areas of HCO and HFO samples are shown at 7 days (A), 14 days (B) and 21 days (C).

Figure 5. Alkaline phosphatase activity with and without triple supplement.

Following 21 days of culture in triple supplement, both HFO and HCO cells produce large nodules with high levels of ALP activity (dark purple).
2.5.2 Morphological changes of HCOs and HFOs observed during the mineralisation process

The second aim was to observe the sequential process of mineralisation under light microscopy and determine if there were any morphological differences between the HCOs and the HFOs. Both, HCOs and HFOs attached to the plastic surface of the culture wells within 24 hrs. The shape changed from a round unattached cell to adherent cells with multiple extensions or filopodia. Osteoblasts make proteins (collagen type-1 and osteocalcin are the main two secretory proteins), which are exported to the extracellular matrix in vesicles. Active osteoblasts are recognized by their basophilic cytoplasm due to the extensive rough endoplasmic reticulum and a prominent Golgi apparatus which appears as a clear zone adjacent to the large single nucleus for transporting the proteins into extracellular matrix (Scherff and Groot, 1990). No distinct difference in cell morphology was observed between the HCO and HFO cells (Figure 6).

![Figure 6: Morphological appearance at day 3 of HCOs (A) and HFOs (B) in monolayer culture.](image)

The osteoblasts have attached to the plastic and have extended their filopodia to form a network with each other and with the surrounding extracellular environment. Scale bar 100 µM.
Retraction was visible in both cell lines as a peeling of the cellular layer from the walls and the floor of the culture well towards the middle, which was visible microscopically and macroscopically. This process occurs following confluency as the cells fold over and form a band, retract from the culture plate periphery and aggregate together towards the middle to make a dense mineralised structure (Figure 7).

Figure 7. Process of retraction and nodule formation.
Retraction is observed for both the HCOs (A) and HFOs (B). The osteoblasts first form a band of retracting cells which folds over as the cells migrate towards the middle before forming nodules. Scale bar 1000 μM. The osteoblasts form multiple extensions or filopodia (arrow) (C) aiding in the retraction process. Scale bar 100 μM.

The cells were checked daily and the process of nodule formation was recorded. There was minimal variation between the six wells seeded for each cell line. However, there was a distinct difference in the commencement of retraction and nodule formation when comparing the HCO and HFO cell lines. Both the HFO and HCO cells were cultured under the same conditions with cells plated at 50,000-cells/cm² (passage 3) in the presence of triple supplement. Nodule formation was observed in the HFO cell cultures as early as day 9, while the HCO cell cultures formed visible nodules 10 days later (Table 1). However, by day 21 there was no difference; both cell lines arrived to a similar end point and 100% of the HFO and HCO cells had macroscopic nodules (Figure 8).
Table 1. Microscopic observation of retraction and nodule formation.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Day initial retraction visible</th>
<th>Day first nodule visible</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCO</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>HFO</td>
<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>

Figure 8. Nodule formation by HCOs and HFOs at 21 days.
Calcium-rich stained Alizarin Red nodules were present in 100% of the HCO and HFO samples.
2.5.3 Formation of calcium rich regions

After 21 days, the samples were stained for calcium-rich areas with Alizarin Red. Light microscopy showed red mineralised nodules (Figure 9).

Figure 9. HFOs cultured for 21 days and stained with Alizarin Red for calcium-rich nodules (dark red).

Scale bar 100 μm.
2.5.4 Hydroxyapatite deposition

The OsteoImage™ (Lonza, PA-1503, Walkersville, MD) end point hydroxyapatite assay was used to provide a relative quantification of hydroxyapatite deposition in the HCO and HFO cell cultures over a 21-day period. A fluorescent plate reader was set at 492 nm excitation and 520 nm emission (Synergy 2 Multi-mode microplate reader (Biotek, USA) (Appendix 6.10).

There was a strong effect of time on production of hydroxyapatite (F 25.8; p=0.001). Consistent with the macroscopic observation of the mineralisation process (Table 1), the initial deposition of hydroxyapatite was detected earlier in the HFO cell cultures compared with the HCO cell cultures with differences in hydroxyapatite detection evident at day 7 (p=0.01) and at day 14 (p=0.002). However, by day 21 similar end points had been reached in both the HCO and HFO cell cultures (p=0.435).

Figure 10. Hydroxyapatite deposition by HCOs (red) and HFOs (blue). A time dependent increase in amount of hydroxyapatite was seen for both cell lines over 21 days. Results are expressed as mean fluorescence +/- SEM for six wells. *p<0.05; **p<0.01; ****p<0.0001.
2.5.5 Proliferation rates of HFO and HCO cells

There was a significant interaction between time and growth and proliferation of cells over time (F=15.1, p<0.001). HCOs and HFOs showed different rates of proliferation over the 21-day period (Figure 11). Analysis of polynomial contrast indicated a quadratic term, suggesting that there is an increase followed by a decrease in growth and proliferation. There was significant increase in proliferation in the first 7 days HFOs (p<0.0001) and 14 days for HCOs (p<0.0001), after which a decrease in proliferation was observed by day 21. The decrease in proliferation in both cell lines coincided with an increase in the production of hydroxyapatite and the formation of visible mineralised nodules.

Figure 11. Relative proliferation rates of HCOs (red) and HFOs (blue) over 21 days.
Results are expressed as mean fluorescence +/- SEM for six wells. **p<0.01; ****p<0.0001.
2.6 Discussion

The protocols and data reported in the present chapter form the basis of a preliminary study designed to characterise the behaviour of cultured HCO and HFO cells grown in the presence of triple supplement. Cellular proliferation and the initiation and progression of mineral formation were characterised at selected intervals over a 21-day period. Changes in cellular morphology and the progression of mineralisation were investigated using light phase contrast microscopy.

In their landmark paper, Bellows and co-workers documented the initiation of mineralised nodule formation by rat calvarial osteoblast cultures. It was observed that formation of mineralised nodules was more extensive in cells cultures with triple supplement than in un-supplemented controls (Bellows et al., 1986). β-glycerophosphate (β-GP) is added to the culture medium as a source of organic phosphate for alkaline phosphatase to convert to inorganic phosphate to induce mineralisation of osteoid matrix nodules, without which the osteoid does not mineralise. Removal of β-GP from cultures stops the initiation of mineralisation (Bellows et al., 1991). This explains the colourmetric differences observed between cell cultures grown with triple supplement and their un-supplemented controls, when stained with Alizarin Red. Mineralised nodules were visible under light microscopy as dark red areas, indicating the presence of calcium deposits. The most intense colour was seen for the HFO cells cultured with triple supplement. Our observations confirm the relationship between intracellular calcium phosphate in osteoblasts and their role in mineralising the extracellular matrix (ECM). All further experiments were therefore carried out in the presence of triple supplement.

Changes in morphology of the osteoblasts were observed to accompany changes in cellular function. Distinct changes in cellular morphology were observed as the cells progressed through the stages of attachment, proliferation and mineralisation of the ECM. Light phase contrast microscopy showed that the osteoblasts spread out and flattened with extensive filopodia in early cultures.
before reaching confluency. Filopodia are thin cell extensions composed of bundles of actin. They are known to have multiple functions, the most important being cell-surface and cell-cell attachment as well as migration (Mattila and Lappalainen, 2008). With further proliferation, three zones could be seen microscopically in both HFOs and HCOs within the cell culture wells. At the periphery, osteoblasts were more spread out with filopodia. Towards the middle, the cells were tightly packed with a swirling pattern. At 21 days, the size of the cellular area retracted, with all the cells migrating to the centre forming a macroscopic nodule. In summary, morphological observations have shown that various morphologies exist within a cell culture and this changes over time. No morphological differences were obvious between HCO and HFO cells. These morphological observations are in agreement with other published work (Malaval et al., 1999).

Hydroxyapatite deposition by HCOs and HFOs cells was quantified over 21-days. Macroscopic findings were compared to the fluorescence results in order to validate the use of OsteoImageTM for further work. The HFOs deposited more hydroxyapatite earlier than HCOs and these results matched the observations for nodule formation. Microscopically, it was observed that HFOs started to retract slightly earlier and subsequently formed nodules earlier. Macroscopically, the first visible mineralised nodule appeared at day 9 for HFO and by day 19 for HCO culture. However, the relative amount of hydroxyapatite, at the 21-day point was similar. All plated cells behaved in a similar pattern with only a small variation within a cell line. This experiment should be repeated to account for possible biological variations and to evaluate differences between mineralisation rates of HCOs and HFOs.

Each macroscopic nodule measured 1-2 mm in diameter by day 21. It has also been previously established that the number of nodules correlate to the seeding density (Bellows et al., 1986). In our experiment, the seeding density was 50,000 cells/cm² when cultured in 48-well plates, and one large nodule per well was formed.
The observed slower rate of mineralisation observed in HCO cell cultures is in agreement with work by Dziedzic-Goclawaska and co-workers who studied femoral and calvarial osteoblast mineralisation in samples from 300 human foetuses (Dziedzic-Goclawaska et al., 1988). The calvarial mineralisation rate was gradual and slower than in the femoral bones. In femoral diaphysis of 6-8 week old foetuses, mineral content was high and the mineral showed a high degree of crystallinity. The authors concluded that the differences in the kinetics of the mineralisation process between the weight-bearing bones and bones that are not exposed to strong external forces are most probably genetically conditioned. It must be appreciated that osteoblast proliferation and differentiation is regulated in vivo by a complex array of local and systemic signalling pathways and growth factors, which is not represented in an in vitro environment.

Proliferation was measured as enzymatic activity in the mitochondria. The conversion of resazurin to a fluorescent resorufin is proportional to the number of metabolically active cells (Czekanska et al., 2011). The limitation of PrestoBlue™ resazurin assay is that the intensity of the colour doesn’t only depend on number of viable cells, but also on the incubation time (Czekanska et al., 2011; Xu et al., 2015). The use of standardised incubation time between groups is crucial to allow direct comparison. Subtraction of the background fluorescence of the media and any additional scaffold was undertaken (Munshi et al., 2014). This is because even in the absence of cells, there is interaction between the resazurin dye and cell culture media.

Preliminary results showed that the rates at which the HFO and HCO cells proliferated differed. A more rapid proliferation of HFO cells was observed compared to HCOs. The cells were seeded at equal seeding densities and at 24 hours the proliferation levels were similar. HFOs achieved their peak in proliferation at 7 days, which was followed by a decrease. HCO cells achieved their peak at 14 days. This pattern of increased proliferation, followed by a decrease in proliferation and onset of mineralisation is typical of osteoblasts. The observed pattern of more rapid proliferation in HFO cells may help explain their earlier onset of mineralisation.
The work presented in this chapter provides insights into the differences between human femoral and calvarial osteoblasts cultured in monolayers. Differences depend on cell sample numbers, variation between species, age of the donor, cellular extraction method, passaging methods, as well as passage number. Previous work using rat osteoblasts has shown that rat calvarial osteoblasts have a high proliferative capacity and produce well-defined mineralised nodules in comparison to femoral osteoblasts, which produce a dispersed mineralised matrix. The authors attribute these differences to technical difficulties in isolating osteoblasts from the long bones of rats, possibly affecting the osteogenic potential of the extracted femoral cells (Declercq et al., 2004). Other work carried on foetal human osteoblasts has shown slower mineralisation of calvarial osteoblasts (Dziedzic-Goclawksa et al., 1988), which is in agreement with our preliminary findings. Our study used HCO and HFO cells from two separate donor foetuses aged 20-22 weeks old.

We have highlighted the biological differences between HCO and HFO cells. Results for these cell lines showed that there is a difference in the number of days they are cultured in triple supplement before the mineralisation process starts. Femoral cells started to form nodules earlier than the HCOs. However, both cell lines had formed the same amount of hydroxyapatite after 21 days. In summary, this work confirmed that in our hands the HCOs and HFOs were capable of making mineralised nodules and would be suitable for the future validation of a 3-D model.
2.7 References


Chapter 3
Thiol-modified hyaluronan-gelatin PEGDA cross-linked hydrogel: a study of osteoblast proliferation and mineralisation in 3-D culture

3.1 Introduction

This chapter will examine the use of hydrogel for the purpose of 3-D culturing of osteoblasts. A general explanation of hydrogels is followed by an evaluation of the thiol-modified hyaluronan-gelatin PEGDA semi-synthetic hydrogel used in the experiments in this, and following, chapters. The challenges of determining cell viability in 3-D culture systems will be reviewed.

3.1.1 Requirements for an ideal scaffold for bone tissue engineering

Comparisons of 3-D and monolayer culture methods show that cell behaviour and signalling on plastic or equivalent monolayer surfaces, may be irrelevant to the bio-dynamics of the in vivo extracellular matrix (Cukierman et al., 2001). For this reason, there has been a significant proliferation of literature on 3-D culture systems (Prestwich, 2007). An in vitro 3-D culture system aims to model the natural in vivo extracellular matrix (ECM) scaffold. ECM is a tissue-specific, heterogenous mixture of biopolymers, proteoglycans, fibronectin, type-1 collagen and water. Cells modify the ECM in their vicinity to be suitable for their signalling, protein storage and transport in order to optimize their own specific functions (Pedersen and Swartz, 2005). Cell interaction with the ECM has an effect on cell morphology, growth, proliferation, function and may also shift the phenotypic expression of already differentiated cell types (Masi et al., 1992).

An ideal bone tissue scaffold must be biocompatible, osteoinductive and osteoconductive. Additionally, it should have adequate mechanical properties, pore size and be bioresorbable. Osteoinductivity is the most challenging property to replicate in vitro. It refers to the ability of the scaffold to induce endogenous signalling cascades and attract surrounding non-differentiated cells,
via molecular signalling, to become osteoblasts and stimulate bone formation. Biocompatibility refers to the ability to support normal cellular activity without toxic effects on the host. Osteoconductivity describes the property of a material that allows bone cells to adhere, proliferate and secrete extracellular matrix. Finally, the scaffold should be able to become vascularised, without which bone formation does not occur (Bose et al., 2012).

Mechanical properties of an ideal scaffold should match the in vivo properties of bone (Hutmacher et al., 2000). Bones vary in their compressive strength depending on their function and load they need to bear. For example, trabecular bone has a compressive strength of 2-20 MPa compared to 100-200 MPa for cortical bone. Cortical and trabecular bone have distinct differences in their architecture and vary in their porosity, their levels of mineralisation and in the level of organisation of their matrices (Rho et al., 1998).

The in vitro scaffold should have mechanical strength and stiffness to withstand normal in vivo mechanical stimulation and have adhesive sites to which cells can attach. Matrix stiffness has been shown to affect phenotype, migration, expression of actin and adhesion proteins, spreading, communication proliferation and differentiation of osteoblasts (Marklein and Burdick, 2010; Lo et al., 2000). As an example of how cells sense and respond to substrate mechanics, in an experiment by Ghosh and co-workers fibroblasts showed higher rates of proliferation and more organized actin fibers in response to a stiffer hyaluronan-based hydrogel, while lower adhesion and increased apoptosis was observed in softer hydrogels (Ghosh et al., 2007). It has therefore been recommended to tailor the scaffold to the cell type and, if needed, to modify stiffness and incorporate adhesion sites into the hydrogel (Liu et al., 2006; Saminathan et al., 2015).

Osteoblasts are anchorage-dependent cells that require scaffolds of adequate stiffness. Osteoblasts release an un-mineralised cross-linked collagen matrix, called an osteocalcin-rich osteoid, which becomes mineralised over a period of weeks. Although there is uncertainty in the literature about the thickness of the osteoid and its stiffness, a preliminary analysis showed it is about 350 (+/- 100)
nm thick and has a stiffness of $27 \pm 10$ kPa, similar to type-1 collagen gel (Engler et al., 2006). The replication of tissue stiffness, estimated to be 30kPa for pre-mineralised collagenous bone, is an important consideration when choosing a scaffold for osteoblast culture (Rehfeldt et al., 2007).

Scaffold porosity or the presence of interconnected channels is another consideration in the selection of a scaffold. The degree of porosity has to be balanced with its detrimental impact on the compressive strength of the scaffold. Generally, a 100 µm pore diameter is cited as the minimum requirement for the diffusion of oxygen and nutrients (Bose et al., 2012), but this can vary as tissues have different nutritional and metabolic demands. Cartilage has low metabolic activity and doesn’t require as much oxygen, and thus is able to be engineered into large tissue structures (Yang et al., 2001). In contrast, osteoid ingrowth requires pores of 40-100 µm and mineralised bone ingrowth requires 100-350 µm pore diameter. These figures are approximate, based on rat models, under stationary culture conditions, usually using synthetic polymers, and have not been supported by all studies (Robinson et al., 1995). An optimal pore size for osteoblasts cultured in collagen hydrogels has been reported to be 100-150 µm (O’Brien et al., 2005). However, considering that osteoblasts and their precursors are less than 10 µm in diameter and can move through small spaces in vivo, Whang and co-workers proposed that optimal pore size be re-examined, after conducting a successful experiment using a synthetic polymer with a median pore size as low as 16 µm (Whang et al., 1999). They concluded that the degree of porosity is more critical than the diameter of each pore. In summary, porosity requirements may depend on the materials used and are cell-type specific.

The bioresorbability of the implanted scaffold is a property that allows replacement of the scaffold by the regenerated tissue. The rate of degradation should match that of tissue formation for good healing to occur. Replacement of a scaffold in a timely manner, in synchrony with in-growth of new bone tissue is crucial. Materials that degrade too quickly may result in defects, whereas slow degrading materials may inhibit new tissue formation and prevent proper integration. Therefore, controllable degradation in tissue engineering is important. Controlled bioresorbability remains one of the key challenges in bone
tissue engineering (Bose et al., 2012; Teixeira et al., 2014b).

3.1.2 Hydrogel: a 3-D hydrophilic polymer scaffold

Hydrogel is a class of materials formed from the cross-linking of hydrophilic monomers into a network, allowing accumulation of water. Hydrogels can be made of natural or synthetic polymers or a combination of both. Modifying the polymer with a reactive functional group, such as thiol, forms a covalently cross-linked network (Teixeira et al., 2014a).

Hydrogels are an appealing scaffold because they are structurally similar to the extracellular matrix of many tissues and their structure can be tuned and modified to specific cell culture requirements (Annabi et al., 2014). Changing the base polymer or the crosslinking can modify the stiffness, degradability and morphology of hydrogels (Teixeira et al., 2014b). Hydrogels are also resorbable and so if implanted have a smaller risk of eliciting an inflammatory response (Schneider et al., 2004). The main disadvantage of hydrogels is that they are weak under mechanical load (Hoffman, 2002). Hydrogels have been utilized in a variety of applications such as, tissue engineering, drug and growth factor delivery, as BMP-2 carriers, in the creation of multi-layered co-culture constructs and also as a model to study genetic bone mutations (Drury and Mooney, 2003; Elisseeff et al., 2005; Martínez-Sanz et al., 2011; Kang et al., 2011). Their role as matrices in skeletal tissue engineering has also grown (Elisseeff et al., 2005). In tissue engineering cells can be encapsulated into the hydrogel scaffold during gelation whereas with the hard surface scaffolds the cells remain on top. The two main categories of hydrogels are synthetic polymers and natural hydrogels.

3.1.2.1 Synthetic polymer hydrogel

Synthetic hydrogels are commonly used because their properties are easily controlled and reproducible. Synthetic hydrogel can be made from hydrophilic polymers such as polyethylene glycol (PEG) and polyethylene oxide (PEO). Their cross-linking can be altered and the mechanical properties controlled by varying the molecular weights of the starting polymer, increasing polymer concentration or modifying the reactive groups. The degradability of hydrogels ideally should
match that of tissue formation for good healing to occur. Degradation can be controlled in synthetic hydrogels by enzymatic degradation. However, synthetic hydrogels have a disadvantage: they do not facilitate the adhesion and spreading of seeded cells and need modification with biologic molecules for cells to respond (Bartold et al., 2006; Drury and Mooney, 2003; Teixeira et al., 2014a). This is especially important for the anchorage-dependent osteoblasts. It has been documented that PEG-only hydrogel, if modified with an adhesive peptide, creates a more natural environment, and results in increased adhesion and increased mineral deposition by the calvarial rat osteoblasts (Burdick and Anseth, 2002).

3.1.2.2 Natural polymer hydrogel

Hydrogels made from natural polymers have gained interest from the tissue-engineering field due to their ability to mimic the extracellular matrix. These properties enable survival, proliferation and the maintenance of cell phenotype. Extracellular components such as collagen, hyaluronan, alginate and chitosan have all been utilized as a hydrogel scaffold for tissue engineering (Drury and Mooney, 2003).

Cell culture within type-1 collagen gel was initially tested with fibroblasts (Elsdale and Bard, 1972). Osteoblasts cultured in a collagen-1 gel matrix expressed significantly more alkaline phosphatase, and showed increased colonization of the 3-D network and osteocalcin synthesis. Collagen-1 based matrix systems also showed favourable effects on attachment and growth of osteoblasts, making them a potential candidate for use as a bone graft material (Masi et al., 1992).

Although cells can remodel natural hydrogels, their mechanical properties cannot be changed once gelation has occurred. Further limitations of natural hydrogels include the potential for variability between samples and carry the potential risk of disease transmission (Teixeira et al., 2014a). Therefore, other hydrogel formulations, the properties of which can be modified, have been explored.
3.1.2.3 Thiol-modified hyaluronan-gelatin, PEGDA cross-linked hydrogel: a semi-synthetic hydrogel

Considering the various advantages and disadvantages of the synthetic and natural polymer hydrogels, a combination hydrogel or a semi-synthetic hydrogel mimicking the extracellular matrix has been developed by Prestwich and co-workers (Prestwich et al., 2007). The thiol-modified hyaluronan-gelatin PEGDA cross-linked hydrogel is marketed as Extracel™, or more recently as HyStem-C™ (ESI Bio, USA). The components are available as lyophilized thiol-modified hyaluronan (Glycosil®), thiol-modified gelatin, which is a denatured collagen (Gelin-S®) and a cross-linker polyethylene glycol diacrylate PEGDA (Extralink™). The process of lyophilisation involves the removal of water to improve the physical and chemical stability of the resultant hydrogel. In the present study human calvarial and femoral osteoblasts were cultured in this semi-synthetic hydrogel with and without mechanical compressive strain. The advantage of this hydrogel is that its mechanical properties can be manipulated by varying the quantity of the synthetic polymer. At the same time, the presence of natural polymers hyaluronan and gelatin mimic the biocompatibility properties of in vivo extracellular matrix.

3.1.2.4 Role of hyaluronan and gelatin in the native extracellular matrix

Hyaluronan (HA) forms a major component of extracellular matrix with key roles in morphogenesis, matrix assembly and homeostasis. It is non-immunogenic and serves a variety of biological functions (Knudson and Knudson, 2001; Martínez-Sanz et al., 2011). It is found in high concentrations in the early stages of bone healing and has been shown to support bone growth (Patterson et al., 2010). Although hyaluronan has been used in medicine for over thirty years, its use in tissue engineering has been more recent as an important building block for 3-D cell culture and tissue engineering (Burdick and Prestwich, 2011; Prestwich, 2011). HA-based hydrogels have been shown to maintain the viability of encapsulated cells, although most of the research is based upon the use of fibroblast cell cultures (Patterson et al., 2010; Saminathan et al., 2015). HA can be modified and cross-linked into a hydrogel to form a more stable scaffold.
Modifications of cross-linking also affect the degradation rate (Kwang et al., 2007; Patterson et al., 2010).

Gelatin is produced by hydrolysis of collagen, and it is formed after breaking the natural triple helix of collagen into single strand molecules. While it lacks the fibrous structure of native collagen, gelatin is soluble, inexpensive and easily modified (Hunt and Grover, 2010; Zheng et al., 2003). Because cells cannot adhere to the HA-only hydrogels, adding gelatin to hyaluronan-based hydrogels has been used to improve cellular adhesion (Zheng et al., 2003; Burdick and Prestwich, 2011; Prestwich, 2007).

3.1.2.5 Thiol modification of hyaluronan and gelatin

The mechanical properties of hyaluronan and gelatin can be modified using various methods. These include the addition of reactive functional thiol groups to increase the cross-linking between natural polymers through the modification of the cross-linker concentration or through addition of fillers to provide structural reinforcement (Teixeira et al., 2014b).

Extracellular matrix (ECM) is a heterogenous collection of proteins and glycosaminoglycans, or GAGs, connected by covalent and non-covalent molecular interactions. Covalent bonds connect sulfated GAGs to core proteins to make proteoglycans. Non-covalent bonds exist between proteoglycans and hyaluronan and also between the fibrils that make up the triple helix of collagen.

HA is a linear glycosaminoglycan and is the only non-sulfated GAG present in the ECM. In its natural form, hyaluronan has weak mechanical properties and rapid degradation in vivo. However, being non-covalently bound and non-sulfated allows chemical modification to address these limitations. One such chemical modification is replacing the native carboxylic groups on the hyaluronan backbone with thiol-containing DTPH groups resulting in a thiol-modified biopolymer, which cross-links slowly in the presence of oxygen (Ghosh et al., 2005).
Thiol modification of gelatin increases cyto-adhesiveness of the cells to the hydrogel. Gelatin provides peptide adhesion ligands for integrins on cell surfaces. Addition of at least 40% w/v thiol-modified gelatin into a hyaluronan-based hydrogel significantly improved attachment and cell spreading (Zheng et al., 2003).

The thiol-modified gelatin and hyaluronan hydrogel properties can be altered by changing the amount of either the thiolated hyaluronan or thiolated gelatin or by varying the molecular weight of the acrylate cross-linker, polyethylene glycol diacrylate (PEGDA) (Burdick and Prestwich, 2011; Prestwich, 2011).

3.1.2.6 Polyethylene glycol diacrylate (PEGDA) cross-linker

PEGDA is a biocompatible synthetic polymer used as a cross-linker to rapidly and covalently link thiol modified hyaluronan and gelatin, speeding up the gelation process and decreasing the rate of degradation (Figure 12) (Serban et al., 2008; Prestwich, 2007).

The ability to modify the properties of hyaluronic acid-based hydrogels, such as Extracel™, has made it an attractive material for tissue engineering with most studies carried out on articular cartilage. Having control over gelation rate and the degree of cross-linking are particularly useful properties.

![Diagrammatic representation of a semi-synthetic hydrogel analogue of natural ECM.](image)

**Figure 12.** Diagrammatic representation of a semi-synthetic hydrogel analogue of natural ECM.

3.1.2.7 Applications of thiol-modified hyaluronic acid and gelatin hydrogel

Although there are reports on the use of chemically modified hyaluronan-gelatin cross-linked semi-synthetic extracellular matrix, the inventor of Extracel™/HyStem, Prestwich and co-workers, have contributed most to the field, raising concern about conflict of interest and the potential for biased reporting. In addition, most studies are proof-of-concept in vitro investigations, in vivo studies using small animal models or early exploratory studies on humans. There are no published studies looking at the behaviour of osteoblasts in this hydrogel.

An independent study, which evaluated twelve different hydrogel products, concluded that cells encapsulated in Extracel™ showed the highest maintenance of viability (Murphy et al., 2013). However, in this study, keratinocytes were used and this finding cannot be extrapolated to osteoblasts.

In addition to its use in 3-D cell culture, Extracel™ has been used in other applications. These include drug testing, tissue regeneration, post-surgical adhesion prevention, and repair of hard and soft tissues in in vivo animal models (Prestwich, 2007; Shu et al., 2004). Its efficacy as a cell delivery scaffold was demonstrated in the rabbit model, where it was used to repair defects in cartilage. Fibroblasts were encapsulated into the gel scaffold, allowed to proliferate and the scaffold was later implanted into a cartilage defect. The implanted material integrated with the host tissue, showed in-growth of cartilage and an ability of fibroblasts to remodel the hydrogel (Liu et al., 2006). In skeletal defect small animal models, it has been successfully used as a carrier for BMP-2 (Kim et al., 2007; Patterson et al., 2010) and for demineralised bone matrix (DBM) particles (Liu et al., 2006). However, the lyophilized form rather than the gel form of this material was the most successful delivery vehicle for bone healing. This could be because the lyophilized form is a solidified sponge and offers a better structural scaffold for the anchorage-dependent osteoblasts. In addition, hyaluronan-based hydrogels have been trialled in bio 3-D printing vessel-like constructs (Skardal et al., 2010), as a carrier of BMP-2 and as a biocompatible scaffold for cells in the regeneration of bone in animal models.
with small calvarial bone defects.

3.1.2.8 Mechanical load and hydrogel

The continuous remodelling of bone and cell differentiation depend on presence of mechanical signals. However, hydrogels are too soft to allow mechanical loading and hence require modification. Vanderhooft and co-workers have demonstrated that semi-synthetic hydrogels can have their stiffness and mechanical properties adjusted and recommended the use of lyophilized hydrogel sponges (Vanderhooft et al., 2009; Liu et al., 2006). Saminathan and co-workers used this hydrogel to engineer periodontal ligament constructs under tensile and compressive strain. They found the viability of the fibroblasts was high, which is in agreement with other studies (Murphy et al., 2013). They also reported that the hydrogel used in their study could not withstand mechanical strain. However, they used a soft hydrogel formulation, which did not modify the concentration of PEGDA. They suggested the addition of thin collagen sheets to the hydrogel to improve its resistance to compressive mechanical strain (Saminathan et al., 2015). Other developments have included the creation of hybrid scaffolds and the modification of hydrogels through the addition of other materials. Nano-silicate particles were incorporated into a gelatin cross-linked hydrogel and this gave rise to an increase in surface interactions, an increase in compressive modulus and an increase in mineralised matrix formation (Xavier et al., 2015). Bio-3-D printing of multi-phasic scaffolds is also emerging - with various scaffolds combined to suit individual cell needs while allowing different cell populations to interact with each other (Obregon et al., 2015).

While it is well accepted that the stiffness of a material alters cell spreading, organization, migration, and differentiation, the optimal stiffness and diffusion properties of hyaluronan-Gelin-S-thiol-modified hydrogel for femoral and calvarial osteoblasts are unknown. Cell attachment is complex and surface properties of hydrophobicity, charge, roughness, porosity and stiffness affect cell behaviour (Schneider et al., 2004). Unlike fibroblasts and mesenchymal stem cells, which are the two most common cell types studied in hydrogel research, proliferation and adhesion of osteoblasts has not been investigated using semi-
synthetic hyaluronan-gelatin hydrogels. Osteoblasts are anchorage-dependent cells and a stiffer, hydrophilic, positively charged surface has been shown to favour their spreading and adhesion. Increasing the concentration of PEGDA cross-linker increases stiffness. However, if only the synthetic PEGDA hydrogel is used, cell adhesion and viability may be decreased (Burdick and Anseth, 2002).

### 3.1.3 Determination of cell viability in 3-D cultures

Accurate evaluation of cell viability relies on quantification of living and dead cells (Gantenbein-Ritter et al., 2008). It is also important to account for differences between culture systems when assessing viability. Cell proliferation for monolayers and 3-D hydrogel cultures vary due to differences in geometry, surface area and diffusion kinetics. All these factors make direct comparisons between culture conditions challenging (Bonnier et al., 2015).

Determining cell viability in 3-D culture systems can be challenging and has been the subject of much research. In a study by Gantenbein-Ritter and co-workers, the viability of cells in a 3-D digestible, fibrinogen scaffold was assessed by three methods. All methods performed well in terms of accuracy and reliability with a similar range of standard deviations. However, all had limitations and either under or over-estimated cell viability to some extent (Gantenbein-Ritter et al., 2008).

The main techniques used with 3-D scaffolds to determine dead to live cell ratio can be divided into fluorescence and non-fluorescence staining groups. It is important to understand the limitations of the stains used, and to use a background control to account for the background fluorescence of the culture system. Fluorescence techniques commonly use calcein acetoxyethyl/ethidium homo-dimer-1 (CaAM/EthH) and fluorescein diacetate/propidium iodide (FDA/PI). Additional fluorescent staining techniques include those, which measure cell metabolism. Non-fluorescent staining includes trypan blue staining after cell recovery from the 3D culture system (Gantenbein-Ritter et al., 2008; Boyd et al., 2008).
To determine the dead to live ratio using fluorescent staining, two stains are used which differ in their penetration of the cell membrane. The FDA/PI stains were used in the present study. FDA stains viable cells fluorescent green. It penetrates the cell wall phospholipid bilayer and is enzymatically degraded by esterases producing a fluorescent green stain in live cells (O’Brien and Gottlieb-Rosenrantz, 1970). However, using high concentration can increase the background noise and the green emission can extend into the red spectrum. The background fluorescence can be minimised by using as low a concentration as is necessary for detection, in the final working solution (Boyd et al., 2008). PI stains dead cells. It cannot enter through an intact cell membrane and can only pass through a damaged cell membrane binding to DNA and RNA to form a bright red fluorescent colour, marking the nucleus of the cell (Jones and Senft, 1985).

Using confocal microscopy to count fluorescently stained cells can over-estimate live, dead and the total number of cells (Gantenbein-Ritter et al., 2008). The confocal laser scanning microscope technique therefore might not be appropriate for quantitative purposes, especially in scaffolds where water content, ECM and optical properties may vary between samples. Optical properties are also affected by the size and depth of the sample with a maximum predictable scan depth of 200 µm. However, the use of confocal microscopy has the advantage of a series of images at various depths into the sample providing qualitative information about spatial arrangement within the matrix and about cell morphology (Boyd et al., 2008).

Enzymatic reduction assays are commonly used to assess cell proliferation. The resazurin-reduction assay, PrestoBlue™ was used in the present study. This utilizes membrane permeable solutions that upon enzymatic reduction by viable cells form resorufin, a fluorescent pink compound. Resorufin is excreted outside the cells into the medium, resulting in a visible colour change from blue to pink (Figure 13). The conversion is proportional to the number of metabolically active cells, but it does not provide a quantification of dead cells. The change can be quantified colourimetrically or fluorometrically, but using fluorescence has been shown to be more accurate (Czekanska et al., 2011, Xu et al., 2015). Being a live-cell assay, the cells can continue to be cultured and proliferation monitored at
multiple time points or the cells can be used for further staining (Emter and Natsch, 2015; Xu et al., 2015). While relatively non-toxic, a study by Xiao and co-workers found a reduction in cell survival when cells were cultured in hydrogel if resazurin was used at concentrations higher than 100 µM (Xiao et al., 2010). Additionally, even in the absence of cells, there is interaction between the resazurin dye and cell culture media. To subtract the background fluorescence of the media and any additional scaffold, a parallel no-cell control group has been recommended (Munshi et al., 2014).

![Figure 13. Resazurin and resorufin structures.](image)

Inside cell mitochondria, resazurin is enzymatically reduced and the red resorufin is excreted outside the cells, changing the medium colour from blue to fluorescent pink. Adapted- Czekanska et al., 2011.

The non-fluorescent trypan blue is a dye commonly used to measure the viability of monolayer cell cultures. But when applied to determining viability in 3-D cell cultures, cellular recovery and the digestion of the hydrogel scaffold is required. Therefore, this method does not provide additional information on spatial arrangement of the cells inside the matrix or on morphology. Despite this limitation, Gantenbein-Ritter and co-workers found it to be the most accurate method for determining cell viability and total cell numbers in a 3-D scaffold system. However, this method may not be suitable for long-term cultures requiring scaffold digestion because the already weakened cell membrane in long-term cultures is further weakened by the collagenase digestion step.
Collagenase and hyaluronidase may also affect cell viability if used at high concentrations or for prolonged periods of time (Gantenbein-Ritter et al., 2008).

The kinetics for cell viability assays differ between monolayer and 3-D cultures (Bonnier et al., 2015). The cell viability assays that are in common use were designed for conventional monolayer cultures and have not been validated on more complex models, such as hydrogel constructs (Riss et al., 2014). Therefore, the protocols normally used for monolayer need to be modified and validated for 3-D culture. It is essential to account for the added volume of the hydrogel as this results in dilution effects (Bonnier et al., 2015). The thickness and cell density of the construct, as well as incubation times, affect the results. In addition, the construct itself can have a high affinity for the assay components and background fluorescence controls are therefore necessary to avoid false positive results. A standardization of culture parameters to limit the variables is important when validating an assay (Kijanska and Kelm, 2016). A common finding is poor penetration of the assay components into the centre of the 3-D construct. All these limitations have been recognized and new assay formulations and protocols are being investigated. Some of these formulations have detergents to enhance penetration, however, this can also have a negative effect on cell viability (Riss et al., 2014). Modified protocols include vibrating the construct to enhance contact of the assay components with the cells. Additional correlation of fluorescent assay results to trypan blue staining and manual cell counting using phase-contrast microscopy have been suggested (Gantenbein-Ritter et al., 2008). All of these limitations highlight the importance of validating assays using new culture models prior to their widespread used in experimental investigations.

3.1.4 Summary

This review has summarised hydrogel models currently employed for cell culture and described thiol-modified hyaluronan-gelatin PEGDA cross-linked semi-synthetic hydrogel, which has the ability to be tailored to mimic the in vivo ECM environment for specific cell types. The experiments in this chapter will evaluate the suitability of the semi-synthetic HyStem-C® hydrogel for the culture
of human femoral osteoblasts (HFO) and human calvarial (HCO) osteoblasts and their ability to synthesise a mineralised matrix.

The challenges of determining cell viability in 3-D culture systems were also reviewed. This chapter uses confocal microscope to highlight the limitations of molecular probes.

3.2 Hypothesis

A modified, more cross-linked, stiffer hyaluronan-gelatin PEGDA hydrogel will increase HCO and HFO attachment, proliferation and hydroxyapatite deposition.

3.3 Aims

1. To determine if the proliferation rates of HFO and HCO cells differ for a stiffer, highly cross-linked compared to a softer hydrogel.

2. To compare the morphology and the process of nodule formation of HFOs and HCOs when cultured in hydrogels at two different cross-linking densities.

3. To determine the relative levels of hydroxyapatite deposition for HFOs and HCOs cultured in hydrogel at two different cross-linking densities.

4. Determine the spatial arrangement of osteoblasts using confocal microscopy with fluorescent stains.
3.4 Materials and Methods

3.4.1 Osteoblast cell culture and cell pellet preparation

Prior to their use in experiments, commercial foetal human calvarial osteoblasts (HCOs, #4600) and femoral osteoblasts (HFOs, #4610) (ScienCell Research Laboratories) were cultured and expanded in osteoblast growth medium in T-75 flasks until 80% confluent (Appendix 6.6). Cell trypsinisation and counting were carried out according to the standard protocols (Appendices 6.1 and 6.2). After cell counting, the volume of media containing the defined number of cells was transferred into vials, centrifuged at 220xg for 5 minutes at 21°C to be pelleted. Following centrifuging, gently using a pipette, the medium on top of the pellet was discarded. The pellet was left to rest, after which any remaining medium was discarded. All experiments were undertaken using both HCO and HFO cell lines in parallel, under identical conditions and incubated in mineralisation medium (Appendix 6.6) at 37°C with 5% CO₂/95% air. All experiments were carried out under aseptic conditions in a Class II laminar flow hood.

3.4.2 Hydrogel reconstitution

The HyStem-C® hydrogel kit containing lyophilized forms of Gelin-S® and Glycosil®, Extralink™ PEGDA powder and degassed H₂O were brought to room temperature. Each component was reconstituted in degassed water to give a 1% (w/v) solution according to manufacturer’s instructions. Strict parameters of oxygen exclusion during reconstitution of the lyophilized components were followed (Appendix 6.11).

3.4.3 Hydrogel cell encapsulation

Each experiment included a parallel hydrogel-only (without cells) group to account for background fluorescence. The components for the hydrogel-only control group were reconstituted first to determine the gelation characteristics on the day. Small volumes (no more than 1 mL) of hydrogel were prepared at one time due to its rapid cross-linking. The amount of each component depended on the cross-linking ratio required (Table 2). Gelin-S® (gelatin) and Glycosil®
Hyaluronan were added to each 15 mL vial containing the prepared cell pellet of a known cell concentration, followed by the addition of PEGDA cross-linker. The components were mixed well by pipetting up and down to distribute the cells throughout the gel. Failing to do this would lead to clumping of cells or local variations in gel structure and swelling (Khetan and Burdick, 2009). Using a wide bore pipette tip, dome-like hydrogel globules were pipetted in the middle of each well (Appendix 6.12). After twenty minutes of gelation, 300 µL mineralisation medium was added to each 48-well plate and this was changed three times a week over a 21-day culture period. At days 1, 7, 14 and 21, the constructs were viewed under an inverted light phase microscope (Nikon Eclipse TiU) and cell the morphology recorded.

### 3.4.4 Culture plate layout

Four 48-well culture plates, one for each time point (day 1, 7, 14 and 21) were used for HCO and HFO cells cultured in hydrogel, which will be called constructs. Each plate contained six test constructs (hydrogel plus cells) and six control constructs (hydrogel only) for each cell line. At each incubation time-point, PrestoBlue™ proliferation and OsteoImage™ hydroxyapatite fluorescent assays were undertaken.

A second set of plates was prepared for confocal microscopy experiments using the same protocol and parameters on 8-well chambered coverslip slides (Nunc® Lab-Tek® Chamber Slide system), which allowed cells to be cultured on microscope slides and viewed under confocal microscopy.

### 3.4.5 Modification of hydrogel cross-linking properties

HCOs and HFOs were first encapsulated and cultured in the standard, softer hydrogel formulation (Table 2). Cells were encapsulated at a concentration of 50,000 cells/20 µL hydrogel (25 x 10⁵ cells / mL) in 48-well plates and cultured for 21 days.

In subsequent experiments, the effect of greater cross-linking and a stiffer hydrogel on the osteoblast proliferation rates and the ability of osteoblasts to
form a mineralised matrix was investigated. As large numbers of cells are required for mRNA analysis, a higher seeding density was investigated. The hydrogel cross-linking ratio was modified by increasing the PEGDA cross-linker to hyaluronan and gelatin ratio, HFOs were encapsulated at a concentration of 100,000 cells /20 µL hydrogel (5 x 10^6 cells / mL) and cultured in mineralisation medium for 21 days. These cells were cultured alongside a standard, softer hydrogel group, at the same cell concentration. As in the previous experiment, dome-like 20 µL hydrogel constructs were pipetted in the middle of each well and incubated in 300 µL mineralisation medium per well, which was changed every 3 days.

Table 2. Hydrogel components for either a soft (standard) or a modified, (more cross-linked) hydrogel (1 mL).

<table>
<thead>
<tr>
<th>Hydrogel components (1% w/v)</th>
<th>Standard 3:1 ratio</th>
<th>Modified 1:1 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosil</td>
<td>375 µL</td>
<td>250 µL</td>
</tr>
<tr>
<td>Gelin-S</td>
<td>375 µL</td>
<td>250 µL</td>
</tr>
<tr>
<td>PEGDA</td>
<td>250 µL</td>
<td>500 µL</td>
</tr>
</tbody>
</table>

The 3:1 ratio (which can also be represented as 1.5:1.5:1) represents a standard, softer hydrogel with a higher hyaluronan and gelatin to PEGDA cross-linker ratio.

The 1:1 ratio represents a lower hyaluronan and gelatin to PEGDA cross-linker ratio, resulting in a more cross-linked hydrogel.
3.4.6 Proliferation of femoral and calvarial osteoblasts in hydrogel

Prior to using the PrestoBlue™ proliferation assay on hydrogel, a pilot study was carried out on HFOs to compare the assay kinetics between hydrogel and monolayer culture. Background fluorescence of the hydrogel was also assessed. Six wells each of HCO and HFO were plated as 20 µL cell-hydrogel constructs and monolayers at a concentration of 50 x 10⁴/ mL in 48-well plates and cultured for 7 days in 300 µL media/per well. Hydrogel-only fluorescence background controls were also cultured in parallel. The cells were stained with 30 µL PrestoBlue™ proliferation assay (Appendix 6.9). Continuous measurements were made and the resulting fluorescence was recorded after 1, 3 and 24 hours incubation using a Synergy™ 2 multi-mode microplate reader at 540 nm excitation/ 610 nm emission wavelengths.

Following this initial study of the effect of assay incubation time, all subsequent experiments used a one-hour assay incubation time. The control and test constructs were stained with PrestoBlue™ resazurin-based fluorescent assay at a 1:10 (dye:medium) ratio and incubated for 1 hour at 37°C in the dark. Resazurin-based dyes are able to penetrate the membrane and viable cells are able to enzymatically reduce it to resorufin, a fluorescent pink compound, which is then secreted into the medium. The conversion is proportional to the number of metabolically active cells. The resulting fluorescence was measured using a Synergy™ 2 multi-mode microplate reader at 540 nm excitation/ 610 nm emission wavelengths.

3.4.7 Hydroxyapatite deposition in hydrogel

At each time point, test and control hydrogel samples were removed from the incubator and stained with OsteoImage™ (Lonza, USA) fluorescence assay to detect hydroxyapatite deposition (Appendix 6.10). The resulting fluorescence was measured in a plate reader (Synergy 2, Biotek, USA) at 485 nm excitation and 528 nm emission wavelengths. The arrangement of the deposited hydroxyapatite in hydrogel was also viewed using confocal microscopy.
3.4.8 Viability stain using confocal microscopy

To assess the viability and distribution of live and dead cells in hydrogel, samples were stained with fluorescein diacetate (FDA) and propidium iodide (PI) respectively. Working concentrations of 0.05 mg / mL FDA and 0.02 mg / mL of PI were made according to the protocol in Appendix 6.13. Test and control hydrogel samples were stained with 40 µL each of PI and FDA and incubated for 15 min in the dark, rinsed twice with 100 µL PBS and viewed at 495 nm excitation/ 517 nm emission wavelengths to detect live cells and 560 nm excitation /590 nm emission wavelengths for dead cells (Zeiss LSM 710 Carl Zeiss Microscopy, Germany).

3.4.9 Alkaline phosphatase activity detection in hydrogel

The test and control constructs were stained with one AP tablet (SigmaFast™ BCIP-NBT; Sigma Aldrich) dissolved in 10 mL de-ionized water as outlined in (Appendix 6.7) and photographed using a Canon G10 camera and Remote Capture-DC software.

3.4.10 Alizarin Red staining of calcium deposits in hydrogel

A protocol for Alizarin Red staining, which binds to calcium-rich areas, was developed for hydrogels (Appendix 6.8).

3.4.11 Data analysis

IBM SPSS Statistics Standard Edition v 22 (Il, USA) and GraphPad Prism v 6 software for Mac OS X (Cal, USA) were used for data analysis and presentation. Differences between groups were analysed using the 2-way ANOVA and were considered significant when P<0.05. Post hoc tests were performed with unpaired t-test, where appropriate.
3.5 Results

3.5.1 Introduction

The effect of hydrogel stiffness on proliferation of, and hydroxyapatite deposition by, HCOs and HFOs was investigated. Initial experiments were conducted using a standard, softer hydrogel preparation. As osteoblasts prefer to attach in a rough and rigid environment and the ability to withstand the forces of mechanical strain is a requirement of our hydrogel model, a modified more highly cross-linked hydrogel was also examined. The rates of cell proliferation and hydroxyapatite deposition by human calvarial and femoral osteoblasts were compared when cultured in these two different hydrogel formulations.

3.5.2 PrestoBlue™ proliferation assay: the difference in assay kinetics between cells in hydrogel and monolayers

To better understand differences in cellular proliferation and penetration of the PrestoBlue™ assay between monolayer and hydrogel cultures, a pilot study was carried out using a low cell seeding density of $5 \times 10^5/\text{mL}$ or 10,000 cells per 20 µL of hydrogel. As the cell proliferation and the ability of the assay stain to penetrate the hydrogel differs to those for monolayers, a direct comparison between the monolayer and 3D gel could not be made. A higher rate of proliferation was recorded for HFOs cultured as a monolayer as compared to the 3-D hydrogel model (Figure 14). Further experiments in hydrogel were carried out at a higher cell concentration.
Figure 14. HFO culture in monolayer and 3D-hydrogel, plated at a low seeding density.
A number of PrestoBlue™ assay incubation times were compared for HFO’s cultured in hydrogel (blue) and monolayer (blue cross-hatched). Data is presented as mean +/- SEM (six wells/per treatment of HFO's).

3.5.3 Proliferation of HCOs and HFOs in a soft hydrogel

HFOs and HCOs were encapsulated at 50,000 cells/20 µL hydrogel construct (25 x 105 cells / mL). In addition, 20 µL hydrogel-only samples were set up for background fluorescence subtraction. A time-dependent increase in proliferation for both cell lines was observed over the 21-day culture period. The most rapid increase in proliferation for both cell lines, HCOs and HFOs, occurred from day 7 to 14 (Figure 15).

The grand mean of proliferation, measured as the overall number of fluorescence units regardless of time, was higher for HFOs than for HCOs (F=9.5; p=0.004). Additionally, the interaction term between cell line and time showed a higher growth rate for HFOs (F=4; p=0.014), suggesting faster proliferation.
Figure 15. PrestoBlue™ proliferation assay validation using soft hydrogel.
A time-dependent rate of proliferation for HCOs (red) and HFOs (blue) was observed. The fluorescence levels were recorded following a 1 h assay incubation time. Data are presented as mean +/- SEM (six wells/cell line).

3.5.4 Hydroxyapatite deposition by HCO and HFO cells in soft hydrogel

A statistically significant time-dependent increase in hydroxyapatite deposition was detected for HCOs (p=0.0054) and HFOs (p=0.0008) by day 21. The most rapid increase in the amount of hydroxyapatite deposited was between days 7 and 14 for the HCOs and between 14 and 21 days for HFOs (Figure 16).

The grand mean of hydroxyapatite deposition, measured as the overall number of fluorescence units regardless of time, was not significantly different for the two cell lines (F=0.0; p=0.937).
3.5.5 Microscopic and macroscopic observations of HCO and HFO morphology and mineralisation in the soft hydrogel

The shape, migration, retraction and aggregation behaviour of the osteoblasts and the formation of nodule-like clusters were evident when viewed under an inverted phase contrast light microscope (Nikon-Eclipse TiU). Throughout the 21-day culture period, the osteoblasts had spherical cell morphology, indicative of their reduced ability to attach to the soft hydrogel (Figure 17).
Figure 17. Osteoblasts cultured in a soft hydrogel at day 14.
The HCOs (A) and HFOs (B) appeared spherical and had failed to form characteristic filopodia for spreading and attachment (Ph1 phase contrast 20x objective). Scale bar: 100 μm.

By day 14, mineralisation of the hydrogel matrix by the HCOs and HFOs was observed microscopically and macroscopically. These observations were in agreement with the OsteoImage™ hydroxyapatite deposition assay results. Nodules could be seen microscopically as large aggregations of cells (Figure 18).

Figure 18. Matrix mineralisation and nodule formation in a soft hydrogel.
Large nodules could be seen following 21 days of culture in a soft hydrogel (A) HCO and (B) HFO (Ph1 phase contrast 20x objective). Both cell lines produced a large number of nodules (C) HCO and (D) HFO (PhL phase contrast 2x objective). Scale bars: A, B 100 μm and C, D 1 mm.
The stained mineralised nodules could also be seen clearly macroscopically. The nodules that were seen macroscopically stained positive for mineralisation with Alizarin Red and alkaline phosphatase, as shown (Figure 19).

**Figure 19. Macroscopic observation of nodule mineralisation.**

Both HCO (A) and HFO (B) produced large macroscopic nodules, which can be seen clearly before staining (left). The nodules stained positive for calcium-rich regions with Alizarin red (upper right) and for alkaline phosphatase activity (lower right).
3.5.6 Effect of an increased amount of cross-linker in the hydrogel on osteoblast proliferation and mineralisation

The ratio of PEGDA cross-linker to hyaluronan (Glycosil®) and gelatin (Gelin-S®) was increased making a more cross-linked, stiffer hydrogel. HFOs were used in a pilot study which compared cellular proliferation and hydroxyapatite formation in cell cultures using two hydrogels of different composition and stiffness (Figures 20 and 21 respectively). The proliferation of HFOs in the stiffer hydrogel was compared to that in the standard soft hydrogel, in which HFOs were cultured in parallel, under identical conditions. Cells cultured in the soft hydrogel tended to proliferate at a faster rate than those cultured in the stiffer hydrogel over the 21-day period. This was statistically significant at day 7 (p=0.0001). However, at day 1 (p=0.096) and by days 14 (p=0.061) and 21 (p=0.716) the differences were not statistically significant. The most rapid period of proliferation occurred between 7 and 14 days.

Figure 20. Effect of increased hydrogel crosslinking on the proliferation of human femoral osteoblasts

The HFOs were cultured in a more highly cross-linked, stiffer (blue hatched) or a standard, softer (blue) hydrogel and the proliferation rate measured over 21 days using the PrestoBlue™ assay. Data are presented as mean +/- SEM (six wells/per treatment of HFO’s). ***p<0.001.
Hydroxyapatite deposition by HFOs was measured with the OsteoImage™ fluorescent assay at days 14 and 21 (Figure 21). There was a significant increase in hydroxyapatite deposition by HFOs in the soft hydrogel (p<0.001) and the more cross-linked hydrogel (p=0.0072) between 14 and 21 days. The comparison between two hydrogel compositions showed no significant effect on hydroxyapatite deposition (F=0.34; p=0.56), indicating that HFOs can deposit hydroxyapatite in both hydrogel compositions. The graph shows background fluorescence (grey bars), highlighting the importance of adequate rinsing, especially in early mineralisation cultures, where a false positive reading may occur due to background fluorescence.

**Figure 21. Hydroxyapatite deposition by HFOs in a hydrogel with greater cross-linking.**

The HFOs were encapsulated and cultured in a standard, soft (blue) and stiff (blue hatched) hydrogel with greater cross-linking and the amount of hydroxyapatite deposition measured at 14 and 21 days. Background fluorescence is shown in grey. Data are presented as mean +/- SEM (six wells/cell line). **p<0.01; ***p<0.001.
3.5.7 Proliferation and mineralisation of HFOs and HCOs seeded in a hydrogel with greater cross-linking and at high seeding density

The effect of seeding the osteoblasts at a high cell density was investigated, as large numbers of cells were needed for mRNA expression analysis. In this experiment, both HCOs and HFOs were encapsulated at 100,000 cells per 20 µL of highly cross-linked hydrogel (5x10^6 cells / mL). The HCOs and HFOs increased in their proliferation at this density over 21 days. The osteoblasts proliferated slowly for the first 7 days and the rate of HFO proliferation decreased by day 7. The most rapid rate of proliferation occurred between days 14 and 21 for both cell lines with no difference detected between HCO and HFO at day 21 (p=0.910) (Figure 22).

The grand mean of proliferation, measured as the overall number of fluorescence units regardless of time, was higher for HCOs than for HFOs (F=6.2; p=0.016). The interaction term between cell line and time was not significant (F=2.5; p=0.067), suggesting similar proliferation rates over time between HCO’s and HFO’s. At day 7, there was a significant decrease in proliferation in HFO’s (p=0.0002). Proliferation increased from day 1 to day 21 in HCO (p<0.0001) and HFO (p=0.0016) cell lines.
Figure 22. Proliferation of HCOs and HFOs cultured at a seeding density of 100,000 cells per 20 µL in a hydrogel with greater cross-linking.

The HCOs (red) and HFOs (blue) were cultured in hydrogel with greater cross-linking and the proliferation rate measured over 21 days using the PrestoBlue™ assay. Data are presented as mean +/- SEM (six wells per cell line). **p<0.01; ***p<0.001 ****p<0.0001.
Hydroxyapatite deposition increased in both cell lines from day 14 to day 21: HCO (p=0.0108) and HFO (p=0.042) (Figure 23). There was no difference in how the two cell lines behave over time (i.e. interaction cell line x time; F=2.7; p=0.115). There was an almost statistically significant difference in the total hydroxyapatite deposited by the HCOs and HFOs, measured by the total relative fluorescent units (p=0.053), indicating both cell lines can deposit hydroxyapatite in the modified stiff hydrogel.

**Figure 23. Hydroxyapatite deposition by HCOs and HFOs at a seeding density of 100,000 cells per 20 µL in a hydrogel with greater cross-linking.**

The HCOs (red) and HFOs (blue) were cultured in a hydrogel with a greater cross-linking and the amount of hydroxyapatite deposition measured at 14 and 21 days. Background fluorescence was subtracted. Data are presented as mean +/- SEM (six wells/cell line). *p<0.05
3.5.8  Morphology and mineralisation of HCOs and HFOs cultured at high seeding density in a highly cross-linked hydrogel

HCOs and HFOs encapsulated into a highly cross-linked hydrogel (Figure 24) behaved quite differently compared to those encapsulated in the standard, softer, gel (Figure 17). Osteoblasts cultured in the highly cross-linked hydrogel formed networks, with the growing filopodia extensions indicative of their increased ability to attach and anchor themselves to the modified hydrogel. Inverted phase contrast light microscopy was used to view the cells encapsulated in hydrogel (Figure 24).

**Figure 24. Osteoblasts cultured in a highly cross-linked hydrogel at day 14.**

When the HCO (A) and the HFO (B) were encapsulated into a highly cross-linked hydrogel they behaved quite differently when compared to those encapsulated in the standard softer gel. The HFOs formed more dramatic filopodia extensions with the formation of more net-like networks compared to the HCOs (phase contrast 20x objective). Scale bar: 100 μm.
The process of mineralisation with the deposition of hydroxyapatite detected at day 14 in the previous OsteoImage™ experiment could also be seen microscopically in the highly cross-linked hydrogel. At day 21, the HCOs and HFOs had made large nodule-like structures in the hydrogel matrix. Inverted phase contrast light microscopy was used to view the cells encapsulated in hydrogel (Figure 25).

![Figure 25. Matrix mineralisation and nodule formation in a highly cross-linked stiffer hydrogel.](image)

Magnified images (Ph1 phase contrast x20 objective) of nodules following 21 days of culture (A) HCO and (B) HFO. Many nodules could be seen at a smaller magnification (PhL phase contrast x2 objective) for both (C) HCO and (D) HFO, dispersed throughout the hydrogel scaffold. Scale bars: A, B 100 μm and C, D 1 mm.
3.5.9 Confocal microscopy observation of viability and mineralisation of osteoblasts cultured in hydrogel

FDA is commonly used for the identification of viable cells grown in monolayer. When employed for the quantitation of viable cells cultured in 3-D, however, the assay incubation time needs to be extended to ensure the stain reaches the centre of the hydrogel construct. Using confocal microscopy and an incubation time of 15 minutes, the FDA dye appeared to only bind to viable cells at the outer edge of the hydrogel (Figure 26 A). The suitability of the Osteolmage™ reagent, which binds hydroxyapatite, for use with hydrogels was also examined. A similar result was observed with the fluorescent stain binding only to hydroxyapatite at the outer region of the hydrogel constructs (Figure 27). However, visual observation and Alizarin Red staining showed that mineralised nodules could form in the centre of the hydrogel (Figure 19). The limiting factor for the use of these fluorescent molecular stains with a hydrogel matrix appears to be their ability to penetrate the hydrogel as the thickness increases.
Figure 26. HFOs cultured in a soft hydrogel for 21 days and fluorescently labelled with FDA/PI.

Serial sections of constructs were photographed using a confocal microscope comprising 15 slices with a depth of 1081.6 µm at 72 µm per slice. After an incubation of 15 minutes no FDA labelled viable cells (A) or PI labelled dead cells (B) could be seen in the centre of the hydrogel. A: Scale bar: 2 mm.
Figure 27. Fluorescently labelled HFOs cultured in a highly cross-linked, stiffer, hydrogel for 21 days.

Serial sections were photographed using confocal microscopy. After an incubation time of 30 minutes, no hydroxyapatite could be detected using OsteoImage™ in the centre of the hydrogel constructs. Scale bar: 2 mm.
3.6 Discussion

3.6.1 Introduction

Osteoblasts are anchorage-dependent cells, and when grown as a monolayer they are able to adhere to the surface of a plastic culture plate. When grown in 3-D hydrogel, which most closely mimics the natural cellular environment, the signalling and cellular functions are enhanced through matrix adhesion and osteoblasts maintain their phenotype (Grigoriou et al., 2005; Cukierman et al., 2001; Cukierman et al., 2002; Wang et al., 2009). However, commercially available hydrogels are not well suited to the introduction of mechanical loads to the culture environment (Geckil et al., 2010). Hence, the present study examined the effect of increased the cross-linking and stiffness of the hydrogel constructs in order to evaluate its suitability for the attachment, proliferation and hydroxyapatite deposition by the osteoblasts.

Experiments conducted in this chapter demonstrated that the semi-synthetic HyStem-C® hydrogel offers a suitable environment for the culture of HCOs and HFOs. Both cell lines proliferated and deposited hydroxyapatite over a 21-day culture period. In the stiffer hydrogel, microscopic observation of osteoblast morphology showed that the cells were elongated with filopodia suggestive of attachment.

3.6.2 Evaluation of the proliferation of HCOs and HFOs in hydrogels

3.6.2.1 Protocol development

To assess the viability and proliferation rate of cells encapsulated in the hydrogel, a commercially available resazurin-based proliferation assay was used. Intended for use with cell monolayers, a protocol for the assay's use with 3-D hydrogel cultures was developed. Bonnier and co-workers have previously shown that protocols developed for use with monolayers may need modification for 3-D culture (Bonnier et al., 2015).
To determine if it was feasible to use the PrestoBlue™ assay in a cell-hydrogel model, cell proliferation in hydrogels was compared to that in monolayers as a positive control. The results showed a significantly higher number of proliferating cells in the monolayer relative to the hydrogel. To improve the detection of proliferating cells, the number of cells seeded into hydrogel constructs was increased to improve the signal to noise ratio and ensure a sufficient number of cells survived the process of being encapsulated in hydrogel. A decrease in the number of proliferating cells measured at day-7 was possibility due to the additional stress the cells experienced during the encapsulation process, thus the initial cell numbers needed to be increased compared to monolayers to achieve sufficient cell numbers in the constructs. Background fluorescence was observed in the cell-free hydrogel control assays, indicating the potential for false positive observations. This is in agreement with another study in which background fluorescence was detected in cell free cultures which contained only culture media (Munshi et al., 2014). Based on these results, experiments measuring fluorescent dyes in hydrogels should always include controls for background fluorescence. It was also evident that monolayer and hydrogel groups require different incubation times to allow the dye to reach the cells. Multiple differences between monolayer and hydrogel culture systems were demonstrated in these pilot experiments and provided valuable information for the development of protocols using the hydrogel model.

3.6.2.2 Proliferation rates of HCOs and HFOs in a stiffer, more cross-linked hydrogel compared to a softer hydrogel

Proliferation of HCOs and HFOs increased in both the soft and the stiff hydrogels over a 21-day culture period. The observed trend towards greater proliferation in the softer hydrogel was not statistically significant. The HFOs proliferated more than the HCOs in the softer hydrogel, but with no difference in the stiffer hydrogel. The lowest proliferation in both hydrogel compositions was observed in the first 7 days, possibly due to an acclimatisation phase. However, cell death was not quantified and an apoptosis quantification assay is recommended in future experiments. In the softer hydrogel, the maximum rate of proliferation was achieved earlier, between days 7 and 14. The greatest total fluorescence
signal was at day 21 in both groups. It is possible that decreasing the amount of hyaluronan and gelatin components in the stiffer hydrogel reduced proliferation. It is also possible that the culture medium did not penetrate as well into the more highly cross-linked hydrogel. It has been reported that increasing the concentration of PEGDA cross-linker increases stiffness, but being a synthetic component, it can decrease osteoblast adhesion and viability (Burdick and Anseth, 2002). Therefore, it was not surprising to see some decrease in proliferation in a more highly cross-linked hydrogel and this is why synthetic hydrogels are frequently modified by the addition of biologic molecules to enhance cell proliferation (Bartold et al., 2006; Drury and Mooney, 2003; Teixeira et al., 2014). In the case of the HyStem-C® semi-synthetic hydrogel used in this research, hyaluronan and gelatin were added. Hyaluronan is a major constituent of the natural ECM that mediates cell proliferation and differentiation and gelatin provides adhesion sites for the cells.

Cell proliferation in a stiffer hydrogel could potentially be further enhanced by adding a reduced volume of more concentrated PEGDA, rather than the 1% that was used in this experiment, thus increasing the overall proportion and bioavailability of hyaluronan and gelatin.

3.6.3 Morphology of the HFOs and HCOs cultured in hydrogels with different cross-linking

The second aim of this chapter was to compare the morphology of the HFOs and HCOs in hydrogels with different degrees of cross-linking using phase-contrast light microscopy. It was demonstrated that while HCOs and HFOs proliferated in both hydrogel densities, those that were cultured in the modified stiffer hydrogel demonstrated morphology that allowed the cells to express their adhesion properties, which is pre-requisite for anchorage dependent osteoblasts.

When the osteoblasts were cultured in the standard softer hydrogel, their shape remained round. This unorganized network is indicative of inadequate attachment to the substrate and failure of the cells to spread. In contrast, when HCOs and HFOs were cultured in the stiffer hydrogel with greater cross-linking,
their shape progressively changed from round separated cells to a mesh-like network of interconnected cells with multiple filopodia and lamellopodia extensions anchoring them to the hydrogel matrix. Filopodia and lamellopodia are actin filament extension which allow the cells to spread, adhere to and communicate with each other and the ECM (Cukierman et al., 2002; Wozniak et al., 2004; Wang et al., 2009). Being anchorage dependent cells, this closely resembles their normal biological behaviour, which was not observed in a softer hydrogel. To further understand the attachment of osteoblasts in our hydrogel model, focal adhesion kinase and vinculin proteins, which are universal focal adhesion markers, could be investigated in future experiments.

3.6.4 Assessment of mineralisation

The third aim of this experiment was to observe and compare the mineralisation process for HCOs and HFOs in both hydrogel densities. Hydroxyapatite mineral deposition by the HCOs and HFOs within the hydrogel matrix was confirmed using Alizarin Red and OsteoImage™ fluorescence staining. Phase contrast and confocal microscopy was also carried out. There were advantages and disadvantages for each staining method. Alizarin Red is a commonly used stain for the detection of calcium. Calcium is a principal component of hydroxyapatite and positive staining with Alizarin Red served as an indicator of mineralisation. However, because Alizarin Red attaches to calcium binding proteins and proteoglycans, it also stained the hydrogel. OsteoImage™ is a fluorescent assay which is believed to be more sensitive as it binds directly to hydroxyapatite and has been used in 3-D cultures (Wang et al., 2006; Langenbach et al., 2011). Hydroxyapatite deposition was detected in proportion to the cell seeding density in hydrogel constructs and this is in agreement with the classical study of the relationship between cell number and number of mineralised nodules (Bellows et al., 1991). The limitation of using OsteoImage™ was the high background fluorescence detected in the hydrogel-only control group and the importance of washing the hydrogel before taking readings to avoid falsely high measurements; background staining of the hydrogel itself was the main limitation with all the staining techniques employed. Phase-contrast light microscope observations showed cellular retraction and aggregation into nodule-like structures by day 7,
and by day 14 the nodules were also seen macroscopically in the cultures of both cell lines and with both hydrogel compositions. Mineralised nodules were stained with Alizarin Red and seen macroscopically and microscopically in all groups, confirming that the environment is suitable for osteoblasts to deposit a mineralised matrix into the hydrogel.

In summary, HCOs and HFOs were able to deposit hydroxyapatite in both hydrogel compositions. The best assessment of mineralisation was found to be a combination of microscopic evidence of nodule formation and a quantitative assay. The standard errors for the OsteoImage™ assay at each time point were small demonstrating the assay suitability for the measurement of hydroxyapatite in hydrogels. Our results show that the OsteoImage™ assay is appropriate for use in small hydrogel constructs as long as the background signal is subtracted and the manufacturer’s instructions of washing are strictly followed. The question as to whether the assay dye can penetrate through the whole construct could not be answered when the amount of fluorescence was recorded using only a plate reader.

3.6.5 Confocal microscopy

The fourth aim was to use confocal microscopy to investigate the spatial arrangement of dead and live osteoblasts. Propidium iodide and fluorescein diacetate were used for dead and live cell detection respectively. The OsteoImage™ stain was used to locate hydroxyapatite deposition. Confocal microscopy enables visualization of cells in 3-D samples and provides morphologic information otherwise not obtainable with conventional fluorescence microscopy. Our results showed incomplete penetration of all stains to the middle of hydrogel cell constructs. This was especially evident in thicker and denser constructs. Theoretically, hydrogels have the benefit of better nutrient exchange compared to other 3-D cell culture systems, but a constant medium flow through the culture may be required for optimal growth and staining. This is why bio-reactors and constant flow models have been used in other systems (Vunjak-Novakovic et al., 1999; Hung., 2005), and may be required for thicker hydrogel constructs.
One of the limitations of the present study was that the commercially available assays for proliferation and mineralisation are more commonly used for monolayer cultures and have not been validated on more complex 3-D culture models, such as hydrogel. These limitations have been recognized recently and new assay formulations are being investigated. Some of these assays have detergents incorporated into the dye to enhance penetration. However, detergents can also reduce cell viability. Other methods to enhance assay component penetration include agitation of the constructs and other forms of physical disruption (Riss et al., 2014; Kijanska and Kelm 2016). To answer the question of whether the lack of fluorescence is due to lack of hydrogel penetration or to true loss of cellular viability in the middle of the constructs, it is recommended to try different incubation times and assays containing detergents.

In summary, it is important to validate viability and mineral deposition assays prior to utilising them with new culture models to understand the kinetics and mechanisms of cell death in each culture system and to appreciate the limitations of using conventional assays on 3-D constructs. Further validation of assays is needed to investigate the cellular activity inside the hydrogel.

3.4.6 Summary

This chapter examined the effect of a more highly cross-linked hydrogel on HCO and HFO morphology, proliferation and hydroxyapatite deposition. These experiments allowed the selection of a hydrogel model for the next phase of the study, which involved analysis of mRNA levels following application of cyclic mechanical compressive strain. A highly cross-linked hydrogel at a seeding density of 100,000 cells per 20 µl hydrogel (5 x 10^6 cells / mL of hydrogel) was chosen as the most suitable hydrogel model for investigating the effect of compressive strain on gene expression.
3.7 References


Chapter 4
Culturing osteoblasts in hydrogel in a mechanically active environment

4.1 Introduction

The importance of culturing cells under mechanical strain has been recognised since the 1900's. Multiple systems for the application of mechanical strain to cell cultures have been developed and these will be discussed in this section. Challenges are posed by complexity of re-creating physiological patterns of mechanical loading \textit{in vitro}. Current understanding of bone and bone cell responses to mechanical stress is incomplete due to the complex signalling pathways involved. The differences in response to mechanical strain between calvarial and limb bones are also largely unknown.

This review will outline the differences in response to mechanical strain between calvarial and limb bones and summarise the experimental evidence for bone functional adaptation using the commonly used tension and compression models. Osteoblast gene expression studies following mechanical load will be introduced and fundamentals of RT-qPCR will be discussed.

4.1.1 Differences in response to mechanical strain between calvarial and limb bones

Calvarial and limb bones do not demonstrate the same response to dynamic mechanical strain and to investigate these differences, Rawlinson and co-workers provided two hypotheses (Rawlinson \textit{et al.}, 1995). The first was that the low load experienced by the skull leads to a deactivation of strain-related adaptive responses. The second hypothesis was that low mechanical loading \textit{in vivo} on calvarial cells renders them sensitive enough to respond to low functional strains, while the same functional strain would be trivial to cells originating from the limbs. Their work on a rat model showed marked
differences between the loads experienced by different bones. The recorded strains on the calvarial bone from feeding did not exceed 30µε (micro strain) in tension and compression. Rat ulna on the other hand, experienced up to 1000-1300µε from various normal physiologic activities. The group also found an increase in prostaglandins and glucose 6-phosphate dehydrogenase activity by femoral but not by calvarial bone cells in response to mechanical load. They hypothesised that each part of the skeleton has a genetically determined form and minimum mass that can be modified in response to mechanical strain. Calvarial bones exist in an apparent paradox in that they maintain their bone mass despite their exposure to low levels of mechanical strain. If these low strain levels could be replicated in any other region of the skeleton, profound bone loss would ensue. The question as to how calvarial bones preserve their bone mass, despite their exposure to such low levels of physiological strain remains unanswered.

Human studies have also shown differences in maximum strains experienced by calvarial and limb bones. Hillam and co-workers (1996) measured this with strain gauges applied to the parietal bone of the skull and to the midshaft of the tibia. Regardless of the load, there was close to a ten-fold difference between tibial and calvarial bone strain. Their study suggested that the skull bones are either insensitive to the effects of disuse or that there is a different threshold for perception of optimal mechanical strain in the skull compared to the limbs (Hillam et al., 1996). The latter is in agreement with the hypothesis that each bone has its own mechanostat.

4.1.2 Mechanostat hypothesis

The mechanostat hypothesis was introduced by Harold Frost, an orthopaedic surgeon, to describe the relationship between bone mass of a single bone and magnitude of applied mechanical stimulus (Figure 28). Harold Frost is today known for his early use of tetracycline labels to study the dynamics of bone modelling and remodelling (Frost, 1969). In 1987 Frost proposed the mechanostat hypothesis, in which normal bone mass is maintained within the boundaries of two thresholds of mechanical strain. If the upper threshold is
exceeded, then either new bone is formed or a fracture results, depending on the magnitude and duration of the mechanical stimulus. Below the lower threshold of the mechanostat equilibrium, disuse osteopenia results where bone mass is lost (Frost, 1987; Roberts et al., 2006).

Figure 28. The idea of a mechanostat regulating bone mass was introduced by Harold Frost in 1987.

For each bone in the skeleton there is an adapted state within the boundaries of which normal bone mass is maintained. If the bone strains exceed a certain critical threshold (> 1500με) the result is bone formation - reduction below another threshold value (< 1000με) will lead to bone loss and osteopenia. Adapted –Frost, 2000: The Utah Paradigm of Skeletal Physiology.

Since its original description, the mechanostat hypothesis has been modified and continues to evolve to account for the multifactorial nature of bone homeostasis. Multiple unique thresholds, or unique mechanostats, have been demonstrated for different bones and for different individuals with no single threshold value (Skerry, 2006). In 2003 Frost outlined thirty-two bone responses based on the mechanostat hypothesis, many of which have been validated in the literature.
Frost also described the important role of hormones which modify the effects of mechanical loading by enhancing or inhibiting the response of bone to mechanical loading (Frost, 2003). The original concept of the mechanostat is only valid when the only variable is magnitude of the mechanical strain. For a particular unit of bone, the threshold at which osteogenesis occurs is determined by a combination of the frequency and magnitude of the applied load. Additionally, as the bone adapts to its environment, it becomes less responsive to routine loading signals (Turner, 1998).

4.1.3 Bone functional adaptation

4.1.3.1 Bone remodeling

The skeleton continuously undergoes remodelling throughout life and contributes to calcium homeostasis, repair of micro-fractures and adaption to mechanical loading. Disruption or uncoupling of the bone resorption and apposition sequence is apparent in a number of bone diseases. Bone adapts to variations in mechanical load at both cellular and tissue levels resulting in changes in shape of the bone in response to new demands (Roberts et al., 2006).

Bone remodelling (turnover) is a coupled process involving the coordinated activity of osteoclasts and osteoblasts known as the activation-resorption-formation sequence (ARF) (Frost et al., 1969). In the late 1990’s cytokines within the TNF family were identified as the essential components of a signalling system that are required for bone remodelling, known as the receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) triad (Simonet et al., 1997; Yasuda et al., 1998).

4.1.3.2 Evidence of bone functional adaptation

Investigations of bone adaptation to mechanical loading have a long history. In 1867, anatomist Herman von Meyer proposed that the trabeculae in cancellous bone were laid down along the lines of maximal compressive and tensile stress lines or ‘trajectories’. The structural similarities of bone trajectories to a crane were recognized by a Culmann, an engineer (Roesler, 1987). In 1892 Wolff, an
orthopaedic surgeon published a theory, stating that bone is morphologically 
adapted to its function according to mathematical principles, resembling a crane, 

obtaining maximum strength with minimum material. This became known as 

Wolff’s law (Wolff, 1892) (Figure 29). Nearly a century later, Bertram and Swartz 

(1991) pointed out that Wolff’s law as a pure mathematical approach to 
determine the structure of bone is too simplified and has a number of 
deficiencies. It does not differentiate between cortical and trabecular bone 

(Turner, 1992), and it only takes into account the positive effects of mechanical 
loading, ignoring the fact that mechanical strain can also have negative effects on 

bone mass (Meikle, 2002). With mounting research evidence, it became clear 

that bone is a complex system, subjected to multiple processes and mechanical 
stimulation is not the only factor responsible for orientation of trabeculae 

(Bertram and Swartz, 1991). Therefore, rather than calling it a law, the term 

“bone functional adaptation” is more appropriate (Ruff et al., 2006).

Figure 29. A: Trajectories calculations made by Culmann in his comparison between a femur and a curved crane. B: Illustration of a proximal end of the human femur showing trabecular pattern of bone.

When Wolff saw illustrations in (A), he hypothesised that mathematical laws must exist. Wolff’s Law in 1892 was based on the mathematical principles where trabeculae are laid down along the lines of maximal compressive and tensile mechanical stress and enables bone to provide maximal strength with minimal material. Adapted – (A)Wolff, 1892 (translated in Wolff, 1986) and (B) Meikle, 2002.

In the 1930’s a series of classical in vitro experiments were conducted to provide evidence for the functional adaptation of bone to mechanical loading. Using the first dynamic cell culture system, Glucksmann cultured endosteal tissue samples
on rib explants; this caused compression to the sample as the ribs were drawn toward one another with muscle tissue degeneration and subsequent contraction. He stated that the effect of pressure on ossifying tissue depends on the stage of differentiation and the degree of maturity and calcification of the developing bone. Glucksmann concluded that mechanical stress stimulates osteogenesis *in vitro* (Glucksmann, 1939).

In the 1970’s quantifiable methods started to be developed. Rodan and co-workers applied 80g/cm² load at a controlled duration and frequency using pressure pistons to compress chick bones. They found a reduction in glucose utilization and an immediate change in the levels of cyclic nucleotides, which were hypothesised to be a potential messenger in mechanotransduction (Rodan and Bourret, 1975; Rodan *et al.*, 1975).

The classic work by Hěrt and co-workers in Czechoslovakia has shown that continuous and intermittent mechanical loads affect bone remodelling differently. Long-term (up to 403 days) external continuous load was applied on young and mature rabbit tibia. Rigid Kirschner wires were inserted through the tibial metaphysis and following a healing period, an electromagnetic field was used to load the bone (Figure 30) (Hert *et al.*, 1969). They concluded that continuous loading is not a stimulus for activation of osteoblasts. This was in agreement with earlier work by Liskova and co-workers on rabbits, who also showed that a continuous load of 0.15-3.65kg/mm² provided no stimulus for osteoblast activation (Liskova, 1965). Following this, Hert and co-workers studied intermittent tensile and compressive force. Intermittent load applied over several weeks generated an increase in bone formation compared with continuous loading particularly in younger rabbits. Both intermittent tension and compression lead to bone formation, suggesting that cells are unable to distinguish between tensile and a compressive mechanical strain (Hert *et al.*, 1971). However, they did not examine differences in outcomes between different vectors of force application in relation to bone tissue planes. As can be seen on Figure 30, both compression and tension applied in this plane would contribute to shear stress between trabecular planes. The outcomes may have
been different if force was applied perpendicular to trabecular planes, as demonstrated in distraction osteogenesis work by Ilizarov (Ilizarov, 1990).

To show that bone can adapt and reorganise, Lanyon and co-workers removed the ulna from the leg of a mature sheep and observed the changes of the remaining radius bone under normal function. They found that following ulna osteotomy, new bone formed on the radius, thus equilibrating strains (Lanyon et al., 1982). In a similar experimental design, using a turkey ulna model (Figure 31), Lanyon and Rubin observed that intermittent tension and compression load produces an increase in cross-sectional area and that a short exposure to loading is sufficient to maintain bone structure (Lanyon and Rubin, 1984; Rubin and Lanyon, 1985).

![Figure 30. Radiographic set up for application of intermittent loading on the rabbit tibia.](image)

Hērt and co-workers inserted 2.0–2.5 mm Kirschner wires into the right tibiae of rabbits, which were attached to an electromagnet that permitted approximation, or separation of the wires, which flexed the bone. Adapted – Hert et al., 1971.
Figure 32. Turkey ulna-load model and three microscopy photographs showing bone mass changes with application of different stress loading regimens.

A: Maintenance of bone structure average daily loading strains (100 consecutive 1 Hz load cycles producing peak strains of 1,000με). C: Isolation from load for 8 weeks causing a decrease in bone mass. D: Application of higher than average daily loading (100 cycles/day with high peak strains of 3,000 με) causing an increase in bone mass. Adapted- Lanyon et al., 1984.

McDonald and co-workers investigated which load characteristics are important in bone remodelling. They hypothesised that the type of load applied may be more important than the load magnitude. Earlier conclusions made by Hert and
co-workers that increased load leads to more bone formation were re-examined as they did not account for the micro-fractures caused by high intermittent load and subsequent bone formation from the healing process may have affected their results. Modified orthopaedic pins were gently screwed into the bone, and following a healing period, compressive load of a variety of magnitudes and frequencies was applied for 45 minutes per day for 28 days to immature and mature tibia of White New Zealand rabbits. Using tetracycline epifluorescence staining, greater deposition was observed in younger rabbits, suggesting other factors aside from mechanical load were causing a biological response. They also concluded that response was frequency dependent and intermittent force produced more bone formation than continuous force. However, all regimens, including continuous load, led to a net increase of bone with osteoblastic activity outweighing osteoclastic activity (McDonald et al., 1994).

In summary, within the limitations of surgical in vivo animal and in vitro models to study the effects of mechanical load on bone remodelling, the results have shown that loading must be cyclic to stimulate new bone formation and that cells cannot distinguish between cyclic compressive or tensile force, with shear stresses on the cell membrane being the main event (Figure 32). Intermittent and high frequency dynamic forces have a greater effect and that small force magnitudes are sufficient to increase bone remodelling in response to mechanical load (Sandy et al., 1993; Duncan and Turner, 1995; Turner, 1998). Additionally, aging greatly reduces the osteogenic effects and hormones may interact with local mechanical signals to change sensitivity of the bone cells to mechanical load (Duncan and Turner, 1995).
Figure 32. Effect of intermittent loading on bone cell membranes.

Intermittent bone loading results in fluid flow through bone canaliculae causing flow-related shear stresses on the cell membranes. This creates streaming potentials within bone and interpretation of the mechanical signals through mechanotransduction pathways. The effects of mechanical loading are dependent on magnitude, duration and frequency of the applied mechanical load. Adapted – Duncan and Turner, 1995.

The effects of reducing mechanical load below a particular threshold have also been investigated and this leads to a non-pathological reduction in bone volume called disuse osteopenia. Disuse osteopenia is observed in the bones of subjects exposed to the low gravity environment of space flight, as a consequence of subthreshold physiological loading (Turner, 2000). The concept of shielding has been demonstrated in orthopaedic surgery following a hip replacement where the rigid implant shields the bone from its normal load strain, causing resorptive processes, although individual variations in response have been noted. Flexible femoral implants have been trialled as a potential solution to the problem of stress shielding and its consequences (Huiskes et al., 1992).
Appliance induced osteopenia was also demonstrated by Meikle and co-workers in an orthodontic rat tooth movement model. They found that a cross arch expansion spring resulted in a reduction in alveolar bone mass. They concluded that stress shielding of the interradicular bone by the rigid orthodontic appliance, provided a plausible explanation for the observed changes in alveolar bone mass. The splinting effect of the orthodontic appliance had caused mechanical loading to fall below the threshold levels, required for the maintenance of normal bone architecture (Milne et al., 2009).

4.1.4 Modern in vitro mechanical load models

Regardless of the system used, in vitro models always combine elements of mechanical strain with the artefact of flow. Flow related shear stresses acting on the cell membrane are a potential cause of cellular deformation. This makes it impossible to pinpoint whether it is the fluid flow or the mechanical deformation, which contribute to the actual mechanotransduction signal (Jansen et al., 2004).

4.1.4.1 Tensile loading systems

Longitudinal stretch systems have evolved since the 1970’s. One of the first studies to note a biochemical change in response to mechanical load used periosteum cell cultures by Harrell and co-workers. They used an orthodontic screw to distort a culture plate, by applying 10kg/cm² and an almost immediate increase in prostaglandins in response to mechanical stress was detected (Harell et al., 1976; Somjen et al., 1980). Further innovative techniques in those times included mounting and stretching rabbit cranial sutures with a force of 20-30 grams with a spring (Meikle et al., 1979). Leung and co-workers used elastin membranes from bovine aortas as extensible substrates for cell growth (Leung et al., 1977). Following this idea, polyetherurethane urea membranes were used as an in vitro circulatory system to mimic the in vivo mechanical environment of endothelial cells (Ives et al., 1986). The first study to use a uniform biaxial cyclic strain on human osteoblasts was done using elastic silicone dishes in which the whole dish and the cell culture were deformable (Neidlinger-Wilke et al., 1994). This study found that a silicone membrane leads to a decrease in proliferation
and that osteoblast proliferation depends on the magnitude of the load, with the highest proliferation at 1% strain. However, there was large individual variation between the 15 donors. The main limitation with substrate distension was the non-homogenous stretching of the sample.

To apply a more homogenous stretch, out of plane substrate distension systems were developed. Hasegawa and co-workers used Petri dishes with an elastic bottom which were loaded from the top over a uniformly shaped convex template (Hasegawa et al., 1985).

The system used in the present study and one of the most common systems used today is the FlexCell® (Flexcell International Corporation, Hillsborough, NC, USA), which was developed by Banes and co-workers. The FlexCell® system is computer regulated system to apply a defined, controlled static or cyclic mechanical load under conditions of tension or compression. It uses culture plates with stretchable and flexible membranes. Tension is applied using suction and positive air pressure is used to apply compression (Banes et al., 1985). Numerous studies have been carried out using this system to study cellular response to mechanical load and on expression of osteogenic genes using monolayer and 3-D culture models (Wescott et al., 2007; Li et al., 2013). It was found that cells experience approximately 50% of the applied strain (Wall et al., 2007; Bieler et al., 2009). A number of experiments have demonstrated that strain distribution was homogenous in the central well area, but not on the periphery of the membrane (Wall et al., 2007; Bieler et al., 2009). Additionally, extended use of the membrane and high cycle numbers lead to permanent changes in the membrane resulting in differences between programmed and experienced strain magnitudes. When programming the required loading regimen, Saminathan and co-workers accounted for the 50% reduction of applied strain (Saminathan et al., 2015), and this was also accounted for in the present study.
4.1.4.2 Compressive loading systems

In the present study, axial compression is achieved through a direct platen abutment (the compression mode of the FlexCell® system). This technique has become widely used in scientific experiments on cartilage and bone tissue cultures. One of the main advantages of this system is that the frequency and magnitude of the applied mechanical strain can readily be modified. However, the influence of friction acting at the interface between the sample and the platen is one of the potential disadvantages as this can lead to the introduction of heterogeneous strains, which operate in different parts of the sample. Additionally, due to the confinement of the sample to a platen, there is decreased free flow of nutrients and of waste exchange, causing most of the cells to adhere to the peripheral surface closer to the nutrition media (Brown, 2000). To overcome this limitation, bioreactor systems with a continuous flow of nutrient media have been developed. Recent developments use range of bi-axial regimen profiles combining multiple systems together to mimic the complex 3-D combination of compressive, tensile and shear deformations that occur in vivo (Yusoff et al., 2011).

Other compressive loading systems include hydrostatic pressurisation and fluid induced shear force, and a combination of the above. Hydrostatic pressure can be employed to apply static and intermittent load to a culture medium by pressurising it. However, because of partial pressures, the nutrient medium would require extra treatment steps. Additionally, such a method would not mimic the physiologic pressures experienced by cells in vivo (Brown, 2000).

In summary, it is unlikely that bone cells can distinguish between tensile and compressive stimulus (Hert et al., 1971; Sandy et al., 1993) and the change of shape with deformation of the cell membrane appears to be the key event in the cell-signalling cascade. In vitro, both tension and compression systems are commonly used. It is not possible to subject cells to a purely tensile or compressive strain because deformation is accompanied by shear strains. While in vitro systems have contributed significantly to an understanding of cell
signalling in response to mechanical strain, *in vivo*, cells experience a diverse range and types of mechanical forces, which cannot be replicated entirely in a culture system.

### 4.1.4.3 Summary

Despite many years of development, *in vitro* models, which can adequately replicate normal physiological conditions, are yet to be identified.

It is known that mechanical loading can have both, positive and negative effects on bone mass depending on the threshold. If bone cells are cultured without mechanical stimulation, they are in a physiological default state, equivalent to disuse osteopenia. Differences in response to mechanical load between calvarial and limb bones have been recognized and need to be considered when studying bone cell culture *in vitro*. The mechanisms by which calvarial bone preserves its bone mass, despite its exposure to low levels of physiological mechanical strain are still largely unknown. The complexity of the 3-D *in vivo* system as yet cannot be replicated and this is the main limitation to studying cellular responses to mechanical strain *in vitro*.

### 4.1.5 Mechanotransduction

If a culture system is to mimic the *in vivo* environment of skeletal tissues, mechanical stimulation is necessary. As a result of this requirement, tissue engineering research has been focused on understanding mechanosensing and signalling pathways, a process called mechanotransduction (Pedersen and Swartz, 2005; Rubin *et al.*, 2006).

Mechanotransduction is the conversion of a mechanical signal to a biological response. It is a complex and not entirely understood process involving interaction between extracellular fluid forces, extracellular matrix and cells (Hsieh and Turner, 2001).

It is evident that there is no single signalling pathway (Price *et al.*, 2011). It is
hypothesized that mechanical stress deforms the bone matrix and increases soluble mediators like calcium, prostaglandins and nitric oxide which send signals for recruitment and differentiation of osteoblasts (Turner and Pavalko, 1998). Differentiated osteoblasts when embedded in mature bone are called osteocytes and have been hypothesized to have a key mechanosensory role due to their extensive dendritic network (Boneyald and Johnson, 2008; Bonewald, 2011). Architectural differences in osteocyte networks between mouse limb and calvaria bones have also been noted and this may explain the differences between calvarial and limb bones in their response to mechanical load (Himeno-Ando et al., 2012; Vatsa et al., 2008). In cell culture, mechanical stimulation of osteoblasts leads to increased matrix formation. However, in vivo, only 5% of the adult bone is lined with active osteoblasts and the rest are osteocytes (Duncan and Turner, 1995). For this reason, Bonewald and co-workers have recommended generation of a cell line with the properties and function of the mature osteocyte to study responses to mechanical load at a cellular level in vitro (Boneyald and Johnson, 2008).

4.1.6 Mechanical load regimens

A multitude of intermittent tensile and compressive loading regimens have been employed for in vitro studies on osteoblasts. These include variability in frequency, magnitude of strain, number of applied loads, incubation time and the source of osteoblasts (Yu et al., 2016). Variability of these factors between studies makes it challenging to identify an optimal mechanical load regimen.

While there are a range of loading frequencies (measured Hertz as cycles of load per second) to which the osteoblasts will respond with increased bone formation (Rubin et al., 1990; Turner et al., 1995), current studies indicate that high frequencies, rather than high load magnitudes are more effective at stimulating bone formation. The most common loading frequencies used on osteoblasts in vitro are 0.5-3Hz (Neidlinger-Wilke et al., 1994, Kaspar et al, 2000; Tanaka et al., 2003).
4.1.7 Osteoblast gene expression following mechanical loading

A change in gene expression of the osteogenic markers, alkaline phosphatase and osteocalcin, has been observed after the application of a mechanical load. Zhu and co-workers (2008) have shown that increased expression of alkaline phosphatase and osteocalcin may depend not only on the stage of mineralisation, but also on the magnitude of load applied. They recorded an increase in ALP activity with lower magnitudes of tensile strain, whereas higher magnitudes enhanced expression of osteocalcin (Zhu et al., 2008).

The stage of differentiation of cultured osteoblasts affects their response to mechanical load in vitro. Weyts and co-workers (2002) investigated the reason why mechanical stimulation has been found to increase cell proliferation in some studies, whereas other studies reported decreased osteoblast proliferation. They stated that it is likely due to the differences between functional stages of osteoblast development, which express different osteoblastic markers in a time dependent sequence. Lian and Stein (1992) also found a similar phenomenon, in that osteoblasts have a regulated, sequential gene expression pattern and a coupled relationship between proliferation and differentiation into matrix maturation and mineralisation. In vitro human foetal osteoblasts, on average, exhibit the highest ALP activity at day 14 (matrix maturation phase) and mineralisation of the matrix at approximately 21. Therefore the timing of mechanical load application affects osteoblast activity differently. Weyts and co-workers (2002) observed that in the first 7 days, regardless of the type of strain regimen applied, a decrease in cell proliferation was observed with an increase in apoptosis. In contrast, stimulation applied from day 11 to 14, resulted in increased cell numbers and proliferation. Application of strain from day 18-21 did not affect cell proliferation (Weyts et al., 2002). These findings indicate that there is a window for maximal proliferation and an optimal timing for the application of mechanical strain. This is in agreement with a recent study done by Li and co-workers (2013) who showed that osteoblast differentiation and mineralised nodule formation could be enhanced by periods of cyclic mechanical strain between days 7 and 13 (Li et al., 2013). Alteration in gene expression
involved in proliferation, osteoblast differentiation and matrix production after mechanical stimulation has been shown by Ignatius and co-workers (2005). Human femoral osteoblasts were cultured in a 3-D collagen-1 gel scaffold. A cyclical regimen at a frequency of 1 Hz and a magnitude of 1% uni-axial strain (10,000 micro-strain) was applied for 30 minutes a day for three weeks and mRNA levels of histone H4, core binding factor 1 (cbfa-1), alkaline phosphatase (ALP), osteopontin, osteocalcin (OCN) and collagen -1, (Col-1) were analyzed. An increase in cell proliferation in response to mechanical stimulation was observed. A significant increase in ALP gene expression was measured initially in the mechanical strain group. However, by the end of the culture period there was no difference in ALP gene expression between the mechanical strain group and unstrained controls. The authors concluded that mechanical load promoted proliferation and differentiation of osteoblastic precursor cells in a Collagen-1 matrix.

Many of the studies investigating the effect of mechanical load on osteoblasts used collagen-1 gel constructs as a suitable cell scaffold. As discussed in Chapter 3, collagen-1 is a major organic component of ECM gel and has an important role in the expression of the osteoblastic phenotype. The main limitation is the dimensional stability of the gel construct which has been shown to undergo 50% shrinkage by day 7 (Ignatius et al., 2005). Semi-synthetic hydrogel systems incorporate the benefits of collagen combined with a synthetic component to modify its mechanical properties. This study uses a hyaluronan and gelatin (denatured collagen) based semi-synthetic hydrogel.

In summary, while an optimal loading regimen has not been identified, current studies indicate that high frequencies and low force magnitude are most effective at stimulating bone formation. Osteoblast cell lines grown in culture undergo a time dependent maturation process. Hence, the application of mechanical loads to osteoblasts at different time points will yield different cellular responses. This is supported by the observation of differences in the pattern of gene expression for genes involved in osteoblast proliferation, differentiation and matrix production at different time points.
Quantification of mRNA using real-time reverse transcription polymerase chain reaction (RT\(^2\)-qPCR) is a fluorescence based, enzyme driven technique used to amplify DNA to a level which can be detected and the associated signal used for quantification. RT\(^2\)-qPCR consists of three steps: 1) reverse transcriptase (RT) dependent conversion of RNA into cDNA, 2) amplification of cDNA using PCR and 3) detection and quantification of amplified cDNA in real time (Figure 33). By amplifying samples at the same rate, a calculation can be made to determine which group of samples has the highest copy of the target gene (Nolan et al., 2006). Although RT-qPCR is a commonly used technique, it is highly sensitive and the quality of the outcome is dependent upon a number of variables. These include the quality of the mRNA extract, the quantity of the cDNA template, the detection chemistries used, the standardisation of the transcription reaction and the interpretation of the data. The MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines have been published to standardize the reporting protocols (Bustin et al., 2009).

By using a specific gene primer, a specific region of DNA is amplified. During RT\(^2\)-qPCR, changes in temperature are used to control the activity of the enzyme and the binding of primers. The process begins with a temperature at 95°C at which the double stranded DNA separates into single strands. A polymerase enzyme is then used to synthesise complementary sequence of bases to these single strands of DNA.
Figure 33. RT² qPCR amplification plot.

The y-axis represents the fluorescence level associated with the amplified DNA. The threshold line is where the signal is above the background noise signal. The point where the threshold line crosses the fluorescence signal is known as the quantitation cycle (Cq). Most commonly 40 cycles are performed. Adapted: Bio-Rad Laboratories Real Time PCR Applications Guidelines, #5279.

4.1.8.1 Fluorescent reporter dyes

RT²-qPCR uses fluorescent reporter dyes to detect a signal, which is proportional to the amount of DNA produced during each PCR cycle. Two detection chemistries are available. SYBR® Green uses an intercalating dye and TaqMan® uses a hydrolysis probe. With both, fluorescence is generated during PCR, allowing a real time monitoring of the reaction.

SYBR® Green is an intercalating fluorescent dye and in the presence of double stranded DNA, it binds to the double helix, the level of fluoresce is proportional to the number of copies DNA amplified. The major disadvantage of SYBR® Green is the non-specific nature of the dye, which binds any double stranded DNA requiring further analysis to interpret and confirm results. Only one gene can be amplified at a time with SYBR® Green. However, it is an appropriate method to use if the target of interest is abundant and a direct comparison is not required.
Unlike SYBR®Green, the use of TaqMan® hydrolysis (used in the present study) allows two or more fluorescent probes labelled with different reporter dyes to be used, which bind to specific gene targets. This allows for detection of two target genes in a single reaction. The unbound 3’ carbon of each probe has a reporter molecule such as FAM and VIC attached, and when cleaved by the polymerase enzyme, fluorescence can be detected at characteristic wavelengths (Figure 34). Hydrolysis probes have the advantage over SYBR®Green in that they are very specific and a signal will only be seen when the primer and the probe bind correctly, making data very reliable (Heid et al., 1996).

Figure 34. Diagrammatic representation of TaqMan® hydrolysis probes.
TaqMan® probes (purple) contain two dyes, a reporter (R) and a quencher (Q). The quencher suppresses the fluorescence signal of the reporter. If the gene of interest is present, the probe fragments are displaced from the target, resulting in increased fluorescence of the reporter dye. If the target sequence matches to the probe, specific amplification in fluorescence occurs. Adapted: Life Technologies - Technical Resources.
4.1.8.2 Efficiency of PCR reaction

PCR efficiency is important when reporting mRNA concentrations for target genes in relation to the reference genes (Bustin et al., 2009). The difference in Cq values between the target and reference genes is calculated. To determine how efficient the PCR reaction is, a series of dilutions are carried out, with a minimum of two replicates, with ideally a 5-log dilution series, which are then plotted on a standard curve. The calculated slope gradient should be between -3.3 and -3.6, which reflects a 90-110% efficiency.

4.1.8.3 Use of appropriate reference genes

Normalization of the expression of the gene of interest is required to control for experimental error. Commonly, a reference gene (also known as a housekeeping gene) is used without validation, assumed to remain constant between experiments. It is important to use a reference gene, which has been validated. The use of the conventional gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been found to be too variable with a large expression error, with an increase in noise of the assay and detection of small changes (Dheda et al., 2005). If the experimental method alters the reference gene, then the results become incorrectly interpreted and invalid. Therefore, it is important to validate reference genes with each experimental method. Godoy-Zanicotti and co-workers showed B2M to be suitable for osteogenic gene detection (Godoy-Zanicotti, 2015).

4.1.8.4 Summary

In summary, the use of fluorescence based quantitative PCR has been widely used in nucleic acid quantification. The use of a TaqMan® hydrolysis probe offers significantly increased specificity for the target gene over other fluorescent qPCR methods. The Minimum Information for Publication of Quantitative Real-time Experiments (MIQE) guidelines provide information regarding performing and reporting qPCR data (Bustin et al., 2009).
4.2 Hypothesis

The exposure of osteoblasts to mechanical strain over a 21-day period will result in a decrease in their proliferation rate, with a concurrent increase in the expression of key osteogenic genes and the initiation of mineralisation.

4.3 Aims

1. A FlexCell® machine will be used to expose hydrogel encapsulated HFOs and HCO to an intermittent (cyclic) compressive load for 21 days.

2. The proliferation rates of HCOs and HFOs with and without cyclic mechanical compressive load will be measured over the 21 days using a resazurin based fluorescence assay, PrestoBlue™.

3. To examine the suitability of the mechanically loaded hydrogel model for the study of osteoblast mRNA expression. Three key osteogenic genes, alkaline phosphatase (AP), osteocalcin (OCN) and bone morphogenetic protein 2 (BMP2) will be analysed. After 21 days of mechanical load osteoblast RNA will be extracted and mRNA levels measured using RT²-qPCR.
4.4 Materials and Methods

To investigate cellular responses to mechanical load, HCOs and HFOs were encapsulated into the highly cross-linked hydrogel (previously validated in Chapter 3). The test group was subjected to a programmed cyclic compressive load for 21 days using the Flexcell® FX-4000™ Compression System (Flexcell® International Corporation, Burlington, North Carolina) (Appendix 6.14).

At day 21 the mRNA levels of an early mineralisation marker alkaline phosphatase (ALP), a late mineralisation marker bone gamma-carboxyglutamate protein (BGLAP) also known as osteocalcin (OCN) and bone morphogenetic protein-2 (BMP2), were determined. The expression levels were normalised to the reference gene beta2-microglobulin (B2M). The B2M gene has been previously shown to be a suitable for the normalisation of osteogenic gene expression (Godoy-Zanicotti et al., 2015).

4.4.1 Osteoblast hydrogel encapsulation and preparation of FlexCell® BiopPress™ culture plates

Hydrogel components were reconstituted according to the protocol developed in Chapter 3, using a 1:1 hyaluronan-gelatin to PEGDA ratio value (Appendix 6.12, Table 5).

For each experiment, cells were cultured until confluent, trypsinised and counted as described (Appendices 6.1 and 6.2). The cells were divided into individual vials containing 40x10^5 cells and centrifuged to form a pellet of a known cell concentration. The aim was to encapsulate 800,000-1000,000 cells per 250 µL of hydrogel. To avoid premature gelation the cell encapsulation was performed in two batches. Each time, 1000 µL hydrogel was reconstituted and added to the pellet of cells. Using a wide bore pipette, 250 µL each of Gelin-S and Glycosil and 500 µL PEGDA was added to a cell pellet and pipetted up and down to create a homogenous solution. Two 125 µL hydrogel-cell layers were pipetted separately. Dividing the 250 µL into two 125 µL layers decreased the chance of the cells sinking to the bottom and improved the homogenous distribution of cells. The
first layer was allowed to set for fifteen minutes before the second layer was added on top. Following a further 30-minute gelation period inside the 13mm well (Figure 35), mineralisation media (4 mL) was added to each well and incubated at 37°C CO₂/95% air. The culture medium was changed twice a week.

**Figure 35. BioPress™ culture plate and platens.**

The hydrogel-osteoblast preparations were transferred, using a pipette, to the centre of the 13mm diameter foam holder (B) and left to set. After 24 hours, the compression platens (A) were placed on top of the set constructs (C).
4.4.2 Experimental design

Two FlexCell® BioPress™ plates were used for each experiment (E), one test and one control plate. The experiment was repeated three times with a total of six plates and this is represented as E1-E3 (Figure 36). Each plate included encapsulated HCOs and HFOs in duplicate wells for RNA extraction and one well of each cell line for proliferation (PB) measurement. The three independent experiments (E1-3) were run using three different vials of cryo-preserved passage 3-5 HCOs and HFOs. RNA was extracted from 24 samples.

![Figure 36. Representation of BioPress™ plate layout for the three independent experiments.](image)

HCOs (red) and HFOs (blue) were encapsulated into 250 µl hydrogel, plated into 13mm diameter wells and incubated for 21 days with or without cyclic mechanical compressive strain, after which RNA was extracted. Proliferation (PB) was measured continuously as a live-point assay at days 1, 7, 14 and 21.
4.4.3 Preparation of BioPress™ plates for mechanical load

The compression platens were fitted to the top of each well following a 24 hr incubation post-plating and gelation. Using an equation provided by the manufacturer, which includes provision for sample height, a calculation was made which indicated the each platen was to be rotated twice. Each BioPress™ plate was placed on the FlexCell® baseplate, the clamping pads tightened and the assembled compression system was placed into a 37°C CO₂/95% air incubator. The control non-mechanical load group was prepared in the same manner in the BioPress™ plates and incubated in parallel.

4.4.4 Compression regimen programming

The minimum force programmable, which is recommended by the FlexCell® Corporation is 0.5 lbs-force (FlexCell® Compression Manual, 2009). As only approximately half the applied force is transmitted to the cells (Wall et al., 2007; Bieler et al., 2009), and because of the delicate nature of hydrogel, a 1 lbs-force was used. The 21 day regimen was programmed as a cyclic compression of 1.0 lb-force every second (1 Hertz) for 5 secs every hour for 6 hrs (Appendix 6.14). The load was applied through positive pressure underneath the BioPress™ plates against the stationary platen on top of the sample holder. The pressure applied was dependent on the surface area of the 3-D sample to which the force was being applied. Compressive pressure was calculated to be to 0.0334 mPa or 33.4 kPa (340.6 gm/cm²) using a formula provided by the manufacturer (Appendix 6.14).

4.4.5 Effect of compressive load on cell proliferation of HCOs and HFOs

The relative rates of proliferation were continuously measured using the PrestoBlue™ proliferation assay with recording made at day 1, 7, 14 and 21. At each time point the test and control BioPress™ plates were removed from the incubator and the stationary compression platens were lifted and the old media replaced with 3 mL of fresh culture medium. Each well required 325 µL of PrestoBlue™ dye (1:10) ratio. The additional 25 µL of PrestoBlue™ dye was added to account for the volume of the hydrogel construct (250 µL) (Bonnier et
To determine a suitable incubation time for the volume of hydrogel, recordings were made at 2 and 5 hrs following incubation at 37°C. As the BioPress™ plates could not be used in a fluorescence plate reader due to their size and shape, following the assay incubation time, the supernatant (200 µL) was transferred to a 48-well plate. The resulting level of fluorescence was measured using a Synergy™ 2 multi-mode microplate reader at 540 nm excitation/ 610 nm emission (Biotek, USA) (Appendix 6.9). Following the proliferation assay, each construct was replenished with fresh media to 4 mL and returned to the incubator. The compression group was placed back into the compression unit in the incubator.

4.4.6 Extraction of RNA using TRizol® from osteoblasts cultured in hydrogel

TRizol® contains guanidinium thiocyanate and phenol to break down cells and tissue and for complete dissociation of nucleoprotein complexes while maintaining RNA integrity (Chomezynski and Sacchi, 1987; Chomezynski and Sacchi, 2006). The suitability of TRizol® reagent as a direct method of cell recovery from hydrogels has been successfully established in other studies and is more efficient than the alternative collagenase digestion method which involves an overnight incubation (Khetan and Burdick, 2009; Yu et al., 2013; Damaraju et al., 2014).

The RNA was extracted from a total of 24 constructs consisting of mechanical load (test): 6 HFOs and 6 HCOs; no mechanical load (control): 6 HFOs and 6 HCOs. The constructs were recovered from each well using TRizol® according to manufacturer instructions (Appendix 6.16). Homogenates were stored in 1.5 mL microfuge tubes at -80°C until RNA extraction.

4.4.7 RNA quality determination

RNA was extracted according to manufacturer instructions using the RNA Pure Purification Kit (Life Technologies). The protocol includes a phase separation, a step to bind the RNA to a silica membrane inside a spin-column followed by repeated washing steps before the purified RNA is eluted from the silica membrane. The quality and yield of RNA was determined by spectrometry using
a NanoVue™ (GE Healthcare, USA). The concentration of RNA can be quantified by measuring the UV absorption at 260 nm. The Beer-Lambert Law is applied to predict a linear change in absorbance, which then corresponds with nucleic acid concentration. An A260 reading of 1 is approximately 40 µg/mL of RNA. Pure RNA has an A260/A280 ratio between 1.8 - 2. Because contaminants like the aromatic amino acids absorb light at 280 nm, ratios < 1.8 may indicate contamination with proteins, and components from TRIzol® containing guanidine isothiocyanate and aromatic substances like phenol. The A260, A260/A280 ratio and concentration were recorded for each of the 24 RNA samples, presented (Appendix 6.17).

4.4.8 Reverse transcription of mRNA to cDNA

The purified total RNA (300 ng) was used to synthesise complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription kit for Real-Time PCR (Applied BioSystems, Life Technologies, USA). A reverse transcription master mix was prepared by combining RT buffer, dNTP Mix, RT Random Primers, Multiscribe Reverse Transcriptase and nuclease-free water in a sterile 1.5 mL microfuge tube. To check for contaminating genomic DNA, the same set of samples without reverse transcriptase (RT-ve) was also included and the volume of RNA was replaced with nuclease-free water. Each reaction was prepared in a PCR tube and included the master mix (10 µL) and RNA (300 ng/10 µL). The reaction tubes were centrifuged for 30 sec before being placed in the PTC-100® Thermal Cycler. The cDNA was synthesized following a 10 minutes 25°C incubation followed by 120 minutes of incubation at 37 °C. The enzyme was inactivated at 85 °C following a 5 seconds incubation.
4.4.9 Relative mRNA level determination using qPCR

A TaqMan® Duplex qPCR assay (Life Technologies) was used to determine the relative expression levels of Bone Morphogenetic Protein-2 (BMP-2) (Hs00154192_m1) osteocalcin (BGLAP) (Hs01587814_g1); and alkaline phosphatase (ALP) (Hs00154192_m1) mRNA. The reference gene Beta2-microglobulin (B2M-Primer Limited) (Hs00187842_m1) was used to normalise the mRNA expression data. The reference gene should be validated to ensure that expression of the gene is not affected by the experimental method (Livak et al., 2001).

Relative gene expression was determined using the $2^{-\Delta\Delta C_T}$ method (Livak et al., 2001). The two most common methods of data analysis used for real time qPCR experiments are absolute and relative quantification. Absolute quantification relates the PCR signal to a standard curve. Relative quantification relates the PCR signal to a control. The determination of the fold-change value is a convenient method for the determination of relative gene expression and can be used instead of the calculation of an absolute transcript number. For this to be valid, the amplification efficiency of the target and the reference must be approximately equal. qPCR assay amplification efficiencies (\(E = 10^{(-1/Slope)-1}\)) were calculated for each assay using a three-point serial dilution of template cDNA (10ng, 1ng and 0.1ng) and run in duplicate.

Each TaqMan® Duplex qPCR assay contains cDNA (5 µL) and a TaqMan® master mix (15 µL). The master mix for each assay contains TaqMan® FAST qPCR master mix (2x; 10 µL), gene of interest qPCR assay (20x; 1 µL), reference gene qPCR assay (20x; 1 µL) and H₂O (3 µL). The qPCR assays were conducted on a QuantStudio6® PCR system (Life Technologies) in 96-well plates using cycling condition of 95°C for 20 sec, 40 cycles of 95°C for 3 sec and 60°C for 30 sec. Threshold value was set at 0.05 and the background cycles were set at 3-10 cycles.
4.5 Data analysis

Cq values were normalized against the reference gene and the fold-regulation calculated using the ΔΔCq method (Livak et al., 2001). A fold-change greater than one indicates an up-regulation. A fold-change of less than one represents a down-regulation and is the negative inverse of the fold-change.

Statistical analysis and graphing was performed using GraphPad Prism 6 (GraphPad Software Inc., CA, USA). Differences between groups were analysed using the non-parametric Mann-Whitney test which does not assume a normal distribution and were considered significant when \( P < 0.05 \).
4.6 Results

4.6.1 Effect of mechanical load on proliferation of HCOs and HFOs

PrestoBlue™ fluorescence readings following incubation of the osteoblasts with the dye for 2 or 5 hr showed similar results. However, by 5 hrs the 250 µL hydrogel was completely saturated and the fluorescence signal had increased suggesting more cells had been exposed to the dye (Figure 37 A and B). The 5hr incubation time (Figure 37 B) showed, all the hydrogel-encapsulated cells were viable at 7 days and at 14 days the HFO were proliferating at a higher rate than the HCOs. The data from two replicate experiments (E1 and E2) are presented, a third proliferation experiment (E3) was not included as it was evident that the gelatinisation step for the gel/cell construct to be used for the proliferation assay had failed to produce a gel as stiff as observed in the first two experiments (E1 and E2). It was decided therefore not to include the data. The results from the first two experiments showed the cells proliferated over 21 days when encapsulated in the hydrogel.

![Figure 37. Osteoblast proliferation rates when exposed to a cyclic mechanical load over 21 days.](image)
(A) PrestoBlue™ assay 2 hr incubation. (B) PrestoBlue™ assay 5 hr incubation. Proliferation rates were measured at 1, 7, 14, and 21-days. HFO (blue) showed higher rates of proliferation compared to HCO (red). Mechanically loaded osteoblasts (dashed line) proliferated at a slower rate compared to controls (no load) (solid line). Independent experiments (E1 and E2) data is presented as +/-SD.
By 21 days, aggregation and retraction of osteoblasts into nodules could be seen macroscopically in the transparent hydrogel, in both, the control group and the mechanical load test groups (Figure 38 A and B). In the control plate, the HCOs showed a more condensed retraction in all three biological replicates. This could be indicative of a faster onset of mineralisation. This was a consistent observation, although a subjective assessment, it provided information regarding the HCOs biological activity and viability as the proliferation assay suggested some cell death.

Figure 38. Nodule formation at day 21.
Nodules can be seen in when the hydrogel and osteoblast constructs, are viewed on a light box. Plate (A) shows a control plate and (B) a cyclic mechanical load group. Aggregations of cells and the start of osteoblast retraction into a nodule can be seen in most wells.
4.6.2 RNA quality

The RNA was extracted from the encapsulated cells hydrogel and converted to cDNA successfully. The concentration of RNA extracted from 24 samples (8 samples per experiment) ranged from 0.3 to 6.53 µg (avg. 3.03 µg)/ 1 x 10⁶ seeded cells. The purity of RNA was measured spectrometrically with majority of the samples falling within the 1.8-2.0 A260/A280 ratio range. Two samples were outside the range, and possible contamination may have been from the TRIzol® used during purification (Appendix 6.17). The cDNA was standardized to a concentration of 1 ng/ µL for qPCR.

4.6.3 Relative levels of mRNA expression

4.6.3.1 TaqMan® duplex RT-PCR assay sensitivity

To measure qPCR amplification efficiency, a serial dilution curve was performed and the slope calculated. A slope of -3.3 indicates a qPCR assay with 100% efficiency. A 90 - 110 % efficiency is considered a good working range.

The detection range for each duplex assay was determined using a dilution series of osteoblast cDNA. A 93.8% efficiency was achieved for ALP (slope -3.48, R² 0.998) and 86.6% for reference gene B2M (slope -3.69, R² 0.996) (Figure 39A). A 61.5% efficiency was achieved for BMP2 (slope -4.8, R² 0.981) and 69.1% for its reference gene B2M (slope -4.38, R² 0.998) (Figure 39B). High levels of amplification for the reference cDNA were achieved for the OCN duplex assay, however no OCN was detected in any of the test or control samples. All RT minus cDNA samples resulted in no amplification (Cq 40), indicating no genomic DNA contamination.
Figure 39. TaqMan® duplex RT-PCR assay sensitivity.
(A) An amplification efficiency of 93.8% was achieved for ALP (●) and 86.6% for reference gene B2M (○). (B) Low amplification efficiencies were achieved for both BMP2 (●) (61.5%) and the reference gene B2M (○) (69.2%). The qPCR assays were run in duplicate. Data is expressed +/- SEM.
4.6.4 Relative levels of mRNA following mechanical load

4.6.4.1 Alkaline Phosphatase (ALP) mRNA expression

The level of ALP mRNA expression was measured using RT²-qPCR and the fold-regulation calculated for each cell line. Although not significant, 21-days of mechanical strain resulted in a reduction of ALP expression for both cell lines (HFO; 17.4-fold down-regulation, Mann-Whitney p=0.132, HCO; 24.8-fold down-regulation, Mann-Whitney p=0.243). There was no significant difference between the cell line control groups in their expression of ALP mRNA after 21-days (6.6 fold; p=0.0649). However, this may be the result of the wide range of HCO mRNA expression levels found across the three experiments making a comparison difficult. A higher level of ALP mRNA was expressed in the HFO mechanical strain test group compared to the equivalent HCO group (p=0.0022), however this could be due to the higher level of endogenous ALP expression (Figure 40 and Appendix 6.18).

Figure 40. Relative ALP mRNA expression levels in HCOs and HFOs, cultured in hydrogel with and without mechanical load for 21 days. Data is expressed as a linear transformation of the Cq (2^ΔCq). Three independent experiments: E1: black; E2: purple; E3: green are shown. The box-whisker plot shows the minimum and maximum range of ALP mRNA detected. The dashed line indicates limit of detection.
4.6.4.2 Osteocalcin (BGLAP/OCN) mRNA expression

OCN was not detected in any of the 24 RNA samples after 40 qPCR cycles. Adequate levels of the reference gene B2M were detected in all of the samples indicating good RNA quality. A Cq value of 40 was assigned to samples with no detectable mRNA (Appendix 6.20).

4.6.4.3 BMP2 mRNA expression

The relative levels of BMP2 mRNA expression were determined and the fold-regulation calculated for both the HCO and HFO cell lines. BMP2 mRNA was not detected in either the control or test group for the HCO cell lone. However, a highly significant 53.9 fold up-regulation of BMP2 mRNA expression (p=0.0043) was observed in the HFO test group (mechanical load) (Appendix 6.19). As the qPCR efficiency for the BMP2 duplex TaqMan® assay was low these results should be interpreted with caution.

![Figure 41](image)

**Figure 41. Relative mRNA expression of BMP2 levels in HFOs, cultured in hydrogel with and without mechanical load for 21 days.**

Data is expressed as a linear transformation of the Cq (2^-ΔCq). Three independent experiments: E1: black; E2: purple; E3: green are shown. The box-whisker plot shows the minimum and maximum range of BMP2 detected mRNA levels. **p=0.0043. The dashed line indicates limit of detection.
4.7 Discussion

4.7.1 The effect of mechanical load on proliferation of HCOs and HFOs

To measure proliferation, PrestoBlue™ was again used. The advantage of PrestoBlue™ proliferation assay is that it is not an end-point assay allowing the evaluation of metabolic activity at multiple time points. While PrestoBlue™ proliferation assay was successfully employed using 20 µL small hydrogel constructs in Chapter 3, a larger volume of 250 µL hydrogel was required for effective compression, with no less than a 1mm thick sample required. It was therefore necessary to validate the PrestoBlue™ incubation time for a larger hydrogel construct volume. Fluorescence measurements were made following 2 and 5 hr incubation with the dye. As the purple dye turns pink as it is metabolised, the colour change could be seen to saturate the gel after 5 hr incubation time.

Recommendations for future research include increasing the number of samples. Additionally, comparing the PrestoBlue™ readings to the expression levels of histone H4, a chromatin associated structural protein would validate the proliferation data. The lower rates of cellular proliferation observed in cell cultures exposed to mechanical load is thought to be due to an increase in the rate of apoptosis. This could be confirmed in future studies designed to evaluate the patterns of expression of a cluster apoptosis related genes.

4.7.2 Visual observation of the retraction process by HCOs and HFOs

Culturing the HCOs and HFOs in the non-transparent BioPress™ plates, made it not possible to view the samples under a light microscopy. However, aggregation and retraction of cells was visible by day 21, demonstrating the same retraction processes had occurred in the thicker hydrogel as was previously observed in the smaller hydrogel constructs in chapter 3.

Observation of retraction, which precedes the nodule formation process, provided valuable insight into the biological activity of osteoblasts, without
removing and damaging the samples. The laying down of the collagenous matrix becomes evident with retraction of osteoblasts and formation of mature osteoblast colonies called nodules, which are areas of osteoid mineralisation with a histologic appearance of woven bone (Liu et al., 1997; Malaval et al., 1999). It would have been interesting to extend the incubation time to continue to observe the nodule formation process.

In future experiments, scanning electron microscopy (SEM) could be used to examine morphological changes that may occur when the HCOs and HFOs are exposed to a mechanical load. Saminathan and co-workers (2015), cultured periodontal ligament cells in Extracel™ (HyStem-C®) hydrogel, and used SEM to observe the attachment of the cells to the hydrogel matrix (Saminathan et al., 2015). However their constructs became dehydrated under the heat of the scanning electron microscope resulting in a collapse of the hyaluronan and gelatin fibrils and hydrogel matrix failure. As long as this limitation is considered, SEM studies could further our understanding of the ability of osteoblasts to spread and attach to extra-cellular matrix and may explain the observed differences in the morphology of cells in the test and control groups.

4.7.3 Quality of RNA extracted and accuracy of mRNA detection

A 93.8% efficiency was achieved for the ALP qPCR assay. However, only a low 61.5% qPCR efficiency was achieved for the BMP2 assay indicating a less reliable result might be achieved. The assay should be further validated with additional cDNA samples. A duplex BMP2 qPCR assay achieved a 85.2% efficiency for BMP2 when combined with the GAPDH reference gene and a cDNA template synthesised from RNA recovered after exposed to a mechanical load for 7 days.

The accuracy of mRNA detection by qPCR also depends on the expression levels of the reference gene. A validated reference gene B2M was used. Good levels of the mRNA reference gene B2M were identified for every sample, confirming that the extraction protocols worked efficiently. The B2M reference gene used in this study was validated by Godoy- Zanicotti (2015) at the University of Otago and shown to be suitable for the study of osteogenic genes. Yang and co-workers
(2012) showed variable results in the expression levels ALP and OCN mRNA can be measured if unstable reference genes are used for the detection of mineralisation markers. They have recommended using caution when interpreting the data from studies using non-validat​ed reference genes.

### 4.7.4 Detection of ALP, OCN and BMP2 mRNA

ALP and OCN are common markers of osteoblast phenotype and bone formation (Pavlin et al., 2001). There is considerable evidence demonstrating the function of BMP2 as an inductor of osteogenesis with important roles in skeletal development and bone formation (Marie et al., 2002).

#### 4.7.4.1 Detection of ALP and OCN mRNA

The ALP enzyme has an important role in the liberation of organic phosphate making it available for the deposition of calcium phosphate salts or hydroxyapatite (Bellows et al., 1991; Aubin, 1998). As the mineralisation cascade progresses and mineral is laid down, cellular proliferation and ALP enzyme levels start to decrease and OCN levels rise. ALP is an early marker of mineralisation and was detected in both the HCO and HFO cell cultures. The application of mechanical strain to the test cell cultures was associated with a down-regulation ALP gene expression (HFO 17.4-fold; HCO 24.8 fold), however this finding was not statistically significant. Further studies are needed to review this finding with larger sample sizes, as there is a known inverse relationship between proliferation and mineralisation. It may be that the reduced rate of proliferation and down-regulation of ALP gene expression observed in the cell cultures exposed to mechanical strain is related to an earlier onset of mineralisation of the hydrogel matrix. Further experiments are required to assess the differences in the responses of calvarial and femoral osteoblasts to mechanical strain.

No late mineralisation marker OCN was detected at 21 days in any of the extracted RNA samples. As bone marker proteins are expressed at different times during the mineralisation process, it may be that at 21-days there had not been sufficient progression of the mineralisation process for OCN to be
expressed (Liu et al., 1997).

OCN is a major non-collagenous calcium binding protein in bone. It is expressed specifically in mature post mitotic osteoblasts and is considered a late mineralisation protein marker, unlike ALP, which is an early mineralisation protein marker. An inverse relationship exists between proliferation and OCN (Aronow et al., 1990; Lian and Stein, 1992; Malaval et al., 1999) and this may explain the absence of OCN, as the cells were still proliferating at 21 days. Additionally, variability may still be seen as the osteoblasts may be in different maturational stages, ranging from proliferating osteoblasts to mature hydroxyapatite secreting cells (Aubin and Liu, 1996).

There are further potential reasons why OCN was not detected. Expression depends on multiple factors including the type of osteoblasts used, passage number, individual osteoblast donor variations, as well as the environment the cells are cultured in. To control for these variables, a commercial line of foetal HFOs and HCOs was used from two donors and passage numbers were kept to no more than 5. Furthermore, unlike alkaline phosphatase, which is expressed by all osteoblasts, mature osteoblast markers like osteocalcin are heterogeneously expressed depending on the site they are harvested from and maturity of the donor bone (Candeliere et al., 1998). Put simply, osteoblasts are not a homogenous class of cells and show marked variation in their patterns of gene expression. The osteocalcin gene is one marker which has been shown to be variably expressed (Liu et al., 1997).

The timing of the different phases of maturation and mineralisation may also differ depending on the culture system used. In a monolayer culture after approximately 14-21 days, the proliferation decreases as the osteoblasts deposit hydroxyapatite and become embedded in the mineralised matrix, unable to proliferate (Coelho et al., 2000). However, because hydrogel offers a different environment, the rate of proliferation and the timing required for mineralisation to start may need to be longer. Additionally, high levels of hyaluronan in the hydrogel may affect differentiation pathway of the osteoblasts.
4.7.4.2 Detection of BMP-2 mRNA

A statistically significant increase in BMP2 gene expression was observed in the HFO cell cultures for the test group compared to the control (Fold-change value 53.9). No BMP2 was detected in HCO samples.

The osteoinductive properties of bone are due to the presence of bone morphogenetic proteins, belonging to transforming growth factor-beta (TGF-β) superfamily. BMPs 2, 4, 6, 7 and 9 are considered as osteogenic proteins with roles in skeletal development, proliferation, differentiation and maturation of osteoblasts (Marie et al., 2002; Cheng et al., 2003). BMP2 has been shown to induce expression of ALP and thus facilitate the process of mineralisation (Rawadi et al., 2003). Matsubara and co-workers (2008) showed that BMP2 induces Osterix (Osx), an osteoblast specific transcription factor, by up-regulating ALP, Msx2 and Runx2 genes required for osteoblast differentiation and bone formation. Future studies could elaborate further on this observation by conducting assays to determine the patterns of expression of Osx, Msx2 and Runx2.

The measured expression of BMP2 which was limited to the HFO cell cultures is consistent with the findings of previous in vivo studies which have shown a difference in expression of BMPs between intra-membranous calvarial and endochondral long bones (Suttapreyasri et al., 2006). Suttapreyasri and co-workers showed variability in the levels of BMPs within intramembranous bone. BMP6 was detected at the highest levels followed by BMPs2, 5 and 7. Endochondral bone has been shown to express high levels of BMP2, followed by 5 and 6. This may explain the BMP2 expression in the HFOs but not in HCOs. These differences in BMP expression may play a role in bone-graft healing and may be responsible for bone healing. Because the type of bone formation at a graft site depends on its origin, endochondral bone formation occurs when the donor comes from endochondral bone, while intramembranous bone formation is noted when the donor comes from intramembranous bone.
As discussed (Chapter 3) the ability of a scaffold to retain BMPs for over-time is one of the important prerequisites for its use as a graft material. The up-regulation of BMP-2 in the mechanical strained HFOs suggests that osteoinductive properties are present, which is a further step to engineering a complete bone matrix.

The effect of mechanical stimulation on the expression of BMPs was studied by Kopf and co-workers (2012). They used a 3-D collagen scaffold model for the culture of osteoblasts and showed that cyclic mechanical compressive load activated mechanotransduction pathways, increased BMP2 signalling pathways and stimulated bone formation. This may explain why BMP2 mRNA levels were up-regulated in the HFO test group and not in the control group in the present study and indicates a relationship between BMP expression and mechanical loading of HFO cell cultures over a period of 21 days.
4.7.5 The limitations of mechanically active in vivo culture systems

It has been reported that osteoblasts may lose most of their ability to express osteoblast-characteristic genes in the first 48 hours of in vitro culture, possibly due to the changes in cell-cell and cell-matrix communication (Krebsbach et al., 1993). While hydrogels mimic the extracellular environment and offer suitable properties required for cell-matrix signalling (Cukierman et al., 2001; Tibbitt and Anseth, 2009), the culture system and the expressed mRNA represents only a small part of what occurs in the complex in vivo environment.

A range of materials are commercially available for use as in vitro cell culture substrates, and with continuing developments in this area, the number of available substrates is only likely to increase. There is also significant variation in the experimental application of mechanical loads to cell cultures as loading regimens may vary in their character, magnitude, frequency and time course. The duration of studies currently reported in the literature can range from a few hours to a few weeks of mechanical stimulation (Li et al., 2013). Similarly, incorporation of growth factors into osteoblast culture to stimulate proliferation and mineralisation will also have a different effect depending on the distinct stages of cell maturation (Debiais et al., 1998). All these variables make any comparison difficult. The present study is the first to use the hyaluronan-gelatin hydrogel on osteoblasts in a mechanically active environment.
4.7.6 Observation of mechanical properties of hydrogel

Based on the observations made in our pilot study, HyStem-C® hydrogel is an appropriate substrate for mechanically active osteoblast cell culture studies of short duration. Consideration has to be given to the potential for time-dependent degradation of the hydrogel substrate as a result of incubation in the presence of cyclic mechanical loading. In the present study, the 3-D architecture of the hydrogel has been preserved following 7 days of cyclic loading in mechanically active culture. By day 21, the 3-D architecture remained intact, but there had been a loss of dimensional stability, as the gel had become thinner and less dense (Appendix 6.21; Figure 50B). An important feature of the hydrogel used in the present study is the ability to modify the PEGDA concentration thus changing the mechanical properties, making it more suitable for long incubation times.

Vanderhooft and co-workers (2009) have carried out rheological tests on the hyaluronan-gelatin hydrogels used in the present study and showed modification of PEGDA concentration had a significant effect on gel stiffness, which ranged from 11Pa to 3.5kPa. The stiffness of the scaffold alters expression of osteoblast adhesion molecules affecting spreading and signalling behaviour (Engler et al., 2004) and future experiments to assess the effect of different PEGDA concentrations on osteoblast proliferation and osteogenic mRNA protein levels should be conducted. Rheology testing can be conducted to assess the strain/stress and elastic modulus characteristics on the selected models, in collaboration with Biomedical Engineering and Chemistry Departments, as was done in previous studies (Ghosh et al., 2005).

To increase the ability of hydrogel to withstand longer incubation times under a cyclic compressive load, Saminathan and co-workers (2015) suggested incorporating thin collagen-1 sheets to form a more robust scaffold. Taking their recommendations, a pilot study was undertaken during the planning stages of this project. Thin collagen sheets were combined with the cell-populated hydrogel and incubated for 7 days. As collagen-1 is the major component of the extracellular matrix, incorporating thin sheets of collagen, rather than a synthetic material offered a biologically compatible method to strengthen the scaffold.
Permacol™- medical grade 0.5mm thick collagen-1 sheets, were compared to a control group of hydrogel-only. Due to the ability to retain its shape, the hydrogel only model was deemed suitable for the purposes of this project, and the combined collagen sheets and hydrogel construct was not ultimately used. However, the results of the pilot experiment confirmed it was possible to construct a layered model that would encapsulate the cells and provide a stiffer scaffold. The results of this pilot study are presented in Appendix 6.21.

4.7.7 Summary

The results of the present study showed that mechanically loaded osteoblast cell cultures had reduced rates of proliferation and a tendency towards the down-regulation of ALP compared to their un-loaded controls. The mechanically active HFO cell cultures up-regulated their expression of BMP lending further support to the growing body of evidence, which suggests that many of the central regulatory mediators of bone metabolism are mechano-responsive.

The decreased proliferation and down regulation of ALP following mechanical load, although not significant, suggested the mechanically loaded group was ahead of the control group in the mineralisation cascade. OCN was not detected which suggests that the late phase of the mineralisation had not been reached. It is now well accepted that osteoblasts grown in in vitro cell cultures are responsive to the specifics of their cell culture environment. It may be that longer incubation periods are required to allow for maturation of the mineralisation process to the point at which OCN could be detected. In future studies, to compare the mechanical strain response of calvarial and femoral osteoblasts the sequential expression of bone marker proteins related to proliferation, maturation and mineralisation should be measured over an incubation period of 4 – 6 weeks with data collected at multiple time points.
4.8 References


Chapter 5:
Summary

This present study was designed to help lay the foundation for the future generation of an in vitro mineralised bone matrix. This engineered bone matrix material, when implanted, would act as a biocompatible, osteoconductive and osteoinductive framework for ingrowth of host cells and mimic the remodelling behaviour of normal bone matrix.

The specific aims of the present study were to: 1) determine if a thiol-modified hyaluronan-gelatin-PEGDA cross-linked hydrogel is a suitable scaffold for the study of osteoblast proliferation and hydroxyapatite deposition in vitro; 2) to validate assays for the measurement of osteoblast proliferation and hydroxyapatite deposition in hydrogels; and 3) examine the suitability of the hydrogel model for the study of mRNA expression in cells under mechanical strain.

Since mechanical loading is an important determinant of bone mass and architecture, and mechanical strain has been shown to promote osteogenic differentiation in vitro (Jagodzinski et al., 2004; Sittichokechaiwut et al., 2010; Li et al., 2013), the osteoblast/hydrogel constructs were cultured in a mechanically active environment.

In chapter 2, experiments were conducted to validate the traditional 2-D monolayer osteoblast culture methods and staining techniques. Commercial human foetal osteoblasts from two embryologic origins (femoral and calvarial) were cultured in triple supplement mineralisation medium (dexamethasone, ascorbic acid and B-glycerophosphate) (Bellows et al., 2986). Mineralised nodule formation was confirmed with Alizarin Red and a quantitative fluorescent method (OsteoImage\textsuperscript{TM}) to detect hydroxyapatite deposition. Both cell lines produced nodules, with a slightly faster onset of proliferation and mineralisation observed with the HFO line.
In chapter 3, experiments were conducted to evaluate the suitability of hyaluronan-gelatin-polyethylene glycol diacetate cross-linked semi-synthetic hydrogel matrix (ExtraceTM/HyStem-C®) as a scaffold for culturing osteoblasts in a 3-D environment. This hydrogel had been successfully used for culturing PDL cells in a mechanically active environment by Saminathan and co-workers (2015). The addition of PEGDA to the hyaluronan-gelatin hydrogel allows the cross-linking to be increased. Two modifications of cross-linking were tested with cell proliferation rates and hydroxyapatite deposition quantified over a 21-day culture period. Confocal microscopy was used to assess the spatial arrangement of the osteoblasts within the hydrogel, but limited hydrogel penetration by the fluorescent dyes was observed. Future research should aim to optimise the penetration of the fluorescent dyes by adjusting the incubation times and through the incorporation of detergents to enhance dye penetration. Both osteoblast cell lines were able to grow, proliferate and deposit hydroxyapatite in both hydrogel modifications. However, the more highly cross-linked hydrogel was a better scaffold for osteoblast attachment as judged by microscopic observations of osteoblast morphology. The more cross-linked hydrogel was used for the mechanical strain experiments in Chapter 4.

Chapter 4 examined the ability of HFOs and HCOs to proliferate in larger hydrogel constructs with and without intermittent mechanical compressive loads applied with a FlexCell® unit. The effect of cyclic compressive load on the expression of alkaline phosphatase (ALP), osteocalcin (OCN) and bone morphogenetic protein 2 (BMP2) mRNA levels, relative to the reference gene beta2-microglobulin (B2M) were investigated. Cyclic compressive mechanical load resulted in a decrease in ALP expression (HFO; 17.4-fold p=0.132, HCO; 24.8-fold, p=0.243), which was not statistically significant, and an up-regulation in BMP2 expression in the HFO group (53.9-fold; p=0.0043). These results support previously published findings of mechanotransduction and BMP signalling pathways working synergistically (Kopf et al., 2012). No OCN mRNA was detected in either cell line, which could be the consequence of OCN being a late stage marker of mineralisation. As the proliferation and mineralisation sequence appears to commence later for the present model, longer incubation
Periods may be required for the process to mature sufficiently for OCN to be expressed. Detection of ALP mRNA should also be performed at multiple time points. Further experiments are required to assess the full osteogenic profile and the differences in the responses of calvarial and femoral osteoblasts to mechanical strain. Additionally, more mechanical regimens should be trialled at various time points.

In summary, the overall objective to evaluate whether it is possible for osteoblasts to proliferate and produce a mineralised matrix in a thiol-modified hyaluronan-gelatin-PEGDA cross-linked hydrogel was achieved. The hydrogel was found to be a suitable scaffold for the study of calvarial and femoral osteoblast proliferation and hydroxyapatite deposition in vitro. Assays for the measurement of osteoblast proliferation and hydroxyapatite deposition were validated. This hydrogel model was found to be suitable for studying mRNA expression under mechanical strain.

This study has contributed to the knowledge of how hyaluronan-gelatin-polyethylene glycol diacetate cross-linked semi-synthetic hydrogel matrix (Extracel™/HyStem-C®) can be used to study osteoblast proliferation and mineralisation processes in a 3-D mechanically active environment, which aim to reflect the in vivo environment better than conventional 2-D models. Possible directions for future research include further modifications of hydrogel cross-linking properties; combining hydrogel with thin collagen sheets; and culturing osteoblasts in sequential layers of hydrogel to simulate the appositional bone growth in vivo.
Chapter 6:
Appendix

Table of Protocols

6.1 Passaging of adherent cells (osteoblasts) using trypsin
6.2 Cell counting using a haemocytometer
6.3 Cryopreservation and storage of cells
6.4 Thawing cryopreserved cells
6.5 Preparation of triple supplement
6.6 Osteoblast growth and mineralisation medium preparation
6.7 Alkaline phosphatase staining protocol
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6.21 Pilot study : Permacol® sheets with hydrogel

All cell culture procedures were conducted in a laminar flow hood using sterile conditions.
6.1 Passaging of adherent cells (osteoblasts) using trypsin

Reagents and materials

- Phosphate buffered saline (PBS) without Ca\(^{++}\) and Mg\(^{++}\) (Gibco, Life Technologies, Cat No 14190250)
- 0.25% Trypsin/EDTA (Gibco, Life Technologies, Cat No 25200056)

Procedure

1. Using an inverted light phase contrast microscope check if HCOs and HFOs have reached 60 - 80 % confluency. Pre-warm PBS, culture media, trypsin in 37°C water bath.

2. Remove cell culture medium from culture flask. Wash cells with appropriate volume of pre-warmed PBS and discard.

3. Add adequate volume of 0.25% trypsin to cover the entire based of the flask. Incubate for 2 min at 37°C and check for cell de-attachment from the flask. This will be evident as the cells will appear round and will be moving freely in the trypsin.

4. Gently tap the flask with the palm of your hand to dislodge any remaining adherent cells. Inactivate the trypsin with the addition of 10 mL culture media. The FBS will inhibit the trypsin.

5. Evenly distribute the cells in the media by pipetting the cells and media up and down gently and transfer the required number of cells to new cell culture flasks containing fresh media.

Table 3. Useful cell culture flask information.

<table>
<thead>
<tr>
<th>Flask</th>
<th>Surface area mm(^2)</th>
<th>Seeding density</th>
<th>Cells at confluency</th>
<th>Trypsin 0.25%</th>
<th>Media (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-25</td>
<td>2,500</td>
<td>0.7x10(^6)</td>
<td>2.8x10(^6)</td>
<td>3</td>
<td>3-5</td>
</tr>
<tr>
<td>T-75</td>
<td>7,500</td>
<td>2.1x10(^6)</td>
<td>8.4x10(^6)</td>
<td>5</td>
<td>8-15</td>
</tr>
<tr>
<td>T-160</td>
<td>16,000</td>
<td>4.6x10(^6)</td>
<td>18.4x10(^6)</td>
<td>10</td>
<td>15-30</td>
</tr>
</tbody>
</table>
6.2 Cell counting using a haemocytometer

Reagents and materials
- Filtered 0.4% trypan blue solution (Gibco, Life Technologies, Cat No 15250061)
- 70% ethanol (Thermo Scientific, Cat No R2470110)
- Filter, sterile eppendorf tubes, pipettes (Gibco, Life Technologies)

Equipment
- Light microscope (MRC/B146; Olympus, Hampton, Tokyo)
- Neubauer Haemocytometer (Cat No. BS-748; Hawksley)

Procedure
1. Clean haemocytometer with 70% Ethanol. Moisten the shoulders of the haemocytometer and affix the coverslip.
2. Mix cells gently by pipetting up and down. Transfer cells (15 µL) and tryphan blue (15 µL, 1:1) to a 1.5 mL microfuge tube.
3. Pipette 15 µL of the resulting solution onto a haemocytometer using capillary action with the end of the pipette tip at the edge of the coverslip. Repeat to fill the second chamber.
4. Use 10x magnification to bring the grid lines of the haemocytometer into focus. Count the live cells (clear cells with blue halos). Dead cells appear dark blue.
5. Count the cells in each of the four large squares. Each large square is made of 16 small squares. Calculate average for four large grids. Multiply by 2 to adjust for 1:1 dilution with tryphan blue. Total cell count is equivalent to \( \frac{x \text{ cells/mL}}{10^4} \).
6.3 Cryopreservation and storage of cells

Reagents and materials

- Phosphate buffered saline (PBS) without Ca++ and Mg++ (Gibco, Life Technologies, Cat No 14190250)
- 0.25% Trypsin/EDTA (Gibco, Life Technologies, Cat No 25200056)
- Cryopreservation media; containing 10% dimethyl sulphoxide (DMSO) (Cat No D-4540; Sigma Aldrich) and 90% FBS
- Sterile cryovials clearly labelled

Equipment

Centrifuge swinging bucket rotor (Beckman Coulter)
Cell freezing Device (Nalgene Labware Mr Frosty™, Rochester, USA)

Procedure

1. When cells have reached 60-80% confluent they can be frozen until required. One T-75 flask at 80% confluency has sufficient cells to be spilt into three cryovials.
2. Make cell freezing/cryo-media and place at 4°C.
3. Trypsinise the cells as per passaging adherent cells using trypsin. Centrifuge at 220xg for 5 minutes at 21°C to pellet cells. Remove supernatant.
4. Add 3 mL of cryo-media to the pelleted cells, pipette up and down to make a homogenate suspension. Transfer 1 mL into each 1.5 mL labelled cryovials.
5. Place the cryovials into cell freezing device, "Mr Frosty™" (kept at room temperature) and place at -80°C to freeze cells at 1°C/minute. Transfer to liquid nitrogen after 24 hours for long-term storage.
6.4 Thawing cryopreserved cells

Reagents and materials

- Culture flasks
- Pre-warmed complete culture media (DMEM with 10% FBS, Anti-Anti and gentamicin)

Equipment

- Light microscope (MRC/B146; Olympus, Hamption, Tokyo)
- Water bath 37°C

Procedure

1. Prepare a culture flask containing pre-warmed media, ready to receive the cells.
2. Cryopreserved cells are delicate. Remove vial from liquid nitrogen. Immediately thaw the cells in a 37°C water bath and rotate gently until the frozen contents have just melted.
3. Gently tip the contents of the cryovial into the culture flask.
4. Check for the presence of cells under the microscope. Place flask in 37°C, 5% CO₂ humidified incubator. Do not disturb so as to allow attachment of the cells to the flask surface. At 24 hours, change media to remove residual DMSO and unattached cells, then every 3 days until 80% confluency is achieved.
6.5 Preparation of triple supplement

Reagents and materials

- PBS without Ca²⁺ Mg²⁺; sterile MQ H₂O
- 2-phospho L-Ascorbic acid (Sigma 49752, soluble in water at 0.3 g / 10 mL)
- Dexamethasone (Sigma D2915, soluble in water at 25 mg / mL)
- β-Glycerophosphate (Sigma G9422, soluble in water at 10 mg / mL)

Equipment

Five-digit balance; Vortex mixer

Procedure

1. Make stock solutions. Weigh out the required amounts (see table below).
2. Dissolve reagents in H₂O and sterile filter using a 0.22 μm filter attached to a sterile syringe.
3. Aliquot working stock of dexamethasone, ascorbic acid and β-glycerophosphate into 1.5 mL microfuge tubes and freeze at -20°C.

Table 4. Triple supplement reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>MW (g/mol)</th>
<th>Stock concentration</th>
<th>Volume required for 50 mL media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone (for growth)</td>
<td>392.4</td>
<td>10 mM (3.9 mg/1 mL)¹</td>
<td>Working stock: 100 μM (1:100) (50 μl/5 mL media)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Final: Use 50 μl (1:1000) 10⁻⁷ M (0.1 μM)</td>
</tr>
<tr>
<td>2-phospho-L-Ascorbic acid (for nodules)</td>
<td>322.05</td>
<td>100 mM (322 mg/10 mL)¹</td>
<td>Final: Use 100 μl (1:500) 2 x 10⁻⁴ (0.2 mM)</td>
</tr>
<tr>
<td>β-Glycerophosphate (for mineralisation of nodules)</td>
<td>216.04</td>
<td>1 M (2160 mg/10 mL)¹</td>
<td>Final: Use 500 μl (1:100) 10 x 10⁻³ (10 mM)</td>
</tr>
</tbody>
</table>

¹ Dissolve in sterile H₂O
6.6 Osteoblast growth and mineralisation media recipes

Reagents and materials

• DMEM, high glucose, GlutaMax™ supplement (Cat No. 10566032; Thermo Fisher Scientific)
• Foetal Bovine Serum (FBS) (Cat No. 10091148; Invitrogen)
• Antibiotic-antimycotic 10,000 U / mL Penicillin; 10 mg / mL Streptomycin; 25 μg / mL Amphotericin B (Cat No. 15240-062; Invitrogen)
• Gentamycin 0.25 μg / mL (Cat No. 15710064; Invitrogen)
• Triple supplement reagents 2-phospho L-Ascorbic acid (0.2 mM), dexamethasone (0.1 μM), β-glycerophosphate (10 mM) (see section 6.5).

Procedure

1. Bring reagents to room temperature.
2. For osteoblast growth media: Remove 57.5 mL from a 500 mL volume of DMEM media and replace with 5 mL antibiotic-antimycotic, 2.5 mL gentamycin and 50 mL FBS.
3. For mineralisation media, per 50 mL of growth media, add 50 μL dexamethasone; 100 μL 2-phospho-L-ascorbic acid; 500 μL β-glycerophosphate.
4. Turn media bottle upside down to mix ingredients well. Store at 2-8 °C, protected from light.
6.7 Alkaline phosphatase staining protocol

Reagents and materials

- BCIP/NBT Alkaline Phosphatase tablet (SigmaFast™ BCIP-NBT; Sigma Aldrich, Cat No B5655)
- Distilled water (10mL)
- Wash buffer (100 mL, PBS containing 0.05% Tween 20, pH 4.2, Sigma Aldrich, Cat No 11332465001)
- 10% neutral buffered formalin

Equipment

pH meter

Procedure

1. Dissolve one BCIP/NBT tablet in 10 mL distilled water and mix by gentle inversion and allow to completely dissolve for two minutes.
2. Remove culture media and wash the samples with wash buffer.
3. Using 10% formalin fix monolayer cells. Fixing is not required for hydrogel constructs. Use a volume of formalin which covers the cells. Incubate for 1 minute. Rinse with wash buffer.
4. Add BCIP/NBT substrate (100μL/well). Incubate at room temperature for 5-30 min and check for colour development after 10 min.
5. Remove the substrate solution, add wash buffer and incubate at room temperature for 2 min.
6. Examine under a light microscope. Osteoblasts which are AP positive are dark blue/violet. The higher the AP activity, the more intense the color.
6.8 Alizarin Red staining protocol

Reagents and materials

- Alizarin Red (S) powder (Sigma Aldrich, Cat No 130223)
- Wash buffer (PBS containing 0.05% Tween 20, pH 4.2, 100 mL)
- 0.45 μm filter
- Sterile PBS 10% neutral buffered formalin

Equipment

- pH meter

Procedure

1. Dissolve 1g Alizarin Red S powder in PBS pH 4.2 (100 mL, 1% solution). Mix using vortex. Filter the dark-brown solution and store at 2-8°C in the dark.

2. Wash the cells 2-3 times with Dulbecco’s PBS at pH 4.2 without Ca++/Mg++. Fix cells with 10% neutral buffered formalin for 1 min (if monolayer cultures; no fixation for hydrogel) then wash the cells with wash buffer for 45 secs. Add Alizarin Red staining solution covering the cells. Stain control hydrogel only samples in parallel.

3. Incubate for 10 mins in the dark at room temperature. Wash the cells four times with wash buffer. Undifferentiated human osteoblasts (without extracellular calcium deposits) are slightly reddish, whereas mineralised osteoblasts (with extracellular calcium deposits) are bright orange-red.
6.9 PrestoBlue™ cell proliferation staining protocol

Reagents and materials

- PrestoBlue™ (Invitrogen, Cat No A13261) cell proliferation reagent.
  Supplied as a 10x solution

Equipment

Synergy 2 Multi-mode microplate reader (Biotek, USA) 535–560/590–615 nm (Plate settings: 48-well sample; fluorescence, top 50%; sensitivity 75)

Procedure

1. Add PrestoBlue™ at a 1:10 ratio to media volume. Include a control well with media only to correct for background fluorescence. Incubate for the required time, depending on thickness of construct at 37°C.

2. Using a fluorescence microplate reader with filters set at excitation 535 nm and emission 615 nm, record fluorescence levels. Cell growth is given as a percentage of untreated control (at 100%) at each time point, after correction for background fluorescence.
6.10 OsteoImage™ hydroxyapatite staining protocol

Reagents and materials

- OsteoImage™ kit containing: a staining reagent, a reagent dilution buffer (x100 concentrate) and a wash buffer (x10 concentrate).
- De-ionized sterile H₂O
- PBS without Ca²⁺ Mg²⁺

Equipment

Synergy 2 Multi-mode microplate reader (Biotek, USA) (Plate settings: 48-well sample; fluorescence, top 50%; sensitivity 75)

Procedure

To prepare reagents:

1. Make Wash Buffer working solution by diluting concentrate with sterile H₂O at 1:10. Store at 4°C.
2. Dilute Staining Reagent (1:100) with Staining Reagent Dilution Buffer based on number of samples. Mix well and keep protected from light.

To stain:

1. Carefully remove media from each well using a pipette taking care not to disturb the hydrogel. Gently wash once with PBS.
2. Add a volume of diluted Staining Reagent to cover the cells based on the well size (refer to product sheet for a table).
3. Cover with foil and incubate at room temperature for 30 minutes.
4. Remove the Staining Reagent and add wash buffer and incubate for 5 minutes to remove background fluorescence. Repeat wash step. Replace Wash Buffer before reading fluorescence.
5. Read in a fluorescent plate reader at 492 nm excitation and 520 nm emission or view under a fluorescent microscope.
6.11 Hydrogel preparation and reconstitution

Reagents and materials

- Extracel™/ HyStem-C 2.5 mL kit (Cat No GS313, Esi-Bio, USA) containing degassed ionized H₂O 10 mL, Glycosil (thiol modified sodium hyaluronate) lyophilized, Gelin-S (thiol modified gelatin) lyophilized, and Extralink™ (PEGDA powder) 5 mg stored in the freezer.
- 1 mL disposable sterile syringe (BD Tuberculin syringe)
- Needle tip (BD Precision Glide™ Needle 18G; 1 ½ TW 1.2x38mm)

Equipment

- Shaker (set at 60 cycles a minute) (IKA®-Schlutter MTS4 electronic)

Procedure

1. Bring hydrogel kit components to room temperature.
2. Minimise exposure to oxygen: do not uncap the Glycosil and Gelin-S until you are ready to use it.
3. Reconstitute lyophilized Gelin-S and Glycosil with degassed water: pierce the rubber septum of the degassed H₂O vial, turn the H₂O vial upside down and pierce its rubber septum with a syringe tip. Draw up 1 mL water without any air bubbles. Transfer to the Glycosil vial by piercing its rubber septum. The pressure inside the vial will suck the water in with minimal effort. Add 1 mL of H₂O to Gelin-S and 0.5 mL to Extralink™ (PEGDA) vials using the same process with a new needle.
4. Gently invert vial containing PEGDA solution for 5 minutes.
5. After adding H₂O, to the Glycosil and Gelin-S incubate at 37°C for 30 minutes then place on a shaker at 60 cycles/minute for two hours to achieve a homogenous solution. The rate of mixing will depend on the room temperature. For maximum mixing, horizontal incubation on a shaker has been recommended (Serban et al., 2008). At the end, put the vials towards the light to check for a homogenous and clear mix. Within 4 hours of making the solutions, combine the Glycosil, Gelin-S and PEGDA with cells (see cell encapsulation protocol).
Figure 42. Diagram showing the three components making up the Extracel™/HyStem-C™ hydrogel.

Figure 43. Hydrogel components before, during and after reconstitution with degassed water

(A) Components of the 2.5 mL Hy-Stem-C™ (Esi-Bio) hydrogel kit. From left to right: Extralink™ PEGDA powder, Glycosil® (hyaluronan) and Gelin-S® (gelatin) in lyophilised forms and degassed ionized H₂O. The water is added to all components to make 1% v/w solutions of PEGDA 0.5 mL, Glycosil® 1 mL and Gelin-S® 1 mL, making 2.5 mL hydrogel in total. (B) Glycosil® and Gelin-S® must be secured on a rocker and mixed thoroughly for two hours. (C) A clear, homogenous, slightly viscous solution should be present at the end of mixing.
6.12 Cell encapsulation into hydrogel

Reagents and materials

- Previously prepared solutions of Glycosil® (thiol modified sodium hyaluronate) (1 mL), Gelin-S® (thiol modified gelatin) (1 mL) and Exralink™ (PEGDA) (0.5 mL)
- 48-well plates (CellStar); 8-well chambered coverslip slides (Nunc® Lab-Tek® Chamber Slide system, Thermo Fisher Scientific, Cat No 154534)
- 15 mL sterile vials
- Wide bore pipette tips (200 µL)

Equipment

Centrifuge swinging bucket rotor (Beckman Coulter)

Procedure

1. Count total number of cells with a haemocytometer. Distribute required number of cells in media into 15 mL vials
2. If seeding at 50,000 cells per 20 µL construct, then 1 mL of gel will contain 2.5x10^6 cells.
3. Take the required volume of cell homogenate and centrifuge at 220 xg for 5 minutes at 21°C.
4. Remove all of the media without disturbing the pellet. Any remaining media will dilute the gel.
5. Prepare and label the culture plates. Plate control samples first to determine gelation timing on the day. Place the hydrogel ingredients into a 1.5 mL Eppendorf tube. Mix by pipetting and plate at the required amount under timed conditions. Doubling the Exralink™ PEGDA concentration will decrease the gelation time by ~ 50% (Serban et al., 2008). As the hydrogel forms, the liquid will become more viscous and harder to pipette and plate.
6. Uncap Glycosil® and Gelin-S® vials and remove the required amount based on Table 2 below. Re-cap the vials to minimise exposure to oxygen.
7. Add the Gelin-S® and Glycosil® to the cell pellet and mix the cells into the gel by gently pipetting up and down. Ensure a homogenous mixture
without bubbles. Add the cross linker Extralink™ PEGDA last. Gently pipette up and down for a homogenous mix.

8. Use a wide bore pipette to place dome-like 20 µL gel constructs in the middle of the well (Figure 44). Pipette up and down prior to plating for a homogenous mix as cells can sink to the bottom of the gel. It will take approximately 5 mins before the gel sets. Gelation timing will vary with temperature and amount of cross-linker. Allow 30 minutes at 37°C before adding any media.

Table 5. Proportion of hydrogel components depending on the ratio used based on 1000 µL total volume.

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<td>PEGDA</td>
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Figure 44. Hydrogel constructs

Hydrogel constructs shown cultured in (A) 8-well chamber slide for confocal microscopy and (B) inside a 48-well plate used for PrestoBlue™ proliferation assay and Hydroxyapatite (OsteoImage™) assays. (C) Side view of a hydrogel/cell construct inside a well.
6.13 FDA/PI fluorescence hydrogel stain under confocal microscopy

Reagents and materials
- Fluorescein Diacetate powder (Sigma Aldrich, Cat No 596098)
- Propidium Iodide (Molecular Probes, P3566)
- PBS pH 4.8
- Deionized water
- Osteoblasts cultured in 8-well chamber slides
- Acetone

Equipment
- Confocal microscope (LSM Zeiss 710, Carl Zeiss Microscopy, Germany)

Procedure
Stock and working solutions:
- Propidium Iodide (PI) - Stock solution: prepare 2 mg/1 mL in deionized sterile water. Store at 2-6°C protected from light. Working solution: Add 10 µL stock to 990 µL PBS (Final concentration of 0.02 mg / mL).
- Fluorescein Diacetate (FDA) - Stock: prepare 5 mg/1 mL in acetone. Working solution: Add 100 µL stock to 9.9 mL PBS pH 4.8 (Final concentration 0.05 mg / mL).

Staining:
1. Remove media and wash each construct twice with PBS.
2. Add FDA (40 µL) and/or PI (20 µL) to each construct. Incubate for 15 minutes in the dark. Include control samples of gel only.
3. Wash each sample with PBS (100 µL) twice. Transport on ice.
   View PI stained constructs at 560 nm excitation /590 nm emission and FDA stained constructs at 495 excitation/ 517 emission.
6.14 Application of compressive load on hydrogel constructs

Reagents and materials

- Cells encapsulated into hydrogel
- Mineralisation culture medium
- BioPress™ culture plates (BF-3000C, Flexcell® International Corporation) and stationary platens.
- Sterile tweezers

Equipment

- Flexcell® FX-4000 compression system (Flexcell® International Corporation)
- Compressor (Schneider air systems Silent Master 50 L/min SEM 100-8-6 W-Oil Free), set at 12-15 psi (83-104 kPa).

Procedure

1. Prior to commencing any work, test the compression system for leakage. Place empty BioPress™ plates and run the selected regimen. Compare the readings on the screen from the programmed cycle to the expected parameters. If the parameters do not match, unplug the tubing from the compression plate and connect the tubing together to create a closed circuit and re-run the regimen. If the parameters match, this means there is a leak in the compression plate. Ensure the BioPress™ plates are positioned in the middle of each gasket to avoid gaps. Repeat the regimen.
2. Prepare the BioPress™ culture plates. Discard of the 5mm foam rings. Pipette the hydrogel/cell constructs onto the centre of the 13mm diameter foam holder. Each construct was made up of two layers (125 µL each) with the combined two layers resulting in 1.5-2mm thickness. Based on manufacturer instructions, the ideal minimum thickness of the gel/cell construct is 1mm with a maximum 3mm to ensure compression. The absolute minimum is 0.35mm as this is the thickness of the compressed foam.
3. Placed samples in 37°C CO₂ incubator for 45 minutes to gel before adding mineralisation culture medium (4 mL).
4. Turn the compression platens twice using sterile tweezers. The turns are calculated using the formula \( x = \frac{(3.11 - h)}{0.62} \) to determine number of 180° turns.

5. Load four BioPress\textsuperscript{TM} plate onto the centre of each of the four black rubber gaskets (Figure 46). Follow the user’s manual to achieve proper clamping. Programmed load is applied through positive pressure from underneath the BioPress\textsuperscript{TM} plates against the stationary platen positioned above the cell/gel construct (Figure 47).

The pressure applied is dependent on the surface area of the sample. 

\[ P_{\text{MPa}} = \frac{(5.65 \times \text{Force lbs})}{(D_{\text{mm}}^2)} \]

The force programmed into the software is in pounds. The regimen was programmed for a cyclic compression as 1.0lbs of force every second (1 Hz) for 5 seconds every hour for 6 hours (Figure 45). According to the formula, the applied pressure to the 13mm diameter sample equates to 0.0334 mPa (33.4 kPa or 340.6 gm/cm\(^2\)).
Figure 45. Representation of a 5 second cyclical compression regimen.
Cyclical compression regimen at 1 lb-force (y axis) per second for 5 seconds (x axis). This regimen was repeated every hour for six hours a day for 21 days.

Figure 46. Assembled Flexcell® clamping system.
Four BioPress™ culture plates are loaded before being placed in the 37°C incubator. The tubing delivering the compressive force exits the incubator through custom-made ports and connects to the Flexcell® compression computer unit.
Figure 47. Flexcell® compression loading system.

A stationary platen is fitted to the top of each well and a compressive load is applied via a positive pressure from beneath.
6.15 Cell recovery from hydrogel in BioPress™ plates

Reagents and materials
- TRIzol® RNA Isolation reagent (Applied BioSystems, CA, USA)
- RNAase free pipettes and Eppendorf vials
- Micro-pestle

Procedure
1. Bring TRIzol® to room temperature. Add TRIzol® reagent (500 µL/well) to each hydrogel construct. Incubate for 5 minutes to allow for complete dissociation of nucleoprotein complexes. The volume of TRIzol® reagent required will depend on the area of the culture dish and not the number of cells present (1 mL per 10 cm²).
2. Mix the hydrogel constructs with TRIzol® using a pipette and transfer to the 1.5 mL microfuge tube. Pipette up and down to make a homogenous mix. Use a micro-pestle if necessary.
3. Store the homogenate at -80°C until required.

6.16 RNA extraction with TRIzol®

Reagents and materials
- TRIzol® lysated homogenates stored at -80 °C
- Chloroform
- 70 % ethanol made with 96-100% ethanol and nuclease free water
- TRIzol® Plus RNA Pure Purification Kit (Life Technologies).
- RNase-free pipette tips
- 1.5 mL RNase-free microcentrifuge tubes

Equipment
- Microcentrifuge at 4°C
- Microcentrifuge at room temperature

Procedure
1. Defrost TRIzol® reagent cell/gel lysates at room temperature.
Add 100 µL chloroform per 500 µL TRIzol® reagent used. Shake vigorously for 15 sec. Incubate at room temperature for 2-3 min.

2. Centrifuge sample at 12,000 xg for 15 min at 4 °C. The mixture should separate into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase containing RNA.

3. Transfer 300 µL of the colourless upper phase to an RNase-free tube. Add an equal amount of (300 µL) 70% ethanol to obtain a final ethanol concentration of 35%. Mix by vortexing.

4. Transfer up to 700 µL of the sample to a spin cartridge (with a collection tube). Centrifuge at 12,000xg for 1 min at room temperature. Repeat until the entire sample has been processed.

5. Discard the flow through and re-insert the spin cartridge in the same collection tube. Add 700 µL wash buffer I to the spin cartridge. Centrifuge at 12,000xg for 1 min at room temperature. Discard the flow-through.

6. Add 500 µL of Wash Buffer II to the spin cartridge. Centrifuge at 12,000 xg for 1 min. Discard the flow through. Add 500 µL Wash Buffer II again and repeat this step.

7. To dry the membrane, centrifuge the Spin Cartridge and Collection tube at 12,000 xg for 1 min. Discard of the flow through and the collection tube. Place the Spin Cartridge into the newly prepared recovery tubes.

8. Add 50 µL RNase-free water to the centre of the Spin Cartridge to be as close to the membrane as possible.

9. Incubate at room temperature for 1 min. Centrifuge the Spin Cartridge with the Recovery Tube for 2 min at 12,000xg at room temperature. The Recovery Tube contains the purified total RNA. To perform a second elution, repeat this step. Discard the Spin Cartridge.

10. Store -80°C.
### 6.17 RNA concentration and quality assessment values

**Table 6. RNA recovered from mechanical load experiment hydrogel**

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Red represents the HCO samples  
Black represents the HFO samples  
Shaded grey area represents the mechanical strain group
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**AVE ΔCq** 10.84

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**AVE ΔCq** 14.96

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**AVE ΔCq** 5.73

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**AVE ΔCq** 7.04
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6.21 Incorporating Permacol™ collagen sheets into hydrogel: a pilot study

6.21.1 Background

Permacol™ (Covidien, Cat. No. 5033-50) is a medical grade 0.5mm thick commercially available dermatological graft material obtained from porcine skin. The dermis is stripped of cellular components resulting in an immunologically inert matrix composed predominately of collagen. It is manufactured as thin sheets, ranging from 0.5- 1.5mm thickness. It has been approved to be used to Europe since 1998 and was cleared by the Food and Drug Administration in 2000 to be used in the USA.

Studies investigating the suitability of collagen sheet grafts have been performed in various animal models, making comparisons across studies difficult (Hiles et al., 2009). Several peer-reviewed publications exist evaluating Permacol™ in surgical repairs. Variable experimental methods and follow up time have shown inconclusive results in level of cellular penetration, angiogenesis and remodelling (Sandor et al., 2008; Hiles et al., 2009).

Chemical cross-linking of Permacol™ with isocyanate increases its resistance to fast enzymatic degradation in vivo. Its tough, but flexible nature makes it suitable
for repair defects (Mulier et al., 2011; Sandor et al., 2008). However, it has been revealed that most dermal grafts undergo significant matrix modification and differ considerably from native tissue possibly as a result of processing or sterilization procedures (Sandor et al., 2008).

The benefits of using a natural scaffold is the facilitation of the recipient’s reparative process, which consists of replacing the scaffold by gradually degrading and remodelling it, thus replacing it with native tissue. Integration relies on recipient’s cellular and vascular ingrowth. Cross-linked materials provide tissue re-enforcement and are degraded and remodelled slower providing more time for remodelling to occur (Mulier et al., 2011; Hammond et al., 2008). In an in vivo human study, with follow-up of two years, histological sections showed infiltration and ingrowth of fibroblasts and successful revascularization, with evidence of native human collagen and elastin remodelling throughout the explant (O’Brien et al., 2011). However, short-term studies showed that the extensive cross linking found in Permacol™ may result in loss of biological signals that promote remodelling and inhibition of cellular infiltration (Jarman-Smith et al., 2004). To assess remodelling characteristics of cross-linked collagen materials, longer follow up times may be more appropriate.

Due to the cost of the Permacol™ sheets, a pilot study was undertaken to examine the feasibility of a hybrid model consisting of Permacol™ and hydrogel.
6.21.2 Aims

1) To assess the practicality and handling properties of Permacol™
2) To determine if TRIZol® is suitable for the extraction of RNA from osteoblasts cultured on Permacol™

6.21.3 Methods

The integrity of two hydrogel models was compared following 7 days of cyclic mechanical strain, a hydrogel-Permacol™ hybrid and a hydrogel only construct.

The hybrid Permacol™ - hydrogel construct was made by first soaking the collagen sheets in mineralisation medium for 30 minutes as this has been shown to enhance cell viability (Jarman-Smith et al., 2004). Then using aseptic techniques, five 5x5mm Permacol™ squares were cut out from the 3x3cm sheet and placed in an eppendorf tube (Figure 49A). The hydrogel components (50 µL hyaluronan Glycosil® and 50 µL gelatin Gelin-S®) were added to pelleted osteoblasts (2.5 x 10^6/mL) and pipetted up and down for a homogenous mix. The five Permacol™ squares were then added to the homogenous mix. Following this, the cross linker PEGDA Extralink™ (100 µL) was added and the mixture, and together with the Permacol™ squares, was pipetted up and down. The hydrogel-Permacol™ soaked squares were then transferred to the 5mm culture area of each well on a BioPress™ plate (Figure 49B). The cell-hydrogel preparation (20 µL) was then pipetted onto the top of each square creating a small construct. Once gelation occurred, another layer of Permacol™ was placed on top, creating a sandwich-like Permacol™-hydrogel model. Cells encapsulated in hydrogel only (no Permacol™) acted as a control.
Figure 49. Permacol™-hydrogel constructs in preparation.

(A) Each Permacol™ sheet was cut into 5 squares and soaked in hydrogel. (B) Permacol™-hydrogel constructs inside the 5mm foam well the BioPress™ plate, with compression platens fitted (C).

Constructs were left to gel inside the culture plate for 30 minutes before mineralisation medium (3 mL) was added and the compression platens put in place. The BioPress™ plates were placed inside the FlexCell® machine and incubated for 7 days. An intermittent (cyclic) compressive force of 1lb was applied at a frequency of 1 cycle a second (1Hz) for 5 seconds an hr, for 6 hrs.
6.21.4 Results and conclusions

After 7 days of incubation under cyclic compressive load, both models maintained their 3-D shape. The hydrogel only constructs maintained their integrity demonstrating their suitability for compression experiments (Figure 50B). This model was subsequently used for this study.

Figure 50. Hydrogel only construct.
(A) Gelation of a hydrogel only construct inside the 5mm foam sample in the BioPress™ plate before compression (B) The same construct after 7 days of incubation under a compressive load. Note that the hydrogel maintained its 3-D structure.

The hydrogel-Permacol™ model provided practical information for future development of layered models. Working with small 5x5mm squares was tricky due to the delicate nature of the sheets. Cutting the Permacol™ sheets was more challenging than anticipated. A biopsy circular punch was too blunt and kept twisting the graft sheet. A scalpel blade with a definitive straight cut sterile ruler worked better. Surgical scissors may be best to trial in the future. TRIZol® was used to assess how the collagen sheets would dissolve. Tissue grinders had to be used. It was not possible to achieve a completely homogenous solution and small particles could still be seen. It would be interesting to see if the type-1 collagen present in the sheets gives higher than normal mRNA levels for collagen-1. Having a control group of the Permacol™ only would answer this question.
6.21.5 Future directions

The use of Permacol™ will require further assay validation prior to the assessment of proliferation and mineralisation using the methods employed in this study, with appropriate background fluorescence control groups. Arca and co-workers (2012) have successfully used confocal microscopy to assess viability of human mesenchymal stem cells cultured on Permacol™ sheets. Histology investigation would be beneficial to assess whether osteoblasts remain on the surface or move into the Permacol™ sheet. Finally, successive layers of hydrogel-Permacol™ constructs may strengthen the model making it more suitable for compressive mechanical load experiments carried out of 4 - 6 weeks and enable the sequential layering of culture osteoblasts mimicking the way bones normally grow-appositionally.
6.21.6 References


