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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**INFLUENCE OF CYCLIC COMPRESSIVE FORCES IN
BONE TISSUE ENGINEERING**

**AKHILANDESHWARI RAVICHANDRAN
SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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**INFLUENCE OF CYCLIC COMPRESSIVE FORCES IN
BONE TISSUE ENGINEERING**

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(B.Tech)**

School of Chemical and Biomedical Engineering

**A thesis submitted to Nanyang Technological University
in fulfilment of the requirement for the degree of
Doctor of Philosophy**

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Preface

This thesis is submitted for the degree of Doctor of Philosophy in the School of Chemical and Biomedical Engineering at the Nanyang Technological University, under the supervision of Professor Teoh Swee-Hin and Dr Lim Jing. This thesis has not been submitted for any degree in any university previously. I hereby declare that this is my original work and I have duly acknowledged all sources of information used in this thesis. Parts of this thesis have been published in the international journals and conferences listed in the List of Publications and List of Conferences.

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List of Publications

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2. Ravichandran A, Liu Y, Teoh S-H (2016). "Review: Bioreactor Design Towards Generation of Relevant Engineered Tissues: Focus on Clinical Translation". Journal of Tissue Engineering and Regenerative Medicine (*In Press*)
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List of Scientific Conferences

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3. A Ravichandran, W Feng, J Lim, MSK Chong, JKY Chan, SH Teoh. Evaluation of Multi-mode Bioreactor System for Bone Tissue Engineering. International Bone-Tissue-Engineering Congress, Oct 8 – 10, 2015, Stuttgart, Germany (Poster)
4. A Ravichandran, J Lim, MSK Chong, SH Teoh. Development of a Cyclic Compression and Biaxial Rotation-Combination Bioreactor System for Bone Tissue Engineering. 16th May 2015, Regenerative Medicine Symposium, Singapore (Poster)
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6. A Ravichandran, J Lim, SH Teoh. Cyclic Compressive Loading Bioreactor for Bone Tissue Engineering. The 2nd Symposium on Biomaterials and Tissue Engineering, 22nd September, 2014, University of Science, Ho Chi Minh City, Vietnam. (Oral)
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Abstract

Bone is one of the most transplanted tissues in the world and the current clinical strategies are unable to meet the growing demand for bone grafts. Therefore, bone tissue engineering (BTE) has become one of the potential alternatives to produce highly cellularized and mineralized bone grafts to meet the growing demand in clinics. Despite the advances in BTE, the current *in vitro* methods have not successfully generated viable, voluminous and mineralized 3D constructs. In this thesis, we hypothesized that application of physiologically relevant mechanical forces would enable generation of highly osteogenic constructs. Influence of mechanical stimulation and fluid flow on *in vivo* bone remodeling has been extensively studied in the past. However, there is lack of a systemic study to understand the effect of physiological strains on *in vitro* bone formation. To fill this gap, we developed a multi-chamber compression bioreactor system to apply cyclic compressive forces on cell loaded scaffolds under controlled environment to evaluate osteogenesis and mineralization of cellular grafts. A parametric optimization study conducted by applying a range of strains on Mesenchymal Stem Cells (MSC) seeded Polycaprolactone- β -Tricalcium Phosphate (PCL-TCP) scaffolds demonstrated that physiologically relevant strains resulted in enhanced osteogenic differentiation. Further, a long term study with optimized strain values indicated cyclic compressive loading induced upregulation of osteogenic gene expression. Osteogenic enzyme activity was also highly elevated by the

end of two weeks in comparison to the static cultures. These results suggested the potential of cyclic compressive loading for generating mineralized tissues.

Although, long term cyclic compression of cell seeded PCL-TCP scaffolds resulted in increased osteogenesis and mineralization, stable cellular adhesion was not achieved prior to cyclic mechanical loading and lack of media exchange resulted in poor cellular viability. It was hypothesized that dynamic culture with fluid flow can result in tissues that possess higher cell numbers and hence enhanced matrix deposition, which in turn would enable better mechanotransduction to the cells under cyclic loading. Hence, we developed a multimodal bioreactor system that allows application of physiologically relevant compressive strains on premature bone grafts that are cultured under biaxial rotation conditions. The bioreactor system was integrated with sensors for dissolved oxygen and pH that allow real-time, non-invasive sensing of the culture parameters. The system was used as a platform to study the independent and combined effects of fluid flow and cyclic mechanical stimulation on cellular proliferation and osteogenic differentiation of stem cells on 3D bone grafts. MSC seeded PCL-TCP scaffolds were cultured in the multimodal bioreactor for a period of 2 weeks under four different modes including static, cyclic compression, biaxial rotation and multimodal modes (combination of cyclic compression and biaxial rotation). The combined effects of optimal fluid

flow conditions and cyclic compression led to the upregulation of osteogenic genes. The multimodal culture also resulted in higher cellular proliferation in comparison to the static groups. We have demonstrated the potential of sensor integrated bioreactor system. In summary, we have developed a platform to engineer bone tissues using a multimodal bioreactor system and demonstrated its potential advantages for generation and maintenance of bone grafts under physiologically relevant conditions.

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- Fig 6.11** Expression levels of genes that are associated with osteogenesis and cellular differentiation – Runx2, ALPL, TGFB1, COL1A1, Osteonectin and Osteocalcin under the different modes of bioreactor assessed using RT-PCR (n=3, ** p<0.01, *** p<0.001).
- Fig 6.12** Qualitative staining for calcium and phosphate in the tissues: Week 1, Alizarin red staining for calcium shows the distribution of calcium deposits on the tissue as does the Von Kossa staining for the phosphates.
- Fig 6.13** Qualitative staining for calcium and phosphate in the tissues: Week 2, Alizarin red staining for calcium shows the distribution of calcium deposits on the tissue as does the Von Kossa staining for the phosphates.
- Fig 6.14** Elution of calcium from the TCP of scaffolds cultured under different modes in the multimodal bioreactor.
- Fig 6.15** Osteogenesis and mineralization under different bioreactor modes, A. Osteogenic differentiation measured by ALP activity of the tissues normalized to total protein levels on day 7 and 14 under different bioreactor modes, B. Mineralization levels of the tissues cultured in all the four modes of bioreactor assessed by evaluating the amount of calcium deposition on the grafts by the end of week 1 and week 2 (n=3, *** p<0.001).
- Fig 6.16** Matrix deposition in the tissue grafts under different bioreactor modes, SEM images of the top view, side view and core views (at 30x, 500x magnifications) show matrix deposition on the scaffolds and the pores of the scaffolds under all four modes of bioreactor culture at the end of week 2.
- Fig 8.1** A. Photographs of the uncoated and coated scaffolds (a-c), B. F-actin staining of mesenchymal stem cell seeded on untreated and coated scaffolds. Z-stack images were taken for cells after 7 and 14 days of proliferation on different scaffolds surfaces. Scale bar: 50 μ m, C. Cellular metabolism on untreated (white bar), and Pyr-PEDOT:tos (squared bars) and PPP-PEDOT:Tos (striped bars) coated PCL scaffolds via AlamarBlue® assay (p < 0.05). n.s.: not significant difference (Iandolo, Ravichandran et al. 2016).
- Fig 8.2** A. ALP activity of the stem cells under osteogenic conditions on the uncoated and coated scaffolds, B. Levels of calcium deposition in bone inducing medium on the uncoated and

coated PCL scaffolds.

Fig 8.3 Cellular proliferation of NC-MSC and BM-MSC on PCL scaffolds for a period of 3 weeks.

Fig 8.4 A. Cellular viability (Scale bar: 250 μm) and B. Proliferation of stem cells on uncoated and coated scaffolds in culture and bone medium conditions with and without electromagnetic stimulation.

Fig 8.5 Matrix deposition and mineralization of stem cells on uncoated and coated scaffolds in culture and bone medium conditions with and without electromagnetic stimulation.

Fig 8.6 Proposed multifunctional bioreactor system that allows application of mechanical, electrical and magnetic stimulation on stem cells seeded on macroporous scaffolds.

List of Symbols

2D	Two-Dimensional
3D	Third-Dimensional
AgNO ₃	Silver Nitrate
ALP	Alkaline Phosphatase
ALPL	Alkaline Phosphatase gene
BM-MSC	Bone Marrow derived Mesenchymal Stem Cells
BMP	Bone morphogenetic protein
BMP-4	Bone morphogenetic protein 4
BMP-6	Bone morphogenetic protein 6
BTE	Bone Tissue Engineering
Ca	Calcium
CaCl ₂	Calcium Chloride
cDNA	complementary Deoxyribonucleic Acid
cGMP	Current Good Manufacturing Practices
CO ₂	Carbon Dioxide
Col II	Collagen II
Col X	Collagen X
COL1A1	Collagen, type I, alpha 1
DBM	Demineralized Bone Matrix
dH ₂ O	Deionized water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
dsDNA	Double stranded Deoxyribonucleic Acid
ECM	Extra Cellular Matrix
EPC	Endothelial Progenitor Cell
F-actin	Filamentous-Actin
FBS	Fetal Bovine Serum
FDA/PI	Fluorescein Diacetate/Propidium Iodide
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HA	Hydroxyapatite
hfMSCs	Human Fetal Mesenchymal Stem Cells
ID	Inner Diameter
ITGB1	Integrin-β1
IVD	Intervertebral Disc
MG-63	Human Osteosarcoma Cell line
MLO-A5	Osteoblast cell line
mRNA	messenger Ribonucleic Acid
MSC	Mesenchymal Stem Cells
NaOH	Sodium Hydroxide
NC-MSC	Neural Crest derived Mesenchymal Stem Cells

NiTi	Nickel-Titanium based constructs
NP/AF	Nucleus Pulposus/Annulus Fibrosus
O ₂	Oxygen
OD	Outer Diameter
OPN	Osteopontin
PBS	Phosphate Buffered Saline
PCL	Polycaprolactone
PCLM	Phase Contrast Light Microscopy
PCL-TCP	Polycaprolactone- β -TricalciumPhosphate
PDGF- β	Platelet-derived growth factor subunit B
PEDOT	poly(3,4-ethylenedioxythiophene)
PET	Polyethylene Terephthalate
PLGA/aCaP	Poly-lactic-co-glycolic acid/ amorphous Calcium Phosphate
PLL	Poly-L-lactide
PLLA	Poly-L-Lactic Acid
qPCR	Quantitative Polymerase Chain Reaction
RT-PCR	Real-Time Polymerase Chain Reaction
Runx2	Runt-related transcription factor 2
RWV	Rotating Wall Vessel
SD	Standard Deviation
SEM	Scanning Electron Microscope
SMC	Smooth Muscle Cell
T	Temperature
TE	Tissue Engineered
TEMP	Tissue Engineered Medical Product
TGFB1	Transforming growth factor beta 1
TGF- β -R1	Transforming Growth Factor- β Receptor 1
VEGF	Vascular Endothelial Growth Factor
β -TCP	Beta-TricalciumPhosphate

1. Introduction

1.1 Background: Need for bone replacement & current strategies

In this ageing world population, there has been a steep increase in the incidences of bone diseases and conditions like physical trauma, tumors, osteoporosis, arthritis and genetic defects creating a constant demand for bone grafts [1] (Fig 1.1). Although bone has a high self-regenerative capacity, this potential is limited when there is extensive damage to the tissue leading to the formation of large critical-sized defects [2]. Further, orthopedic and musculoskeletal products and therapy are estimated to have a market potential of 18 billion dollars by the year 2018 (Source: MedMarket Diligence, LLC).

Fig 1.1 Bone replacement is required for different conditions with extensive bone loss due to trauma, osteoporotic fractures, craniofacial congenital defects, and bone tumor [1].

Current clinical approaches for bone replacement rely majorly on autografts for bone replacement, where the tissue from a healthy site is harvested from the same individual and implanted in the defect site.

Although the autografts exhibit high osteoconductivity and osteoinductivity, limited supply of the graft makes it difficult to perform multiple reconstructions [3, 4]. Further, autograft procedures result in pain, donor site morbidity, and the grafts cannot be customized based on specific requirements [5, 6]. The properties of different bone grafts used for bone replacement are listed in Table 1.1.

Table 1.1 Properties of different bone grafts are evaluated based on the properties of structural strength, osteoconductivity, osteoinductivity and osteogenicity (adapted from [3]).

Bone Graft	Structural strength	Osteo-conduction	Osteo-induction	Osteogenic
Autograft	++	++	++	++
Allograft	+/-	+	+	+
Synthetic grafts (acellular)	+/-	+/-	-	-
Tissue engineered bone grafts	+	++	++	++

On the other hand, allografts are harvested from human donors and involve risks of immune-based graft rejection and transmission of infection or disease from the donor [7]. In addition, acellular synthetic grafts are also gaining attention in the clinics owing to their structural strength and biocompatibility, however, they exhibit poor integration with natural bone. With the ever increasing demand of bone grafts coupled with the shortcomings of current strategies, there has been a shift of

interest towards engineering bone tissues using cells, scaffolds and appropriate cues to obtain functional bone grafts.

1.2 Objective and proposed approach

Classical approaches developed in bone tissue engineering combine scaffolds, cells and soluble growth factors to induce bone growth and development. A functional, engineered bone graft is expected to possess sufficient mechanical strength, osteoinductive and osteoconductive potentials, ability to respond to physiological microenvironment and a degradation rate that matches the rate of formation of new bone [5, 8]. Since bone is a dynamic tissue that constantly renews and responds to mechanical loading through remodelling, the developed bone graft should be exposed to relevant biomechanical forces. This would aid in conditioning the grafts to *in vivo* loads, in extracting osteogenic responses as well as acceleration of tissue maturation. For this purpose, bioreactors have been proposed as controlled platforms to study mechanical forces and understand their effects on tissue growth and development for regenerative medicine.

With respect to bone tissue engineering, several bioreactors have been used to generate 3D bone grafts (Table 1.2). Most of the bioreactor systems including spinner flasks, rotating wall vessel, perfusion bioreactors and biaxial rotation bioreactor system provide the tissues with dynamic fluid flow conditions to induce bone formation. At tissue

level, bone senses and responds to mechanical loads that are compressive, tensile and torsional in nature. Consequently, mechanical bioreactors have also been developed to evaluate the effects of these forces on bone tissue engineering.

Table 1.2 Bioreactors commonly used in bone tissue engineering.

Type of bioreactor	Images	Design and advantages	Remarks
Spinner flask		Stirrer based mixing of oxygen and nutrients in medium, simple set up, relatively low cost	<ul style="list-style-type: none"> • Distribution of shear forces not homogenous, creates turbulence closer to the spinner blades • Piercing of scaffolds for anchorage • Limited to porous scaffolds • Insufficient mass transport
Perfusion bioreactor		Direct perfusion through the entire scaffold, laminar flow	<ul style="list-style-type: none"> • Requires optimization of flow rates to prevent washing cells away from scaffold • Not homogenous cell distribution
Rotating wall vessel bioreactor		Low shear stress, high mass transfer, continuous free flow	<ul style="list-style-type: none"> • Insufficient mass transport to the central core of the scaffolds • Not homogenous cell distribution • Free floating scaffolds can collide which could damage scaffolds and cells
Biaxial rotation bioreactor		Increased velocity and flow within scaffold pore, better cellular homogeneity in scaffolds	<ul style="list-style-type: none"> • Piercing of scaffolds for anchorage • Limited to porous scaffolds
Mechanical force systems: Compression, tension, torsion, shear, hydrostatic pressure		Mechano-stimulation of cells, accelerate proliferation and maturation of tissue graft	<ul style="list-style-type: none"> • Exact mimicry and control of range of mechanical parameters could be a challenge

Legend:

*

Bioreactor studies have demonstrated superior properties of the developed bone grafts when compared to static cultures (Reviewed in detail in Chapter 2). Despite research showing preliminary successes, clinical utility of bioreactors still faces many research challenges. To engineer a specific tissue, an *in vitro* end point for tissue maturation needs to be defined. There are varying degrees of maturation for each type of tissue. If we consider the bone tissue, there are four progressive stages of bone regeneration [9], starting from precipitation of mineralized nodules in the first stage and final stage is defined by formation of dense and compact lamellar bone. Once an end point for the tissue in question has been specified, we need to optimize protocols to reproducibly generate matured tissue grafts.

1.2.1 Bioreactor design for parametric optimization

Throughout the process of protocol development for engineering the specific tissue, several factors and conditions such as varying concentrations and types of soluble factors, cellular density and combination of cell types, different modes and extent of mechanical stimulation and flow rate need to be optimized. Particularly for larger vessels, these studies are expensive, resource and time-consuming and often hinders the progress of research. One way to minimize experimental variations during parametric optimization studies would be to develop an array of scaled-down, multi-chambered bioreactors within one system. Essentially, the presence of multiple chambers with

separate media conditions will allow the study of effects of different intrinsic parameters (different cell sources, scaffold materials or configurations and media components) and also external factors (mechanical, fluid and electrical stimuli) concurrently in one bioreactor run. This would provide time and cost savings in terms of consumables, manpower and resources, and yield faster results, hence help expedite research and development.

1.2.1.1 Proposed design for compression bioreactor

This thesis proposed the use of cyclic compressive forces to mimic the physiologically relevant mechanical strains experienced by native bone. In order to extract favourable osteogenic response, the biochemical and biomechanical parameters need to be optimized. Having highlighted the advantages of multiple chambers within one bioreactor for parametric optimization, this thesis proposed the development of a multichamber compression bioreactor. This platform with independent control of the biochemical and biomechanical environment for each of the chambers will enable time-effective optimization of parameters (Fig 1.3). As a step towards improving functional relevance and clinical applicability of bioreactor generated tissue grafts, studies should move towards physiological biomimicry of native tissues and their microenvironment [10]. Current mechanical bioreactor systems for bone tissue engineering fail to provide mechanical cues that mimic the native bone

microenvironment and hence, we proposed the use of physiological strains for inducing accelerated bone formation.

Fig 1.2 Proposed multichamber compression bioreactor system for parametric optimization studies.

1.2.2 Bioreactor design for multimodal studies

In addition to application of physiologically relevant stimuli, generation of effective bone grafts depends on uniform cellular distributions on clinically sized scaffolds [11]. Dynamic fluid flow is necessary for improving mass transport within critical sized grafts for uniform cellular distribution on 3D tissue grafts. Therefore, there is a need to develop bioreactor systems that can provide dynamic fluid flow conditions and appropriate mechanical stimulation to generate clinically relevant bone grafts with high cellularity and osteogenicity.

1.2.2.1 Proposed design for combination bioreactor system

The thesis proposed the development and evaluation of a combination bioreactor system, having reviewed the independent stimulatory effects of biaxial rotation and cyclic compression on *in vitro* bone formation (in Chapter 2). This combination system also referred to as the multimodal bioreactor system will be designed to allow application of relevant compressive strains on tissue grafts cultured under biaxial rotation conditions (Fig 1.4). No prior studies have been reported the combination of the biaxial rotation and cyclic compression. Further, to tap the potential of luminescence based non-invasive sensors for rapid sensing of environmental parameters in bioreactor culture (further elaborated in Chapter 2), the thesis proposed the integration of dissolved oxygen and pH sensors (PreSens®) with the multimodal bioreactor.

Fig 1.3 Proposed combination bioreactor system to apply cyclic compression on grafts under biaxial rotation.

In sum, this thesis is motivated to develop a multimodal bioreactor platform that allows application of physiologically relevant mechanical stimuli on stem cell seeded bone grafts that are cultured under dynamic culture conditions. This work utilizes MSC seeded PCL-TCP scaffolds as relevant bone grafts. PCL scaffolds have been extensively studied in the past decade for engineering bone tissues [12-14]. The MSCs (human fetal source) used in this project have been well-characterized for their osteogenic capacities in the past [15-17]. Taken-together, the proposed approach involves application of physiologically relevant strains on bio-active PCL-TCP scaffolds seeded with MSCs that are simultaneously cultured under dynamic fluid flow conditions with an aim to produce

grafts with sufficient cells numbers and highly mineralized matrix deposition.

1.3 Specific aims and hypotheses

The main aim of the thesis is to study the influence of cyclic compressive forces on the generation of effective bone grafts for tissue engineering.

Specific aim 1: Develop a cyclic compression bioreactor for parametric optimization in bone tissue engineering.

Specific aim 2: Evaluate the influence of cyclic compression on osteogenic differentiation and mineralization of 3D tissue grafts.

Specific aim 3: Assess the effects of a multimodal bioreactor culture that combines the biaxial rotation with cyclic compression on cellular proliferation, gene expression and mineralization of premature bone grafts.

Hypothesis 1: We hypothesized that the developed cyclic compression bioreactor aids in the selection of appropriate parameters for generating relevant bone grafts.

Hypothesis 2: We hypothesized that application of physiologically relevant cyclic compressive stimuli results in accelerated generation of mineralized tissues.

Hypothesis 3: We hypothesized that the combined effects of biaxial rotation with cyclic compression in the multimodal bioreactor leads to generation of highly cellularized and mineralized bone grafts.

1.4 Novelty and implications

Mechanical loading studies on PCL-TCP scaffolds have been conducted for the first time to elucidate its stimulatory effects on mineralization of these grafts. The novelty of the thesis lies in the proposed approach of a multimodal bioreactor that allows the study of combined effects of biaxial rotation and physiologically relevant mechanical forces on bone tissue engineering for the first time. Results should aid in advancing our understanding of relevance of physiological biomimicry in bioreactors for bone tissue engineering. Further, bioreactors that provide physiological conditions for culturing 3D tissue grafts could be used drug testing, implant testing and creation of *in vitro* 3D tissue models for disease modelling.

1.5 Scope of thesis

Chapter 1 introduces briefly the need for bone tissue engineering bioreactors and also lists the specific aims and hypothesis of this thesis.

Chapter 2 thoroughly surveys the literature to review the need for mechanical stimulation in bone tissue engineering and discusses current approaches of bioreactors for engineering bone tissues.

Chapter 3 describes the materials and methods used for all the research experiments discussed in this thesis.

Chapter 4 describes the development of a multichamber cyclic compression bioreactor and demonstrates the system's ability for parametric optimization studies.

Chapter 5 demonstrates the stimulatory role of application of physiological compressive forces on osteogenesis and mineralization of premature bone grafts.

Chapter 6 discusses the development and evaluation of bone graft generation in a multimodal bioreactor system.

Chapter 7 presents conclusions of this thesis with future work recommendations.

Chapter 8 presents an annexe to this thesis with preliminary results from collaborative studies on different aspects of bone tissue engineering.

2. Literature Review[#]

[#] Partially published in Ravichandran, Liu et al, 2016, Journal of Tissue Engineering and Regenerative Medicine

2.1 Bioreactors in tissue engineering

2.1.1 Introduction

In the past two decades, researchers have been able to generate a wide range of 3D tissues using tissue engineering strategies including biomaterials, cells and appropriate environmental cues (Fig 2.1). Some of these tissue engineered medical products (TEMPs) have also been translated to clinics for *in vivo* tissue repair and regeneration. Despite the emergence of tissue engineering methods as a promising strategy for overcoming the lack of organs for transplantation, its introduction into the clinical setting is limited to mostly thin tissues such as skin.

Fig 2.1 Tenets of tissue engineering. Figure adapted from [18]

One main clinical challenge that is hampering the widespread adoption of tissue engineering strategies is the poor cell survival particularly at the core of thick voluminous grafts [9]. Growth of thick cellular grafts in a

static culture environment is challenged by mass transport (of oxygen, nutrients, waste products and metabolites) that solely depends on passive diffusion within a cellular graft, which is often limited to short distances (100-200 μm) and could result in inadequate nutrient supply [19]. Furthermore, static cultures do not represent the native microenvironment of most tissues. One of the key motivations behind the introduction of bioreactor systems for engineering thick tissues was to mimic the physiological scenario and recapitulate nutrient transport through blood flow in the human body.

2.1.2 Applications

The introduction of dynamic culture conditions using bioreactor systems as compared to conventional methods of cell culture in static dishes have thus far shown significant improvement in the quality of the tissue-engineered graft in terms of better cell growth [20-23]. In static cultures, cells are often non-uniformly loaded onto and within the scaffold and the cellular viability and growth is not even throughout the graft. The flow regime generated within bioreactors helps to overcome the limitations of oxygen diffusion within tissue-engineered grafts which is critical in maintaining cellular survival within the graft and uniform cellular distribution. For instance, Zhang et al showed high levels of cell viability within the core of human fetal MSC grafts using a biaxial rotating system as compared to a necrotic centre that was observed in static grafts [24]. The use of a fluidized bed bioreactor to scale up the culture of liver cell

biomass for a bio-artificial liver machine achieved a highly dense and viable population of cellular spheroids encapsulated in alginate beads (>45 million cells/mL beads) within 11-13 days [25]. In a recent study, Nguyen et al had demonstrated that dynamic bioreactor culture supported stem cell viability and functionality throughout a human femoral sized (200 cm³), engineered bone graft [16]. Other groups have also used bioreactor systems as dynamic cell loading systems for improving seeding efficiency [26-30]. Koch et.al had analysed the cell seeding efficiency and cell distribution after varying fluid flow velocity and perfusion cycle number in a perfusion bioreactor for bone tissue engineering [27]. They had reported cell seeding efficiency of about 50% with the perfusion bioreactor. The studies so far, have demonstrated that dynamic bioreactor culture with improved fluid dynamics can aid in better cell seeding as well as promote tissue maturation.

In addition to providing dynamic fluid flow conditions, bioreactors have now evolved to provide a physiological biomimicking environment for the generation of artificial tissues for human transplantation. There has been increased focus on the mimicry of native microenvironment of the tissue, whether it is biological, physical, electrical or mechanical. Studies are showing that bioreactors offer excellent controlled platforms for providing such biomimicking cues resulting in functionally relevant tissue grafts. Mechanostimulation has been shown to support cellular activity and differentiation for a variety of tissue types [17, 31-33], hence maturing

the graft to the desired extent before implantation. Bioreactors are also being used for recellularization of decellularized organs/tissues. Decellularized organs are acellular substrates of tissues and have shown promising potential in functional organ replacement [34]. Bioreactors are being used as platforms to recellularize these decellularized tissues under appropriate physiological conditions [35-37].

2.1.3 Design considerations

Table 2.1 lists some of the important factors that need to be taken into consideration while designing a bioreactor system. Upon conceptualizing the bioreactor design, the effectiveness of the bioreactor in achieving the specific roles can be verified using prediction methods to better understand the implications of various parameters such as fluid flow regime, oxygen tensions, mechanical force stimulation and their influence on cellular behaviour. Once the structural design is ready, aspects such as chamber construction, biocompatibility of its material, size factors and sterility maintenance have to be decided. This is followed by provision of control and sensing systems for detection and maintenance of the optimal biochemical and biomechanical environment to maximize efficacy of tissue formation. A facility to image the grafts in the bioreactor environment would add value to the system in terms of understanding real time tissue growth and dynamics.

Table 2.1 Key elements of design for bioreactors in tissue engineering.

Criteria	Key elements	Purpose
MODELLING	Modelling and prediction of key parameters	Characterize microenvironment
SYSTEM SETUP	Selection of chamber material	Bio-inert, non-corrosive
	Tight sealing mechanism	Prevent leakage and contamination
	Optically transparent windows	Real-time imaging of cellular grafts
	Versatile design	Accommodate various scaffold configurations and cell types
	Single-use tubing, valves, accessories	Prevent contamination
	Robust system	No fatigue, wear and tear over long term
SYSTEM SIZE	Smaller system	Save resources i.e. media
	Multi-chambered parallel systems	Study of multiple parameters or samples
	Bench top	Easier to handle
	Portability	Logistically easier, in case of transport
FLOW REGIME	Appropriate levels of laminar shear stresses	Increase mass transport, stimulate cells without wash off or damage
ENVIRONMENTAL CONTROL	Control of oxygen levels, pH, temperature, foam levels, gas, flow rate, lighted chamber	Maintain optimal cell growth conditions
PHYSIOLOGICAL BIOMIMICRY	Specific mechanical stimulation of scaffold/ <i>In vivo</i> mimicry	Enhance mass transport and control cell fate processes, mimic physiological loading
SENSORS	Gas levels, pH, dissolved oxygen, temperature	Maintain optimal cell growth conditions
	Media sampling for metabolites	Understand metabolic requirements
	Media sampling for contamination	Sterility check of media
IMAGING	Imaging facility integrated with chamber	Evaluation of scaffolds, cells, viability, tissue growth dynamics
FEEDBACK	Sensor/Imaging based control mechanism	Maintain optimal conditions
	Sensor/Imaging based alerts through alarm, messages, e-mails	Alert user during system errors
	Video Imaging of system	Constant supervision of the system

2.2 Physiological biomimicry

For making tissues of a specific organ, there is a need to identify the most optimal combination of cell/scaffold/bioreactor along with the appropriate environmental cues that include biological, chemical, physical, mechanical and/or electrical cues based on the native physiology of the specific tissue in question. At every step in this entire process, physiological biomimicry of the native tissues and the environment could result in tissues with enhanced functional properties [10]. While biological and chemical cues for the regeneration of a specific tissue have been extensively studied and optimized over the past few decades, other types of physiologically relevant cues such as electrical and mechanical cues have also gained a lot of attention in the past few years. Stimulatory effect of electrical signals on *in vivo* tissue growth and regeneration has been well established in the past. Balint et al has done an excellent review of the physiological relevance of electrical stimuli, the methods of stimulation and resulting cellular effects of electrical stimulation [38]. Non-invasive ultrasound stimulation has also been widely studied as a tool for tissue regeneration especially the bones, cartilage and soft tissues [39].

Static cultures for tissue engineering without appropriate mechanical stimulation may result in poorly cellularized grafts and require longer culture durations or larger initial cell numbers. By physiological mimicry of mechanical forces experienced by native tissues within the body,

accelerated tissue formation has been demonstrated by an increase in proliferation and differentiation of cells. Several groups have reviewed the importance of studying developmental biology of tissues to aid functional tissue engineering [40, 41]. Almost a decade ago, Martin et al had suggested modelling bioreactors based on the natural reactor “uterus”[42]. Our group had developed the biaxial bioreactor system that has the ability to spin (rotate about the z-axis) and tumble (rotate about the x-axis) simultaneously, which mimics the “fetal gyroscopic motion” inside the womb. This biaxial rotation has been reported to aid in increased mass transport through thick scaffolds leading to efficient nutrient and waste exchange [20] and showed increased bone formation and greater neo-vasculogenesis *in vivo* [43]. Recently, Morgan et al performed bioreactor studies for cardiac tissue engineering by mimicking the native biophysical environment during ventricular contraction of the heart. They demonstrated that the delayed electrical stimulation after the start of mechanical stimulation mimicking the *in vivo* scenario promoted generation of functional cardiac tissues in comparison to other modes of stimulation [44]. In another study by Heher et al, bioreactor was used to apply mechanical stimulation protocols that mimic *in vivo* muscle development during embryogenesis for engineering functionally relevant skeletal muscle tissues [45]. The results demonstrated that strain led to aligned myotube formation and also temporal expression of functional genes similar to patterns observed *in vivo*. In this Chapter, amongst the various environmental parameters, we would like to highlight the role

played mechanical stimulation in tissue development and discuss the current trends in mechanical bioreactors for engineering tissues.

2.2.1 Mechanical forces *in vivo*

Mechanical forces in the human body play a role in regulating tissue growth and remodelling processes. Cells respond to mechano-stimulation by changes in cellular alignment, rate of proliferation, differentiation, migration, matrix deposition or by apoptosis. Mechanical stimulation of 2-dimensional (2D) cell cultures showed structural and functional changes like morphology, alignment, polarity, gene and protein expression [46-48]. Literature on single cell studies have aided understanding in downstream signalling events in a mechanically stimulated cell that culminates in differential gene expression, protein synthesis or release of factors which in turn will indirectly control the aforementioned cell-fate processes [49-52].

However, these models do not represent the native 3D environment. Tissues exhibit a hierarchical cellular arrangement based on their physiological functional role which could be closely related to the mechanical forces that act on them. To engineer artificial tissues that resemble the native tissues both structurally and functionally, it would be important to provide appropriate *in vitro* conditions that mimic the native microenvironment of the tissue. This has clearly been neglected in our

long-standing conventional methods of culturing cells in a static environment. In the human body, the tissues are exposed to forces like fluid flow, compression, tension, torsion and hydrostatic pressure. The native biomechanical environment of different tissues in the body is represented in Fig 2.2.

Fig 2.2 Mechanical forces acting on various organs associated with their inherent functionality. (A) Blood vessel, (B) Bone, (C) Cardiac Tissue, (D) Cartilage, (E) Heart valve, (F) Ligament and Tendon, (G) Liver and Kidney, (H) Lungs, (I) Muscle, (J) Skin, (K) Teeth, (L) Urinary Bladder. Shear forces due to blood flow is a common feature in majority of the organs. Other key forces include compression, tension, torsion, hydrostatic pressure [53].

2.2.2 Mechanical stimulation *in vitro*

Engineering functional tissues in conjunction with the use of bioreactor systems focuses on a biomimetic approach where the mimicry of the physiological forces comes into play within the controlled environment in the bioreactor [54, 55]. Table 2.2 lists the various bioreactor studies that have been developed to mimic the physiological loading regimes of the native tissue. Some of the commonly applied mechanical stimuli on cell seeded 3D scaffolds are shear stresses, tension, torsion and compression.

Shear is one of the most commonly studied forces in the bioreactor likely due to the mimicry of blood flow in the human body. Different groups have thoroughly reviewed the role of shear stresses in bioreactor systems, concentrating on different aspects like the current state of organ engineering [56], review on the impact of shear stresses on osteogenesis and angiogenesis [57] and influence of dynamic shear stress in bone tissue engineering [58]. Tensile forces are more commonly studied for tissues like muscles [59], tendons [60], ligaments [61, 62], blood vessels [63] and cardiac tissues [64, 65]. Torsional forces have been used in engineering ligament tissues [66, 67] and intervertebral discs [68]. Research studies that involved compressive forces have mainly focused on engineering bone [69, 70] and cartilage tissues [71].

Table 2.2 Mechanical forces acting on various organs and studies that have utilized such forces in bioreactor.

Organ (<i>Functionality</i>)	Physiological mechanical forces	References
Blood vessel (<i>Blood flow</i>)	Cyclic circumferential and longitudinal stretch, luminal pressure, hydrodynamic shear	[72, 73]
Bone (<i>Physical support, locomotion</i>)	Interstitial fluid flow, cyclic compression, tension, torsion	[24, 69]
Cardiac tissue (<i>Pumping of blood</i>)	Cyclic stretch, hydrostatic pressure, shear	[74, 75]
Cartilage (<i>Joint lubrication and shock absorption</i>)	Compressive, shear, tensile	[76, 77]
Heart valve (<i>Unidirectional blood flow</i>)	Flexure, shear stress due to blood flow, closed valve tension	[78-80]
Ligament and Tendon (<i>Force transmission, joint stability</i>)	Cyclic stretch, shear and torsion	[66, 67, 81]
Liver and Kidney (<i>Excretion</i>)	Shear	[33, 82]
Lung and Trachea (<i>Breathing</i>)	Shear, airflow pressure, cyclic stretch, surface tension	[83, 84]
Muscle (<i>Movement</i>)	Cyclic and continuous strain	[85, 86]
Skin (<i>Protection, excretion</i>)	Compression and stretching	[87, 88]
Teeth (<i>Mastication</i>)	Compression and shear	[89]
Urinary bladder (<i>Excretion</i>)	Cyclic stretch and hydrostatic pressure	[90, 91]

2.3 Physiology of bone

Having reviewed the importance of mechanical conditioning for engineering 3D tissues, this section seeks to understand the relevance of mechanical forces in bone physiology. Bone is a dynamic tissue that responds to mechanical loading through remodelling. There are four major cell types found in bone namely a) osteoblasts, that actively synthesize bone matrix, b) osteoclasts, that resorb bone, c) osteocytes, mature bone cells derived from osteoblasts and d) progenitor cells or stem cells that differentiate to form osteoblasts.

2.3.1 Mechanism of bone remodelling

2.3.1.2 Mechanical stimulation induced bone remodelling

Bone tissue experiences complex strain patterns with varying proportions of compression, tension and torsion. Under physiological loading conditions, bone is in an equilibrium state with the net rate of bone formation equaling the rate of resorption. Upon increased loading, osteocytes recruit osteoblasts to synthesize more matrix and under decreased loading, osteocytes recruit osteoclasts to resorb bone (Fig 2.3).

Fig 2.3 Osteocytes maintain the bone tissue by sensing biochemical and biophysical signals and respond by interacting with osteoclasts or osteoblasts to resorb bone or form bone respectively. Reproduced from [92].

Osteocytes are hypothesized to be the primary mechanosensors that sense the biomechanical forces applied on bone [93]. Osteocytes embedded in the bone matrix are connected to the other osteocytes through gap junctions between their cellular processes extending from the cell body. These cellular processes are enclosed within a canalicular network forming lacuna outside the cell processes which is in turn filled by the interstitial fluid [94]. Tethering elements attach the canalicular walls to the integrins on the cell processes that are in turn connected to the actin filaments within the cells. It is hypothesized that the mechanical strains applied to bone result in pressure gradients that cause interstitial fluid flow within this lacuno-canalicular network (Fig 2.3) [95]. This flow applies hoop stresses on the tethering elements which in turn apply amplified strains to the cytoskeleton of the cell processes of the interconnected osteocytes leading to downstream signalling events resulting in bone remodelling [96]. On osteoprogenitor cells, mechanical loads are experienced as fluid flow or hydrostatic pressure [95, 97].

Fig 2.4 Mechanical load applied on bone causes interstitial fluid flow in the lacuna-canalicular network which is hypothesized to apply stresses on tethering filaments that connect the osteocytes cell body to the canalicular wall, thus amplifying the strains applied to the cell cytoskeleton (Adapted from [53]).

2.3.2 Mechanical loading induced *in vivo* bone formation

As early as 1886, Julius Wolff had stated that mechanical loading leads to adaptation of bone by changes in bone architecture and density [98]. Later, the Mechanostat theory that was proposed by Frost discussed the adaptation of bone to mechanical strains by modelling and remodelling mechanisms [99]. More recently, Rubin and Lanyon had proposed the adaptation hypothesis [100] that describes an optimal strain environment that is maintained by the bone during all the three states of disuse, adapted state and overuse by increase in bone resorption, maintaining bone mass in a dynamic state (equal rates of bone formation and resorption) and increase in bone deposition respectively (Fig 2.5). Each type of bone is genetically programmed to accept a particular strain value as normal and any deviation from this normal value results in bone remodelling [100]. The loading condition in the bone varies from region to region and is also dependent on the kind of physical activity. Daily

activities like walking, running, jogging and jumping apply cyclic strains to the bone in the range of 200 – 3000 $\mu\epsilon$ [101] and frequency of loading can range from 0.5 Hz - 2 Hz.

Fig 2.5 Mechanical usage windows based on Frost's Mechanostat hypothesis (adapted from [97, 99]).

Animal studies have consistently proven the stimulatory effect of dynamic mechanical loading on bone remodelling [102-104]. Turner et al had studied the effects of rat tibial bending for a period of 14 weeks. They observed a rapid increase in bone formation within five days of loading followed by a peak around 2 weeks. A 20% increase was observed in the cross-sectional area of the loaded tibia in comparison to the static controls [103]. By mechanical loading on rat forelimbs for the formation of woven and lamellar bone, McKenzie had demonstrated differential underlying gene expression levels of markers for cell proliferation and angiogenesis [104].

Mechanical loading has also been used in clinical settings to regulate physiological bone formation. Low-magnitude, high-frequency mechanical stimuli were found to increase bone mass densities in children with disabling conditions [105] and improve muscle and bone mass in young women [106]. Another study demonstrated the prevention of postmenopausal bone loss in the spine and femur of older women by application of short-term mechanical loading [107]. Distraction osteogenesis is a clinical technique that exploits mechanical stimulation to reconstruct bone defects [108, 109]. Bone fragments are separated by application of tensile forces which would mechanically stimulate the healing bone callus. This causes the release of growth factors leading to the proliferation and differentiation of stem cells and also aid in angiogenesis [110]. In summary, regulatory effect of mechanical forces on physiological bone formation has been well documented, and mimicry of this biomechanical microenvironment has been surveyed for improving the rate of *in vitro* bone formation.

2.4 Mechanical forces in bone tissue engineering

2.4.1 Four stages of bone formation

The four progressive stages of bone regeneration has been summarized previously [9] and represented in Table 2.3. Essentially, bone tissue engineering strategies aim to develop late-stage engineered bone tissue with highly organized, multilayered bone plates with mechanical properties that resemble native bone. With progressive stages, the focus

has translated from tissue regeneration to tissue remodelling that involves the tissue conforming to external stresses.

Table 2.3 Four stages of bone formation (Reproduced from [9]).

Stages of bone formation	Stage 1	Stage 2	Stage 3	Stage 4
	Mineralization	Woven bone	Trabecular bone	Lamellae bone
Mechanical strength	X	XX	XXX	XXXX
Bone structure	Random distribution of mineralized nodules	Disjointed plate-like networks, low density with large porosity	Highly oriented and joined bone plates, conforming to applied stresses	Dense and compact, multi-layered bone plates arranged in concentric manner conforming to applied stresses
Time interval	1-2 weeks	3-6 weeks	3-6 months	Months - years
Representative figure

2.4.2 Bioreactors for engineering bone tissues

With the introduction of a variety of bioreactor designs, the field is fast expanding with a rising trend in the number of bioreactor-related publications for bone tissue engineering in the recent years (Fig 1.2).

Fig 2.6 Web of science citation report on number of publications and citations in the past 20 years on bioreactors for bone tissue engineering.

As mentioned earlier, bone experiences a combination of compressive, tensile, shear and torsional forces. Over the years, many studies have demonstrated the osteogenic effects of mechanical stimulation on osteocytes, osteoblasts and osteoprogenitor cells. Polbhat et al. had analyzed the changes in gene expression of osteoblast-like cells upon compressive loading and demonstrated increased Collagen I and ALP mRNA and protein expression [111]. Osteoblast stress fiber reorientation and collagen formation was demonstrated by Matsugaki et al. when the continuous cyclic stretch of 4% was applied to the osteoblast monolayers [46]. Application of 2.5% tensile strain on MSCs was shown to enhance proliferation and regulate cell differentiation by increasing the expression of osteogenic genes like Collagen 1A, BMP-2, etc. [112]. To replicate the *in vivo* scenario, mechanical strains were applied on cells grown on 3D scaffolds for engineering bone tissues (Table 2.4). In most of these studies, increased osteogenesis, mineralization or better

mechanical properties have been reported for the mechanically stimulated groups when compared to the unloaded control groups.

Table 2.4 Research studies that applied cyclic compressive loading studies on 3D scaffolds for bone tissue engineering.

Scaffold	Cells/Tissue	Mode	Observed results (in comparison to static conditions)	Ref
Collagen/NiTi	MSC/Callus model	100 μ m, 1 Hz	↑ ECM mineralization, tissue maturation	[113]
Bone Explant	Autologous Bone	1000-4000 μ ϵ , 1 Hz	↑ Osteogenic gene expression, ALP, Ca, mechanical properties	[69]
DBM	EPC/ Bone	5%, 1 Hz	↑ Proliferation and formation of microvascular structures	[70]
Collagen-alginate	MSC/ Bone/ Cartilage	10%, 15%, 1 Hz	↑ Osteogenic (10%) & osteo-chondrogenic gene expression (15%)	[114]
Bone-Fibrin	BMSCs/ Bone	4 kPa, 0.05 Hz	↑ Expression of OPN, TGF- β -R1, ITGB1, SMAD5, PDGF- β	[115]
PLLA	Primary bone cells	1.5%, 1 Hz, 1 h daily	Higher mineralization	[116]
HA composite	MG-63 cells/ Bone	3 Hz (+/-)	↑ Collagen, fibronectin, VEGF gene expression	[117]
Polyurethane	MLO-A5 cells/ Bone	5%, 1 Hz	↑ Collagen, osteopontin, osteocalcin gene expression, stiffness, mineralization	[118]
PCL	Osteoblasts/ Bone	10%/20%, 0.5 Hz	↑ Osteogenic gene expression, ALP and matrix proteins	[119]

Hydrogel based mechanical loading studies have demonstrated the stimulatory effect of compression on the expression of osteogenic genes like osteopontin, TGF- β R1, SMAD-5, etc. [114, 115]. Enhanced mineralization and osteogenic gene expressions were observed in cell-seeded polymeric scaffolds like polyurethane and polycaprolactone (PCL) under cyclic compression [71, 119, 120]. Dynamic compression of EPC seeded DBM scaffolds resulted in higher proliferation and microvascular tube like formation [70]. Zong Ming et al demonstrated that the application of cyclic compression on bone explants induced an increase in the expression of osteogenesis related genes and proteins, resulting in enhanced mechanical properties and mineralization when compared to the unloaded controls [69]. Matziolis et al demonstrated an increase in gene expression that contributed to osteogenic predifferentiation of Bone Marrow Stromal Cells seeded on fibrin matrix on application of pressure of 4 kPa at 0.05 Hz for a day in comparison to controls that were not mechanically stimulated [115].

2.4.2.1 Proposed bioreactor for mechanical stimulation

Literature review of mechanical loading studies on 3D tissue grafts for bone tissue engineering demonstrated the potential of mechanical loading for *in vitro* bone formation. However the experimental designs chosen for most of the studies were not appropriate and physiologically relevant. Hence, we proposed the use of cyclic compressive forces experienced by native bone for engineering bone tissues from clinically

relevant scaffolds. The scaffolds proposed in this thesis are 3D printed, bioresorbable Polycaprolactone- β Tricalcium Phosphate (PCL-TCP) scaffolds and the cells are Mesenchymal Stem Cells (MSC) isolated from human fetal femurs. PCL-TCP scaffolds have been extensively studied in the past decade for engineering bone tissues. *In vitro* experiments and long term *in vivo* implantation studies using different animal models have demonstrated the scaffold's slow degradation rate [9, 121], good mechanical properties [122], biocompatibility and high osteoconductivity [9, 121, 123]. The fetal MSCs used in this study have been shown to exhibit superior proliferative and osteogenic potential along with lower immunogenicity [15, 16]. Studies have also demonstrated the clinical potential of these fetal MSCs for bone regeneration [23, 124]. Gotharstrom et. al., revealed an improvement in skeletal damage in infants with severe osteogenesis imperfecta after an *in utero* implantation of allogeneic fetal MSCs [23]. In addition to the studies that demonstrate the individual contributions of cells and scaffolds to bone regeneration, studies involving dynamic culture in biaxial rotation bioreactor have shown their combined effectiveness in generation of efficient bone grafts [17, 24, 43]. Taken-together, the thesis proposed the application of physiologically relevant strains on bio-active PCL-TCP scaffolds seeded with MSCs to engineer mineralized bone tissues.

2.5 Bioreactors as dynamic tissue culture and sensing systems

2.5.1. Dynamic fluid flow regime

As mentioned earlier, generation of *in vitro* engineered tissues of clinically relevant sizes is impeded by diffusion limited mass transport resulting in insufficient supply of nutrients to the cells. The mass transport limit by diffusion is $<200\text{ }\mu\text{m}$ [19]. Inside the body, capillaries provide nutrient and waste exchange with tissues at a maximum distance of $100 - 200\text{ }\mu\text{m}$ [19]. 3D tissues that exceed this size limitation will demonstrate necrotic cells in the regions farther from the media. By creating dynamic conditions and promoting nutrient transport within the scaffolds, bioreactors can generate tissues with higher cell numbers and homogeneous cellular distribution.

Perfusion bioreactors, spinner flasks and rotating wall vessel (RWV) bioreactors are more commonly used for the creation of dynamic fluid flow conditions to enhance nutrient transport to cells. Perfusion bioreactors enable direct perfusion of nutrient media through the scaffold pores. However, direct shear forces on the cells could wash them off the scaffolds [125]. Spinner bioreactor is characterized by the use of a mechanical stirrer to enable mixing up of nutrients and oxygen within the system and prevent formation of gradients. Although, the set-up is relatively simple and low cost, there is non-homogeneous distribution of shear forces could lead to unequal cellular growth on scaffolds. RWV bioreactors apply least shear stress to the cells and enable thorough

mixing up of fluid. Inherent disadvantage of this system is collision of constructs since these bioreactors do not offer anchorage to the scaffolds [125].

Our group had previously developed a biaxial rotation bioreactor system that can anchor cellular scaffolds and rotate in two independent axes (Fig 2.7A). This biaxial rotation was found to increase fluid velocities and mass transport within the scaffolds [126]. Zhang et al had reported enhanced cellular viability in the core of scaffolds and increased osteogenesis and mineralization of MSC seeded PCL-TCP scaffolds cultured within the biaxial bioreactor (Fig 2.7 B,C,E) [20]. In a head-to-head comparison with other commercially available systems, Zhang et al showed homogeneous cellular distribution and superior osteogenic induction in the scaffolds cultured within the biaxial bioreactor system (Fig 2.7 D,F) [125]. In addition to *in vitro* studies, biaxial bioreactor cultivation also showed enhanced neo-vasculogenesis and greater bone formation when implanted in critical sized rat femoral defects [43].

Fig 2.7 A. Biaxial rotation bioreactor rotates in two independent axes, spinning around z-axis and tumbling around y-axis, computationally proven to enhance fluid velocities within the bioreactor and promote mass transport [126], B. Biaxial bioreactor culture enhanced cellular proliferation in comparison to static cultures, C. Biaxial culture promoted ALP activity and calcium deposition when compared to the scaffolds cultured under static conditions [20], D. Biaxial bioreactor cultured aided in increasing the cellular proliferation and prevention of necrotic core formation as shown by the FDA/PI staining (green cells: live, Red cells: Dead) in a head-to-head comparison with other commercially available bioreactor systems (SF-Spinner Flask, RWV- Rotating Wall Vessel), E. Mineralization was quantified and also determined qualitatively by F. Von Kossa staining and was found to be higher in the biaxial bioreactor cultured scaffolds in comparison to the other bioreactors [125].

2.5.2 Combination of dynamic fluid flow and mechanical stimulation

Several bioreactor systems have explored the combination of mechanical stimulation and dynamic fluid flow conditions for engineering 3D tissues (Table 2.5). Most of these studies have used perfusion

pump as the sole contributor to creating a dynamic fluid environment for tissue culture. Further, the individual and combined effects of mechanical stimulation and dynamic fluid flow have been elucidated only in two studies [71, 127].

Table 2.5 Combination bioreactors that combine compression and dynamic fluid flow for engineering 3D tissues.

Compression	Dynamic fluid flow	Cell/Tissue	Scaffold	Effects	References
10%, 0.5 Hz	10 ml/min	Bone marrow stromal cells/ Bone	Bovine spongiosa disc	Perfusion increases cell proliferation, compression induces differentiation	[127]
0.2%, 1 Hz	Continuous perfusion	NA	Ovine trabecular bone explants	Mechanical strain driving factor for new bone formation	[128]
10%, 1 Hz	5 ml/min	MSC/ Hematoma	Fibrin	Cyclic loading leads to increased oxygen levels in the centre of scaffold	[129]
10%, 5 Hz	5 ml/min	BMSC/ Bone	Decalcified bone matrix	Feedback control of O ₂ and pH levels	[78]
10%, 0.5 Hz	10 ml/min	BMSC/ Meniscus	Polyurethane	Perfusion increases cell proliferation, compression induces differentiation	[71]
10%, 0.5 Hz	12 ml/min	BMSC/ Osteochondral	Collagen I-bone hybrid matrix	Perfusion enhanced Col II expression, compression enhanced Col X expression	[130]
330 kPa, 0.1 Hz	4.8 mL/min.	Fibroblasts and MSC	PLL beads	Design development	[131]

2.5.3 Sensing in bioreactors

In cell and tissue culture, growth media provides the necessary nutrients for cell proliferation and/or differentiation, while metabolites and by-products such as lactate are being released into the media. Slight changes in vital parameters like dissolved O_2 , CO_2 , pH and glucose in the culture media could adversely affect the cellular functions like proliferation and differentiation. With respect to tissue culture in bioreactors, on-line monitoring of culture conditions is important to ensure accuracy and reproducibility of the bioreactor runs. There are three ways of sensing bioreactor culture conditions namely, invasive, shunt and non-invasive sensing [74] (Fig 2.8).

Fig 2.8 Ways of sensing bioreactor culture conditions. Figure adapted from [132].

Shunt sensing is a simple technique where culture media would be sampled at regular intervals and measured off-line using benchtop sensors. This has obvious disadvantages of not being real-time. Direct

invasive sensors that can perform on-line sensing in bioreactors are generally sterilisable/single-use, electrochemical sensors that are in contact with the culture media, mostly restricted to the media reservoir, culture media loop or inlet/outlet of bioreactor chambers [78, 133, 134]. This method is widely popular owing to the abundance in commercially available pH and DO (dissolved O₂) electrochemical probes. In fact, microelectrodes are not only used to measure the surrounding milieu but also inside the growing grafts to assess the conditions of cellular microenvironment [135]. The major limitation with these invasive sensors is the possible risk of contamination in addition to high costs, fouling and consumption of analytes in the culture media [72].

Over the past few years, there has been an increasing interest in the non-invasive imaging modalities, especially luminescence based sensors for sensing environmental conditions within the bioreactor. While direct contact is required for electrochemical sensing, it is possible to non-invasively detect fluorescence based signals through the walls of a transparent vessel. Fluorescence based sensing for detection of media conditions in the bioreactor started almost two decades ago[136]. A number of fluorescent dye based adhesive patches have been developed and available commercially that can be adhered on to transparent bioreactor vessel surfaces. Changes in fluorescence signal is detected using optical probes that are not in direct contact with the sterile culture media [137]. Papkovsky et al have explained the working

principle of these sensors in depth and reviewed the different types of these luminescence based sensors[138]. CellPhase®, HP5 and PSt3 / PSt6 (PreSens®), RedEye® are some of the commercial fluorescent sensor patches that are suited for sensing DO/pH/metabolites inside bioreactor vessels. Some of these sensors come in single-use packages making it safe for clinical usage. Santoro et al had used PSt3® disposable, micro-oxygen sensors to measure the oxygen levels at the inlet and outlet of a perfusion bioreactor used for engineering cartilage grafts [139]. The consumption of oxygen in the bioreactor chamber gave an indication of the number of chondrocytes on the graft which in turn enabled non-invasive evaluation of quantitative quality of engineered cartilage graft. Recently, Raimondi et al demonstrated the use of SF-RPSu4® sensor foils within a miniature perfusion bioreactor system to quantify the oxygen concentrations within the tissue-engineered construct in a non-destructive way [140]. Although these non-invasive sensors exhibit great potential for rapid sensing of environmental parameters in the bioreactor culture, there is a need to address some of its limitations including complexity of optical set ups required for detection and lesser flexibility in aligning the set up with dynamic, moving bioreactor parts.

2.6 Commercial systems and future trends

Currently available commercial systems for generation of artificial 3D tissues provide dynamic flow environment in addition to application of

specific mechanical forces depending on the tissue type. Table 2.6 summarizes a selected list of commercially available bioreactors for generating 3D tissue engineered grafts. The table showcases the current trends in commercial bioreactor design including provision for application of mechanical forces and availability of sensing modalities to sense culture conditions inside the bioreactor system.

Table 2.6 Selected commercially available bioreactors and its associated features that can be potentially used for engineering 3D bone grafts with/without stem cells. All information was obtained from the respective company website.

Commercial bioreactor	Mechanical forces			Sensing and/or control	References
	Fluid flow/ Perfusion	Compression	Tension		
IAXSYS bioreactor		✓	✓	NA	[141]
BioTense Bioreactor	✓		✓	T, Strain, force	[142]
BioDynamic 5500 test instrument		✓		Pressure, dissolved oxygen, T, pH, CO ₂ , force, displacement, metabolites, etc.	[143]
Multi-specimen BioDynamic test instruments	✓	✓	✓		[144]
BioDynamic 5900 Test Instrument	✓	✓			[145]
BioDynamic 3DCulturePro Bioreactor	✓				[146]
TEB 1000 flow bioreactor	✓			Dissolved oxygen, T, pH, CO ₂ metabolites	[147]
TC-3 mechanical stimulator bioreactor	✓	✓	✓	Not specified	[148]
Flexcell Tissue Train Culture System and FX-5000 compression system		✓	✓	Not specified	[149]
Z®RP Bioreactor 500	✓			pH, T, pO ₂ , flow rate	[150]
Quasi-vivo system	✓			pH, T, pO ₂ , airflow, flow	[151]
TisXell Regeneration Systems	✓			pH, T, pO ₂ , rotation speed, flow rate	[152]
Rotatory, perfusion, stem cell culture systems	✓			Speed of rotation	[153] [154] [155]
Osteogen	✓	✓	✓	Flow, pressure, real-time stiffness	[156]

From an engineering point of view, the bioreactor technology has shown significant development over the years, with precise control of the various parameters of the microenvironment and real-time sensing and imaging facilities which allow greater control and reproducibility of the tissue graft generation. In addition, closer interactions among clinicians, scientists, engineers and other stakeholders such as the regulatory bodies and industrial partners are required to integrate the various requirements needed for clinical translation of bioreactors [157]. Future trend of bioreactors lies in an approach directed towards the mimicry of physiological environment which incorporates the key design considerations suggested in this Section 2.1.3 and is envisaged to be of effective clinical utility (Fig 2.9).

Fig 2.9 Schematic showing the ideal workflow of the procedure from bench to transplantation of the bioreactor-cultured TE graft. The laboratory phase comprises of bioreactor design, computational modelling, and followed by *in vitro* and *in vivo* validation experiments. *In situ* imaging systems that allow real-time monitoring of grafts are necessary prior to human implantation [53].

3. Materials and Methods

3.1 Cells

3.1.1 Isolation and culture of MSC

Cells were obtained using the method described by Zhang et al [17]. Briefly, single-cell suspensions were prepared by flushing the marrow cells out of fetal femurs using 22G needle and passed through a 70 μ m cell strainer and plated on a T-75 cell culture flask (NUNC, USA) at 10^6 cells/ml using Dulbecco's modified Eagle's medium (DMEM) with High glucose, Glutamax supplement (GIBCO, USA) (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 mg/ml streptomycin (GIBCO, USA), which will be referred to as D10 medium. Non-adherent cells were removed during media changes that were performed once in 2-3 days. The multi-lineage differentiation ability of the MSCs was confirmed by culturing the cells in adipogenic, chondrogenic and osteogenic induction media and assessing the cells for respective markers.

3.1.2 Multilineage differentiation

Adipogenic differentiation: MSCs were seeded at 2×10^5 cells in 2 ml of D10 media on 6 well plates. The medium was changed every two days until the cells reached confluence. Once the cells reached confluence, three cycles of adipogenic induction medium (3 days)/adipogenic maintenance medium (1-3 days) (Poietics human MSC, Lonza, USA) followed by a week's culture in adipogenic maintenance medium was carried out to induce adipogenic differentiation. The lipid vacuoles

formed on adipogenic differentiation were stained with AdipoRed (Lonza, USA).

Chondrogenic differentiation: 5×10^5 MSCs were suspended in 1 ml of complete chondrogenic medium (Poietics human MSC, Lonza, USA) were split into two 0.5 ml cell suspensions. The 0.5 ml cell suspensions were transferred to 15 ml polystyrene tubes and centrifuged at 150g for 5 minutes. The pellet and supernatant were left undisturbed inside the incubator at 37°C and 5% CO₂ to promote pellet culture for chondrogenic differentiation. The pellet was flicked during every media change once in 3 days. After 14 days of culture, the cells were fixed using 4% paraformaldehyde for an hour and then embedded in paraffin for histological sectioning. 5 µm thickness sections were obtained using a microtome and the glycosaminoglycans (GAG) were stained using Safranin-O stain.

Osteogenic differentiation: MSCs were seeded at 2×10^5 cells/ well using D10 medium on 6 well plates. Once the cells reached confluence, D10 media was replaced by osteogenic induction medium (D10 medium supplemented with 10 mM β-glycerophosphate, 10^{-8} M Dexamethasone, and 0.2 mM Ascorbic acid) also referred to as bone medium. After 2-3 weeks of culture, the cells were washed twice with PBS, fixed with 4% paraformaldehyde for an hour followed by von Kossa staining for

phosphates (2% AgNO_3 in dH_2O) for 10 minutes in the dark and exposed to bright light for 30 minutes.

3.2 Scaffolds

3.2.1 Mechanical characterization

Cylindrical PCL-TCP scaffolds with dimensions 8 mm outer diameter x 3.2 mm inner diameter x 5 mm height were used for this study. The scaffold morphology and $0^\circ/60^\circ/120^\circ$ laydown arrangement pattern was analyzed following sputter coating with Platinum (Sputter current: 20 mA, duration: 100 s) and observed under Scanning Electron Microscopy (JEOL, Japan). The mechanical property of the PCL-TCP scaffolds was assessed to determine the range of stress levels that could be operated on the scaffold without disrupting its mechanical integrity. PCL-TCP scaffolds (wet batch) were soaked in PBS for 24 hours prior to mechanical testing to mimic the bioreactor microenvironment. After 24 h, the dry and wet batches were mechanically tested using an Instron 5500 microtester with a 1kN load cell and a cross-head speed of 0.5 mm/min.

3.2.2 Scaffold treatment and sterilization

PCL-TCP scaffolds were treated with 5M NaOH for 3 hours to increase hydrophilicity followed by two PBS washes and drying by centrifugation. The scaffolds were sterilized in 90% ethanol for 24 hours. The ethanol was removed by two PBS washes and one dH_2O wash. The sterile scaffolds were transferred to low adhesion 24 well plates in a sterile

manner. The excess water was removed by centrifugation and pipetting. The scaffolds were placed in the incubator at 37°C for 24 hours for drying followed by storage in dry chamber until use.

3.2.3 Preparation of cell-scaffold grafts

The dry scaffolds in 24 well plates were loaded carefully with Tisseel solution (Tisseel dissolved in dH₂O and diluted twice with PBS) (Baxter) specifically in to the pores of the scaffolds. Cells were suspended in Thrombin solution prepared by dissolving Thrombin 4 in CaCl₂ and diluting twice with PBS) (Baxter). The cell-thrombin suspensions were seeded on to the scaffolds by repeating the pattern of loading of Tisseel. The scaffolds were left in the incubator for an hour for polymerization to form fibrin polymer and for better cell adherence before adding media.

3.3 Bioreactor systems

This thesis developed and evaluated two bioreactor systems for engineering 3D bone grafts.

3.3.1 Multichamber compression bioreactor system

The multichamber bioreactor comprises of four sterilizable glass chambers each with a capacity to contain 60 ml of culture medium (Fig 3.1). Each chamber can anchor six scaffolds on the compression shaft tightened with spacers and a locking nut and cyclically compress them under controlled environment. The system allows independent control of

loading for the chambers. The bioreactor allows gaseous exchange through gas permeable silicone tubings.

Fig 3.1 Multichamber compression bioreactor system.

3.3.2 Biaxial rotation and cyclic compression bioreactor system

Biaxial rotation bioreactor was modified to include micromanipulator to apply cyclic compression on the growing tissue engineered construct (Fig 3.2). The bioreactor chamber can contain a volume of 1 L and the tubings connect this chamber to a reservoir (500 ml). Media perfusion was maintained at 5 rpm. The chamber and L-bracket rotation angles were kept at 90°.

Fig 3.2 Biaxial rotation and cyclic compression bioreactor system.

3.4 Culture chamber sensing systems

3.4.1 DO sensing system

The SP-PSt3 Oxygen spot sensor (Fig 3.3A) was adhered to the bioreactor chamber surface by using silicone glue and left to cure overnight.

Fig 3.3 A. Oxygen sensor spot back (red) and front (black), B. Working principle of O_2 sensors (provided by manufacturer).

The sensor was calibrated based on the manual instructions. Briefly, air-bubbled media (21% O₂) was used as Cal100 and N₂ bubbled media was used as Cal0 (0% O₂) (Fig 3.4). Following calibration, the chamber was washed with DI water and autoclaved and dried. The dissolved oxygen levels (%) in the chamber media were read out non-invasively as per manufacturer's protocols using an optic fibre (Fig 3.4).

Fig 3.4 Calibration of oxygen sensor spot adhered to bioreactor chamber.

3.4.2 PH sensing system

The flow-through sterile pH sensor (FTC-SU-HP8, Presens) was placed in the media loop at a location that allowed easy access to the detector (Fig 3.5A). The pH of the media was detected from the flow-through pH sensor twice a day in a non-invasive manner as per manufacturer's protocols.

Fig 3.5 A. Single-use flow-through cell pH sensor, B. Working principle of pH sensors (provided by manufacturer).

3.5 Analysis of osteogenesis: Qualitative and quantitative

3.5.1 Gene expression

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) was used to analyze gene expression. Briefly, the mRNA was isolated by rinsing the grafts with PBS followed by treatment with lysate buffer (Buffer RLT (Qiagen) with 2-Mercaptoethanol (Sigma-Aldrich, USA)). The extracted RNA was reverse transcribed to produce cDNA. The qPCR was performed using iQ SYBR Green Supermix (Bio-Rad, Singapore) and Real Time PCR System (CFX Connect, Bio-Rad, Singapore). Table 3.1 lists the primers used in this thesis. Gene expression results were normalized against the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the gene expression was calculated using the $-2^{\Delta\Delta C_T}$ method relative to the undifferentiated MSC controls (n=3).

Table 3.1 Primer sequences for all the genes used for RT-PCR in this thesis.

Gene	Primer sequences	PrimerBank ID/ Reference
GAPDH	F: CATGAGAAGTATGACAACAGCCT R: AGTCCTTCCACGATACCAAAGT	7669492a3
Runx2	F: AGTAGGTGTCCCGCCTCAGA R: CCHGTGGAHAAAAGGACTTGGT	[15]
TGF- β 1	F: GGCAGTGGTTGAGCCGTGGA R: TGTTGGACAGCTGCTCCACCT	[15]
BMP-4	F: GCCAAGCGTAGCCCTAAGC R: GTGGCGCCGGCAGTT	[158]
BMP-6	F: CGGTGCACTAGCCCCCTTCC R: TGCCCAGCAAACCCGCTCG	[158]
ALPL	F: ACTGGTACTCAGACAACGAGAT R: ACGTCAATGTCCCTGATGTTATG	294660769c2
Osteonectin	F: AGCACCCCATTGACGGGTA R: GGTCACAGGTCTCGAAAAAGC	4507171a1
Osteocalcin	F: CACTCCTCGCCCTATTGGC R: GCCTGGGTCTCTTCACTACCT	4502401a3
COL1A1	F: AGGACAAGAGGCATGTCTGGTT R: CCCTGGCCGCCATACTC	[15]

3.5.2 Alkaline phosphatase assay

Cell lysates were tested for ALP activity using SensoLyte™ pNPP Alkaline Phosphatase Assay Kit (AnaSpec, USA) as per the manufacturer's instructions. The ALP activities were normalized to the total protein content determined using the Bradford Protein Assay (ThermoScientific, Singapore).

3.5.3 Calcium content assay

The calcium content of the cellular scaffolds was assessed as described before [15]. Briefly, the calcium deposits were dissolved in 400 μL of 0.5 N acetic acid and determined by a colorimetric assay using calcium assay kit (BioAssay Systems, USA) as per the manufacturer's instructions. Cell-free empty scaffolds were used as negative controls (n=2) to compensate the elution of calcium from the cell-free scaffold.

3.5.4 Von Kossa staining

Von Kossa staining was performed as described previously [17]. Samples were rinsed twice with PBS and then fixed in 4% paraformaldehyde for 1 hour followed by two washes with dH_2O . Samples were then stained with freshly made 2 w/v% silver nitrate (Sigma-Aldrich, Singapore) in dH_2O for 10 minutes in the dark and exposed to ultraviolet light for 30 min. Samples were then rinsed in dH_2O and air-dried before imaging under digital microscope.

3.5.5 Alizarin red staining

Alizarin red staining was performed by fixation of samples with 4% paraformaldehyde for 1 hour followed by rinsing with dH_2O . The samples were stained with freshly made 40mM Alizarin Red (pH 4.2) for 10 mins at room temperature. This was followed by multiple washes with dH_2O

to remove non-specific stains and air-dried before imaging using digital microscope.

3.6 Imaging

3.6.1 Phase contrast light microscopy

Cellular morphology, adhesion and extracellular matrix production were examined by phase contrast light microscopy (PCLM) (Olympus LX71, Japan) daily and imaged at fixed time intervals using an attached camera unit.

3.6.2 Fluorescence microscopy

Cellular viability was examined by Fluorescein Diacetate/ Propidium iodide (FDA/PI, Life Technologies, Singapore) staining as described previously [15]. FDA stains the viable cells green, and PI stains necrotic and apoptotic cell nuclei red and the stains were viewed under fluorescent microscope (Olympus LX71, Japan).

3.6.3 Confocal microscopy

Fixed samples were permeabilized using 0.1% Triton X-100 for ten minutes followed by staining the F-actin using tetramethyl rhodamine isothiocyanate-conjugated phalloidin (1:500, Chemicon) as described previously [159]. Confocal laser scanning microscope (Zeiss LSM 510)

was used to visualize the stained cells and z-stack images were acquired to get the 3D distribution of actin fibers.

3.6.3 Scanning Electron Microscopy (SEM)

Samples were fixed using 4% paraformaldehyde, serially dehydrated (25%, 50%, 75%, 95%, 100%), and sputter coated with platinum (current: 20 mA and sputtering duration: 100 s) and viewed using JEOL JSM6390LA analytical SEM (JEOL, Japan).

3.7 Cellular proliferation

3.7.1 DNA content assay

Harvested scaffolds were incubated with a Collagenase (1 mg/ml, Sigma-Aldrich, Singapore) and Trypsin (0.1 %, Sigma-Aldrich, Singapore) cocktail in PBS for 2 hours to digest the ECM completely and lysed by freeze-thaw method as described before [17]. Total cell numbers in 3D grafts were estimated by quantifying the DNA content in the cell lysate of each scaffold using PicoGreen DNA Quantification Kit (Molecular Probes, USA) as per the manufacturer's instructions. Cell numbers were indirectly assessed by evaluating the DNA quantities.

3.7.2 Total protein assay

Total protein count of the grafts determined using the Bradford Protein Assay (ThermoScientific, Singapore) as per the manufacturer's instructions.

3.8 Statistical analysis

Data were represented as mean $\pm$ SD and compared using Student's t-test and two-way ANOVA analysis. A p value of less than 0.05 was considered to be statistically significant. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

4. Development of Cyclic Compression Bioreactor for Bone Tissue Engineering

4.1 Introduction

The first specific aim for this thesis was to develop a cyclic compression bioreactor for engineering bone tissues. Current clinical approaches for bone defects repair by autologous grafts are unable to meet the demand due to issues like limited supply, donor site morbidity etc. [5, 6]. Allografts suffer from shortcomings including immune-based graft rejection and transmission of infection or disease from the donors [7]. This has led to the shift of focus towards engineering of bone tissues through tissue engineering approaches- using cells, scaffolds and appropriate cues to obtain functional bone grafts. Tissue engineering provides a promising approach for generating bone grafts, whereby biochemical cues are conventionally used to induce 3D tissue development. Once implanted, the engineered bone grafts are expected to support and respond to physiological mechanical loads *in vivo* [54]. This has led to the development of bioreactor systems that can apply mechanical forces on 3D tissue constructs to study the role of mechanical cues on cell-fate processes, accelerated tissue maturation and also predict the *in vivo* functional response of the engineered tissue.

Bone is a highly dynamic organ that experiences cyclic mechanical strains and constantly remodels according to the needs of the body. Consequently, there has been a growing interest to manipulate the cell-fate processes of osteoblasts, osteocytes and progenitor cells to study effects of mechanical forces on proliferation, stress fibers, calcium

deposition, bone markers and osteogenic gene expression levels using bioreactor platforms. Mechanical loading at tissue level include forces like compression, torsion, tension and pressure which have shown to influence the bone modelling and remodelling processes under both *in vitro* [22, 67, 116, 119] and *in vivo* conditions [103, 160]. *In vitro* mechanical loading studies on tissue grafts for bone tissue engineering have demonstrated induction of osteogenic differentiation [161, 162], mineralization of matrix deposition [115, 116, 163, 164] and neovascularization [70]. Although the combined and independent effects of these forces on tissue constructs have shown to significantly influence *in vitro* cellular proliferation and differentiation [68, 69, 114, 117, 165, 166], there is little consensus on the ideal loading parameters for optimal bone formation. The response of bone as isolated osteogenic cells in monolayers or as 3D tissues has been shown to vary with the type of mechanical stimulus, magnitude, frequency and duration of loading [69, 114, 117, 167]. Furthermore, the substrate on which the bone cells are attached should replicate mechanical properties of native osteoinductive environment of bone tissues to extract appropriate physiological response [168, 169]. Ideally, the application of physiological loads on relevant tissue constructs would aid in instructing the cells to behave like their native equivalents, which in turn could enable tissues to exhibit hierarchical tissue structure and function [170, 171]. Native bone tissues experience peak cyclic strains in the range of 0.2%-0.35% [100] and peak loading occurs at a frequencies of around 1 – 3 Hz. Most of the

previous cyclic loading studies have focused on loading parameters (strains ranging from 1% - 10%) that are much different from the physiological range [70, 116, 119, 120]. In attempting to mimic the physiological niche of the bone tissue, several other factors and conditions such as biochemical cues and other mechanical stimulation parameters also need to be optimized. To minimize experimental variations upon optimization of parameters and to operate in a more time-efficient and cost-effective manner, it would be helpful to develop an array of scaled-down bioreactors within one system.

Thus, we developed a multichamber compression bioreactor system to optimize the conditions for application of cyclic compressive loading in bone tissue grafts. We used Polycaprolactone- β Tricalcium Phosphate (PCL-TCP) scaffolds loaded with Mesenchymal Stem Cells (MSC). PCL-TCP scaffold based engineered tissues have been extensively studied in the past and studies have demonstrated their effectiveness in bone regeneration [15, 20, 43, 123]. The mechanical property of the scaffolds was assessed to determine the range of strain magnitudes that can be applied on the scaffolds. The mesenchymal stem cells used in the study were isolated and characterized for their ability to undergo multi-lineage differentiation. Studies were performed to understand the optimal biochemical and mechanical parameters for producing effective engineered bone grafts. We hypothesized that application of physiologically relevant mechanical stimuli on developing tissue

constructs will be beneficial for accelerated maturation of bone tissue grafts.

4.2 Bioreactor design

The compression bioreactor setup consists of four spherical, autoclavable glass chambers that can be secured to a flat metal base of 32 cm x 32 cm x 19 cm size. This setup can be placed within an incubator shelf with optimum humidity conditions, 37°C and 5% CO₂ (Fig 4.1). Each of the bioreactor chambers can be filled with 60 ml of media and possess a chamber lid that can be locked on to the chamber using locking nuts to ensure sterility. The tissue grafts can be loaded to the shafts housed in the chamber lid with stainless steel spacers (assumed incompressible) (Fig 4.1A). Each chamber can accommodate six scaffolds tightened to the shafts of the chamber lid with a scaffold locking nut. The bioreactor components in direct contact with the culture media (chamber, chamber lid with scaffold-anchoring shafts, spacers and scaffold locking nut) can be sterilized by steam sterilization at 121°C for 30 min.

Fig 4.1 Multichamber compression bioreactor system, A. Schematic of a single chamber of the multichamber bioreactor system showing the scaffolds loaded on the shaft of the chamber, B. Side view of the bioreactor chamber with the micromanipulator on the top, C. View showing all the four chambers: 2 loaded chambers with motors (left) and 2 static chambers (right).

Micromanipulators were loaded on to the chambers (Fig 4.1B) to apply cyclic compression on the grafts concurrently in different chambers with independent control of physical parameters including magnitude, frequency and delay periods. The compressive strain application was based on the displacement using the micromanipulator and the feedback

was obtained and saved to the system. Calibrated strain gauge was used to verify the strains applied to the scaffolds in the bioreactor. For this study, the frequency was fixed at 1 Hz to mimic the walking frequency in humans [172]. Media change was done every week such that 10 ml of fresh medium was available per scaffold every week for both static and loaded groups.

4.3 Results

4.3.1 Cell characterization

MSC exhibited a spindle shaped morphology when cultured in tissue culture plate (Fig 4.2A). The stem cells were inducted to form osteogenic, adipogenic and chondrogenic lineages by using the respective inductive media. The von Kossa staining of osteogenic differentiation revealed formation of black extracellular crystals (phosphates) after 14 days of induction (Fig 4.2B). AdipoRed Assay showed red staining of the lipid droplets formed after adipogenic differentiation for 21 days (Fig 4.2C). Safranin O staining of the paraffin embedded sections demonstrated orange stained GAGs representative of the chondrogenic differentiation of those cells after 2 weeks of induction (Fig 4.2D). The potential of stem cells to differentiate into multiple lineages was confirmed by these results.

Fig 4.2 Multi-lineage differentiation potential of MSCs, A. Undifferentiated MSCs under light microscopy, B. von Kossa staining of osteogenic differentiation of MSCs shows the staining of phosphates (black), C. AdipoRed staining of lipid droplets (red) formed due to adipogenic differentiation, D. Safranin-O staining of GAGs (orange) shows chondrogenic differentiation of the pellet culture of MSC.

4.3.2 Scaffold characterization

4.3.2.1. Morphology of scaffolds

The SEM images show the 0°/60°/120° laydown arrangement pattern of the PCL-TCP scaffolds with a porous structure in scaffolds (Fig 4.3).

Fig 4.3 PCL-TCP scaffold morphology, A. Top view (upper) of the PCL-TCP scaffold OD = 8mm ID = 3.2 mm, Side view (lower) of the scaffold Height = 5mm, 70% porosity, B. SEM of the PCL-TCP scaffold showing 0°/60°/120° laydown pattern.

4.3.2.2. Mechanical property of scaffolds

Results from compressive mechanical testing of acellular scaffolds (n=6) (Fig 4.4) indicated that the scaffolds had a Young's modulus of 38.9 ± 6.8 MPa and compressive strength of 0.98 ± 0.07 MPa (Table 4.1). The yield strain of acellular scaffolds was found to be $2.6 \pm 0.3\%$.

Fig 4.4 Mechanical testing of PCL-TCP scaffolds. Compressive stress-strain curves of PCL-TCP scaffolds, both dry and wet groups, show the linear elastic region followed by the process of densification due to an increase in the stress levels as the rods and struts of the scaffold are crushed.

Table 4.1 Results from mechanical testing of PCL-TCP scaffolds.

Condition	Average yield stress (MPa)	Average percentage yield strain	Young's modulus (MPa)	Failure stress (MPa)
Dry	0.96 ± 0.04	2.55 ± 0.3	38.17 ± 4.6	78.98 ± 15.9
Wet	0.98 ± 0.07	2.57 ± 0.4	38.89 ± 6.8	63.64 ± 11.2

4.3.3 Optimization of biochemical cues

Studies were conducted to choose the optimal media conditions for effective *in vitro* bone formation (Table 4.2). MSCs are generally maintained and expanded in culture media conditions (DMEM + 10% FBS) devoid of bone-inducing chemical components (dexamethasone, ascorbic acid and β -glycerophosphate).

Table 4.2 Different media conditions for bone tissue engineering [173].

Media	Components	Remarks
1. Non-induction media	DMEM + 10% FBS	Enables cellular proliferation and sustenance
2. Induction media	DMEM + 10% FBS + Bone induction factors (Dexamethasone + ascorbic acid + β -glycerophosphate)	Enables stem cell differentiation towards osteogenic lineage

Under osteogenic induction conditions, MSCs in monolayer cultures (2D, static) (Fig 4.5A) undergo osteogenic differentiation and show enhanced enzymatic activity (ALP-Alkaline Phosphatase) (Fig 4.5B). By the end of two weeks, there was a 17-fold increase in the ALP activity of MSCs cultured under osteogenic induction conditions when compared to those cultured under non-induction media ($p < 0.001$). With respect to

mineralization, a 3-fold increase in the levels of calcium deposition by day 10 ($p<0.01$) and 7-fold increase in the levels of calcium deposition by day 14 ($p<0.001$) was observed under osteogenic induction conditions when compared to those cultured under non-induction media (Fig 4.5C).

Fig 4.5 Osteogenesis of MSCs cultured in 2D under different media conditions, A. Phase contrast microscope images of MSC under non-induction and osteogenic induction media conditions showing the formation of mineral deposits under induction conditions, B. ALP activity clearly elevated by the second week in the osteogenic induction media in comparison to non-induction media ($***p<0.001$). C. Mineralization in the 2D MSC cultures were significantly higher under osteogenic induction conditions in the 14 day study ($**p<0.01$, $***p<0.001$).

A pilot study was conducted with the multichamber bioreactor system for a period of 2 weeks to check the safety, sterility of the bioreactor run. The study also assessed the role of cyclic loading (0.22%) on the osteogenic differentiation of 3D stem cell grafts in the absence of osteogenic induction factors. Frequency was maintained at 1 Hz and duration of compression was maintained at 4h/day following previous mechanical loading studies that compress for a few hours/day. Compressive load was released after each run and the scaffolds were maintained in the loading chamber for the entire duration of the study. Static groups were also cultured in a separate chamber without loading. The sterility was maintained throughout the culture period. The pH of culture medium was monitored throughout the culture period. Feedback on the applied strains was obtained and saved. Live/dead fluorescence imaging showed that bioreactor cultures allowed maintenance of cell viability on the scaffolds (Fig 4.6).

Fig 4.6 Cellular viability was qualitatively analyzed using live/dead fluorescence imaging and the results showed that the bioreactor culture allowed maintenance of cellular viability (Scale bar: 200 μ m).

When cultured in the absence of osteogenic cues, there were no significant differences in the ALP activities between the static and cyclically compressed groups (Fig 4.7A). Further, results demonstrated that mechanical loading alone did not have a significant effect on the osteogenic differentiation of MSCs in the absence of osteogenic induction factors (Fig 4.7B).

Fig 4.7 Effect of cyclic compression on tissue grafts in non-induction media, A. ALP activities of static and loaded groups over the two week period did not change significantly ($p > 0.05$), B. Calcium content of loaded groups were higher in comparison to that of static culture group on day 14 ($p > 0.05$).

Mechanical loading in the presence of osteogenic induction factors resulted in higher ALP activity in comparison to the static controls on both day 3 and day 14 ($n=3$, $p < 0.001$) (Figure 4.8A). Higher mineralization of the grafts was observed on application of cyclic compression within three days (Fig 4.8B) and it further elevated by the end of week 2 ($n=3$, $p < 0.001$).

Fig 4.8 Osteogenic differentiation upon mechanical loading under osteogenic induction, A. ALP activity was found to be significantly higher in the loaded group by the end of week 2 (n=3, ***p<0.001). B. Calcium deposition assay demonstrated an increase in the levels of mineralization within 3 days of culture in the loaded group. Significantly higher levels of mineralization were observed in loaded scaffolds by week 2 in comparison to that of the static controls (n=3, ***p<0.001).

The number of scaffolds to be inserted per shaft was fixed at three to ensure reproducibility of the studies. One revolution of servomotor shaft leads to a compression of 6.6 μm , the possible combinations of strain magnitudes that can be applied on the grafts were chosen based on the machine parameters (Table 4.3).

Table 4.3 Range of strain amplitudes and frequencies that can be applied on the scaffolds using the cyclic compression bioreactor system.

Revoluti on	Amplitude (μm)	Strain/Scaffol d ($\mu\epsilon$)	Strain/Scaffold (% strain)
5	33	2200	0.22
10	66	4400	0.44
15	99	6600	0.66
20	132	8800	0.88
25	165	11000	1.1

4.3.4 Optimization of compressional strain magnitude

A strain optimization study was performed to optimize the effect of mechanical cues on cell behavior cultured in multichamber bioreactor.

Table 4.4 shows the range of strain magnitudes used in this study.

Frequency was maintained at 1Hz for all the loaded groups.

Table 4.4 Different strain magnitudes used in compression bioreactor for bone tissue engineering studies.

Range	Strain	Frequency	Remark
1. Physiological	2200 $\mu\epsilon$	1 Hz	Physiologically relevant strains experienced by native bone during walking, running, etc. Very few studies have been conducted using physiological strains
2. Supraphysiological	>3000 $\mu\epsilon$ (8800 $\mu\epsilon$, 11000 $\mu\epsilon$)	1 Hz	Much higher than usually experienced by native bone, used in a number of <i>in vitro</i> studies

Results from the strain optimization study clearly showed higher ALP activity in the grafts compressed with 0.22% (physiological) strain in comparison to those under higher strain values (0.88%, 1.8x, $p<0.001$; 1.1%, 1.9x, $p<0.001$) by the end of 14 days culture (Fig 4.9A). Calcium deposition was also found to be higher in the constructs compressed with 0.22% strains in comparison to those under other strains by day 14 ($p>0.05$) (Fig 4.9B).

Fig 4.9 Optimization of strains used on grafts cultured in the compression bioreactor, 3A. ALP assay demonstrated higher levels of ALP activity in the constructs subjected to 0.22% strains when compared to those subjected to 0.88 % (** $p < 0.001$) and 1.1% (** $p < 0.001$) strains, B. Results from calcium assay indicated higher levels of mineralization in the constructs subjected to 0.22% strains when compared to those subjected to other strains by the end of week 2 ($p > 0.05$, n.s: not significant).

4.4 Discussion

A multichamber compression bioreactor system has been designed to apply a range of cyclic compressive loads to scaffolds under sterile conditions. The aim was to study the effect of mechanical loading on cell-seeded macroporous 3D scaffolds for bone tissue engineering. It is well known that bone tissues adapt to mechanical loading and the use of biomechanical forces for *ex vivo* engineering of tissues has been in place since early 1990s [174]. Since then, there have been a number of cell culture systems that have utilized cyclic compressive/tensile strains to mimic the *in vivo* conditions for *in vitro* bone regeneration [69, 114, 120].

Many bioreactor studies for applying cyclic compression for bone tissue engineering have utilized hydrogels as model matrices to elucidate the mechanism of cellular mechanotransduction [114, 115]. These studies have demonstrated the stimulatory effect of compressive loading on the expression of osteogenic genes like osteopontin, TGF- β R1, SMAD-5, etc. Cyclic compression of polymeric grafts like polyurethane and polycaprolactone demonstrated enhanced mineralization and osteogenic gene expression [71, 119, 120]. Previous studies that cyclically compressed scaffolds to induce bone formation have been performed on materials with relatively poor mechanical properties [114, 115, 175]. Other studies used with cyclic compression bioreactor used demineralized bone scaffolds [69, 70] or on scaffolds that lack osteoinductive properties [71, 120]. Some are relatively easy to be compressed and might not suit the needs of an implant for bone tissue that is under constant stress. The final tissue engineered product is expected to possess sufficient mechanical strength and relevant degradation properties to provide support to the defect area till neo-bone formation occurs. It is also advantageous for the scaffolds to have osteoinductive and osteoconductive components that have the capability to recruit osteoprogenitor cells and differentiate them [5]. In our study, we used well-established, clinically relevant PCL-TCP scaffolds seeded with stem cells to elucidate the effects of mechanical loading on these substrates for the first time. The mechanical property of the acellular PCL-TCP scaffolds was assessed to determine the range of strain levels

that could be applied on the scaffold during the cyclic compression studies without disrupting its mechanical integrity. Following analysis of the stress-strain curve, the maximum limit for compressive strain value was kept at 1.1% to represent supraphysiological strains. Cancellous bone has a Young's modulus of 20 – 500 MPa and a compressive strength of 2—12 MPa [176]. Results from the mechanical testing study indicate that the elastic modulus and the compressive strength of the scaffolds used in our study are within the range exhibited by cancellous bone. A strain of 2200 $\mu\epsilon$ was chosen on the basis of optimal physiological strain range as reported by Yang et al. [101] and the frequency mimics the walking frequency observed in humans. We used MSC seeded PCL-TCP scaffolds as relevant engineered tissue constructs and analyzed the effects of compressive strains on cellular differentiation and mineralization.

Previous studies of cyclic loading on 3D cellular constructs for bone tissue engineering have demonstrated the stimulatory effect of mechanical forces on bone formation [116, 117, 119]. However, these systems show limitations in terms of adaptability for parametric optimization studies. As mentioned previously, cells respond to differences in strain magnitude, frequency, duration and even rest periods between loading bouts [62, 69, 117, 177]. Most of the bioreactor systems allow loading of a few samples at a time making it less feasible for conducting statistically relevant studies in a shorter time. Moreover,

systems with capacity for multiple samples hardly provide isolated culture conditions or allow independent control of loading of the samples, making them less suitable for parametric studies. Studies have shown the development of an array of scaled-down, multi-compartment bioreactor systems aid in acceleration of tissue characterization and also help to minimize experimental variations during parametric optimization [81, 178, 179]. In addition to enabling parametric optimization studies, the multi-array configuration could also be potentially used for drug and toxicology studies with disease models [180]. These advantages establish the need to develop bioreactor systems that accommodates parallelization of experiments enabling one to test a wider range of parameters for understanding effects of environmental cues on bone tissue engineering. Our custom-built, multi-chamber compression bioreactor system can apply a range of strains on cellular scaffolds in a semi-high-throughput fashion with independent control of loading and medium condition in each of the chambers. Essentially, the presence of multiple chambers with separate media conditions allow the study and optimization of intrinsic parameters (media components) and extrinsic factors (mechanical cues). Additionally, the multichamber bioreactor system developed in this study has a same chamber for tissue culture and mechanical loading. This design is advantageous as it simplifies the study and prevents any possible contamination that can occur due to transferring of samples thereby saving time and cost in terms of resources. The key constraint with regards to the use of relatively small

chambers in a multichamber setup is in the limited number of scaffolds that can be accommodated in the bioreactor and the need for frequent media change.

From the media optimization studies, loading of the grafts using the bioreactor did not lead to osteogenic differentiation in the absence of osteogenic induction factors. A previous study that analysed the effects of dynamic loading on MSCs seeded on polyurethane scaffolds demonstrated that bouts of loading could produce the same effect as addition of dexamethasone without loading [181]. However, the control media used in their study was supplemented with β -glycerophosphate which is a potent inducer for mineralization alone and in combination with ascorbic acid [182]. Nishimura et al demonstrated similar results where growth media (without osteoinduction factors) did not contribute to osteogenic activity measured by ALP levels in MSC-seeded collagen sheets under both static and dynamic cultivation in a radial flow bioreactor [183]. The results from this clearly showed that the presence of bone inducing factors was necessary for effective osteogenic induction. Having optimized the media conditions for effective osteogenesis, we wanted to test our hypothesis that application of physiologically relevant strains on tissue grafts will enable *in vitro* bone formation. To confirm the same, a strain optimization study was carried out using three different strain magnitudes (0.22%, 0.88% and 1.1%). Osteogenesis measured by ALP levels was found to be much higher in

the constructs loaded with physiological strains than those with higher strains. A previous report demonstrated the effect of different strains on ALP levels in a 3 week study that applied multiple strains (0.1%-0.4%) on rabbit bone explants [69]. In their study, while they demonstrated highest ALP activity in the explants loaded with physiologically relevant strain of 0.2% in comparison to their controls, it was a 1.3-fold increase by the end of 2 weeks. In our study, cyclic loading with physiological strains led to almost 3-fold increase in the ALP activity in compressed constructs. Increased osteogenic enzyme activity could have resulted from activation of signalling pathways and upregulation of osteogenic gene expression upon mechanical loading. Further, calcium levels were also found to be higher in the groups with physiological loading. Zong ming et al had demonstrated a similar increase in the tissue mineral density of bone explants loaded with 0.2% and 0.1%, which are physiologically relevant strains, in comparison to static controls by the third week of loading.

4.5 Conclusion

In summary, we successfully developed a multichamber bioreactor system to apply cyclic compressive loads on engineering bone tissues for bone tissue engineering application. The developed system also allowed us to culture engineering bone tissues made from clinically relevant macroporous scaffolds and MSC under an optimized biochemical environment. This system offered a high-throughput platform to perform mechanical loading studies on multiple scaffolds with varying regimens simultaneously. In line with our first hypothesis, the superiority of physiological strains on functionally relevant engineered tissues over higher strains has been demonstrated. Having determined the optimal conditions to induce osteogenic differentiation, studies were conducted to evaluate the temporal effects of application of cyclic compression for longer durations on cellular viability, osteogenic gene expression and mineralization of the stem cell-loaded PCL-TCP scaffolds.

5. Evaluation of Cyclic Compression Bioreactor for Bone Tissue Engineering[#]

[#] Partially published in Ravichandran et al, 2016, Journal of Biomedical Materials Research, Part B: Applied biomaterials

5.1 Introduction

Having established the superiority of physiological compressive strains in *in vitro* osteogenesis in the previous chapter, Chapter 5 is motivated to analyze the effects of application of long term physiological loading on bio-active PCL-TCP scaffolds seeded with MSCs on cellular proliferation, osteogenic gene expression and mineralized matrix deposition. Application of appropriate mechanical forces on premature tissues has been shown to aid in acquiring relevant structural and functional tissue characteristics [54]. Therefore, it becomes important to identify the specific biomechanical forces that are experienced by habitually loaded tissues and evaluate their efficacy to direct *in vitro* tissue morphogenesis. Although current mechanical loading systems provide cues, the experimental designs were not appropriate. We hypothesized that the application of relevant cyclic compressive strains on premature tissue grafts will result in accelerated generation and maturation of bone tissue grafts. This study could also aid in understanding and projecting the regeneration cascade of tissue engineered bone implanted *in vivo* under physiologically loaded conditions.

Physiological, cyclic compressive strain (1 Hz) of 0.22% was applied to the cellularized scaffolds using the multichamber compression bioreactor system for a period of 4 weeks. Cyclic compression strains led to differential expression of osteogenic genes. Osteogenic enzyme activity

and levels of mineralization were found to be elevated within two weeks of mechanical loading. This study suggested that the direct application of physiological mechanical strain on tissue constructs could lead to accelerated maturation of bone tissue grafts. Poor maintenance of cellular viability upon cyclic loading led us to introduce a pre-static culture period to check if it aided in better cellular adhesion and reduced cellular loss upon mechanical loading. Further, the oxygen levels inside the multichamber bioreactor system were measured using a non-invasive sensing modality to assess the rate of oxygen consumption in order to gauge the availability of oxygen in the chamber media for the cells in one week of bioreactor culture.

5.2 Experimental approach

Scaffolds were harvested on days 0, 7, 14, 21 and 28 and analyzed for cellular viability, mineralization, osteogenesis and gene expression (Fig 5.1). Scaffolds from Day 0 were harvested 24 hours after seeding and just before loading and therefore would act as static controls from day 0. Tissue grafts were compressed for a period of 4 weeks at 0.22% strain and a frequency of 1 Hz with 4 hours of compression every day (Fig 5.2).

Fig 5.1 Experimental workflow for long term compression study. Schematic showing the experimental workflow starting from cellular isolation, expansion and seeding on PCL-TCP scaffolds to bioreactor culture for 4 weeks and analysis of the grafts for cellular proliferation and osteogenic differentiation at given time points.

Fig 5.2 Representative cyclic compression regime, Scaffolds were compressed for 4 h/day followed by 20 hours of rest; magnified look of the dotted lines illustrate the displacement data for a 0.22% strain at 1 Hz frequency for 4 h.

5.3 Results

5.3.1 Cyclic compression led to upregulation of osteogenic gene expression and increased osteogenic differentiation

5.3.1.1 Cellular adhesion and viability

Scaffolds were divided into top, middle and bottom sections to study cellular adhesion and viability in different segments of the scaffold. Phase contrast microscopic images of the cellular scaffolds demonstrated that the fibrin glue with cells occupied the scaffold pores extensively in 24 hours after cell seeding (Fig 5.3). By day 7, the regions occupied by fibrin receded towards the scaffold struts in the compressed scaffolds, contrary to what was observed in static scaffolds (Fig 5.3).

Fig 5.3 Scaffolds were sectioned into top, middle and bottom regions to analyze the cellular adhesion and viability of MSCs on the PCL-TCP scaffolds, phase contrast light microscopy (PCLM) images show the scaffold pores being occupied by cells in fibrin matrix within 24 hours (Scale bar: 200 μ m). Day 7 PCLM images showing the rough outline of fibrin occupied regions within the scaffold pores clearly indicate a decrease in the fibrin occupancy of the loaded scaffolds in comparison to the controls.

Live/dead imaging demonstrated comparable viability of cells on both the compressed and static scaffolds on day 7 (Fig 5.4). Results of DNA quantification using Picogreen assay demonstrated that the cells were able to maintain cell numbers for 28 days (Fig 5.5). A general decreasing trend was observed in the DNA quantities assessed using the Picogreen assay under both static and compression conditions.

Fig 5.4 Cellular viability studies using FDA/PI staining and fluorescence microscopy show mesenchymal morphology of the cells in the fibrin gels in the pores of PCL-TCP scaffolds within 24 hours (Scale bar: 600 μ m). By day 7, we noticed a receding fibrin in the loaded conditions when compared to the static controls.

Fig 5.5 DNA quantification of cyclically loaded samples vs static samples using Picogreen assay revealed a decreasing trend in the DNA quantities for both static and loaded samples.

5.3.1.2 Gene Expression upon cyclic loading

To evaluate the effect of cyclic compression on osteogenic gene expression of MSCs, cell lysates were analyzed using qPCR. Genes that are related to osteogenic differentiation were found to be upregulated upon mechanical loading (Fig 5.6). A 1.6-fold increase was observed in the expression of Runx2 in the loaded scaffolds, while a 2.2x and 2.6x increase was observed in the expression of BMP-4 and BMP-6 respectively (n=2) in comparison to static cultures. TGFB1 showed an increasing trend with time in the loaded groups.

Fig 5.6 Stimulatory effect of cyclic compressive loading was demonstrated by the regulation of genes that are associated with osteogenic differentiation- Runx2, TGFB1, BMP-4 and BMP-6. Cells in the cyclically loaded scaffolds show an increased expression of Runx-2, BMP-4, and 6 in the first week of loading when compared to the static counterparts (**p<0.005, *p<0.05). TGF- β 1 also shows an increasing trend with time under loaded conditions (p>0.05).

Cyclic compression also resulted in upregulation of classic markers of osteogenesis including osteonectin and COL1A1 on day 7, and osteocalcin on day 14 when compared to the respective statically cultured constructs (Fig 5.7, osteonectin (day 7): 1.9x, $p<0.05$; COL1A1 (day 7): 1.6x, $p<0.05$; osteocalcin (day 14): 1.9x, $p<0.05$).

Fig. 5.7 Stimulatory effect of cyclic compressive loading was demonstrated by the regulation of osteogenic genes- Osteonectin, Osteocalcin and COL1A1. Cells in the loaded scaffolds show an increased expression of Osteonectin and COL1A1 in the first week of loading and Osteocalcin in the second week of loading when compared to the static counterparts (* $p<0.05$).

5.3.1.3 Osteogenesis and mineralization

Osteogenesis was quantitatively analyzed by measuring the activity of ALP, an early osteoblastic marker. Loading led to a 3.76x increase in the ALP levels in comparison to its static control ($n=3$, $p<0.05$). ALP levels of

the compressed scaffolds gradually elevated in the loaded samples and peaked around day 14 and then declined in the later time points (Fig 5.8). A clear peak of ALP could not be found for the static samples.

Fig 5.8 Cyclic compression led to higher osteogenesis when compared to its static controls. A. Cyclically compressed scaffolds showed 3.76 x higher ALP activity on day 14 as compared to the scaffolds cultured under static conditions (** $p < 0.001$).

Mineralization was observed by measuring the levels of calcium and the results from calcium assay demonstrated a 2-fold increase in the calcium levels of loaded scaffolds by the second week in comparison to the unloaded scaffolds ($n=3$, $p < 0.05$). Calcium levels saturated in the loaded samples beyond week 2 and the static scaffolds reached similar levels of calcium by the end of the 4 week study (Fig 5.9). Cyclic loading resulted in extensive matrix deposition by the end of week 2 in comparison to the unloaded scaffolds, and static controls demonstrated similar levels of deposition only by the end of 4 weeks (Fig 5.10).

Fig 5.9 Mineral deposition in the loaded samples showed calcium quantities peaking around day 14 as compared to the static samples that reached similar levels of calcium deposition only by the end of the 4 week study. Calcium deposits were two-fold higher in the loaded samples on day 14 (** $p < 0.001$).

Fig 5.10 To visualize the matrix secretion, the scaffolds were sectioned to obtain the top view and the core view. SEM images of fixed, day 14 loaded scaffolds showed extensive matrix secretion on the struts in comparison to the static scaffolds.

5.3.2 Pre-bioreactor static culture to improve cell viability in compression bioreactor

Results from the previous study showed poor cellular viabilities on scaffolds under bioreactor culture. To improve cell numbers on the scaffolds, the scaffolds were maintained under static culture conditions for one week before application of mechanical loading. Results

suggested that one week of pre-bioreactor static led to improved cellular viability and adhesion (Fig 5.11).

Fig 5.11 Maintenance of cellular viability following pre-bioreactor static culture, A. Fluorescence live images of FDA/PI staining show higher cell numbers and better distribution of cells on scaffolds under both loaded and static conditions and there is no observable cell loss under cyclic loading (Scale bar: 250 μ m), B. Quantification of DNA showed that there were no significant differences in the cell numbers till the second week between the static and loaded scaffolds indicating that a pre-static culture helped in better cell attachment before mechanical loading.

Better matrix deposition was also observed on scaffolds on both static and loaded conditions after pre-bioreactor static culture of one week (Fig 5.12).

Fig 5.12 Matrix deposition on scaffolds following pre-bioreactor static culture was observed in both static and loaded scaffolds due to a pre-bioreactor static culture of one week.

5.3.3 Non-invasive oxygen sensing in the cyclic compression bioreactor

The dissolved oxygen levels were monitored every day for a duration of one week in static bioreactor chamber loaded with six cellular grafts. Results from the study demonstrated rapid oxygen consumption by the cells resulting in the dissolved oxygen levels in chamber media dropping to almost 55% of the initial levels in freshly prepared media (DO: 20.8 ± 0.1 % to 11.4 ± 0.1 %).

Fig 5.13 Non-invasive sensing of oxygen levels within the bioreactor chamber, A. Oxygen spot sensor adhered to the inner wall of bioreactor chamber and oxygen level detected using optic fibre. The plot depicts the consumption of the dissolved O₂ levels in chamber media in the first week of culture of cellular grafts under static conditions within the multichamber cyclic compression bioreactor.

5.4 Discussion

In recent years, research studies on bone tissue regeneration have shown intensive interest in the recapitulation of native developmental biology and physiological mechanisms to engineer functional tissues [168, 184, 185]. Bone tissue is metabolically active and can adapt to applied mechanical stresses by remodeling [186]. The adaptation of bone to mechanical loading is primarily achieved by sensing the applied range of strains which is followed by recruitment of osteoblasts and osteoclasts to deposit and resorb bone respectively [99]. Consequently, many studies have focused on the osteogenic effects of *in vitro* mechanical stimulation on osteogenic cells including osteocytes, osteoblasts and osteoprogenitor cells [46, 111, 112]. We had

hypothesized that the application of mechanical strains on stiffer, relevant tissue constructs could aid in the generation of highly-mineralized tissues. Hence, we developed compression bioreactor system and evaluated the effects of cyclic compressive strains on the maturation of engineered bone grafts.

From our results, Runx2 was highly expressed (1.66x) upon cyclic compression along with other key genes associated with osteogenesis, BMP (4, 6) at week 1. Expression of growth factors like BMP and TGF- β was upregulated for the loaded scaffolds as reported by similar studies [69, 115] and these factors are known to activate signalling pathways leading to osteoblastogenesis. BMPs are autocrine factors that act through SMAD signalling pathway to activate expression of Runx2 gene (Fig 5.14) [187]. Runx2 is a master regulator of osteogenesis and is responsible for the downstream activation of osteogenic genes including ALP, COL1A1, osteocalcin and osteonectin. Further, Runx2 has been shown to be upregulated under mechanical stimulation on bovine sternum explants [188] as well as 3D PCL scaffolds seeded with osteoblasts [119]. In a similar study, Gharibi et al had shown an upregulation of osteogenic gene expression upon application of physiologically relevant cyclic loading on MSCs seeded on CaP scaffolds [168]. In their study, they had reported a 1.8-fold increase in the Runx2 expression after 2 hours of loading followed by 22 hours of rest. The temporal expression of BMPs and early osteogenic genes

follow the profiles expressed in the bone regeneration cascade (Fig 5.15) [187].

Fig 5.14 BMP signalling pathway leading activation of Runx2 and other osteogenic genes resulting in bone formation. Reproduced from Chapter 55 Bone Regeneration, Principles of Tissue Engineering [187].

Fig 5.15 Temporal and dosing profile of key biological factors in the bone regeneration cascade. Reproduced from Chapter 55, Principles of Tissue Engineering [187]. Fibroblast Growth Factor (FGF), Transforming Growth Factor b (TGF-b), and Platelet-Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Bone Morphogenetic Proteins (BMP), Interleukins (IL), Endodermal Growth Factor (EGF), Parathyroid Hormone (PTH), CT (Cytokines).

From our results, Osteonectin and COL1A1 were highly expressed upon cyclic compression in the first week, while Osteocalcin expression was found to be almost doubled in the loaded groups in the second week in comparison to static cultures. Osteocalcin is an important marker of osteogenic differentiation of MSCs and has been shown to be upregulated under mechanical stimulation on bovine sternum explants [188] as well as 3D Polyurethane scaffolds seeded with osteoblasts [119, 120].

To further confirm the mechanostimulatory effects, we analyzed the phenotypic expression of osteogenic differentiation by analyzing the levels of ALP and calcium deposition. In our study, cyclic loading with physiological strains led to almost 4-fold increase in the ALP activity in compressed constructs. Functionally, calcium deposition at week 2 was 1.96-fold higher in the mechanical stimulation group as compared to their controls. Previous 3D mechanical loading studies had also demonstrated a similar increase in the mineralization levels after compression [116, 120]. In a frequency optimization study, Tanaka had demonstrated an increase in osteoblastic calcification in collagen sponges upon cyclic compression of 0.2% strain and a frequency of 2 Hz over a period of two weeks [88]. The study demonstrated similar levels of normalized calcium levels by the end of two weeks ($\sim 1.5 \text{ mg/cm}^3$), although the fold increase with respect to unloaded constructs

was 1.5-fold in comparison to the 2-fold increase observed in our study. In another study by Sittichockechaiwut et al appreciable changes in the levels of calcium (4-fold increase) were observed only after 20 days of 5%, 1 Hz cyclic loading on osteoblastic cells in polyurethane scaffolds [120]. We observed earlier onset of mineralization and higher levels of calcium with our physiological strain on relevant constructs. However, it is difficult to compare the above studies that were performed with different types of scaffolds, cells and mechanical loading systems which in turn may have caused discrepancies in the observed results.

With respect to cellular viability, the results revealed a reduction from the initial cell numbers in bioreactor culture with and without mechanical stimulation. This loss of cells could be attributed to poor mass transfer result from lack of dynamic culture to improve nutrient transfer through scaffolds [78]. One possible explanation could be that of lineage commitment and osteogenic differentiation in the loaded scaffolds. A similar loss in cell viability was reported by Kearney et al. in a tensile loading study on MSCs and suggested that the onset of tissue-specific development could have resulted in reduced cellular proliferation [112]. This entry into cellular differentiation marks the end of proliferative cycle which is also supported by the upregulation of gene expression of TGF- β 1, a marker for cellular differentiation, in the loaded scaffolds with time.

Our results also indicated that application of mechanical strain led to a further decrease in the number of cells in the first week while the viability trend remains similar for both the compressed and static control groups after the first week. From PCLM images, we observed that the fibrin matrix was prematurely removed from the scaffold possibly due to effect from the applied mechanical compression resulting in cellular loss in the first week for loaded scaffolds. Such a loss of cell numbers was observed in a similar dynamic mechanical loading study by Baumgartner, where PLGA/aCaP electrospun scaffolds seeded with adipose derived stem cells were cyclically compressed for a period of 9 days [189]. They attributed the reduction in cellular densities to insufficient matrix to support the cells or too strong a compression regime. Hence, we conducted a study to include a pre-bioreactor well plate culture to ensure more stable attachment of cells on to the scaffolds before compression. The results from this study showed much better cellular attachment in comparison to the previous studies and cell numbers were maintained on both loaded and statically cultured scaffolds. Although, loading associated cellular loss from the scaffolds was not observed here, cellular proliferation seemed to saturate by one week of bioreactor culture. Hence, we wanted to ascertain if the media provided enough nutrients required for cellular proliferation throughout the week using the oxygen spot sensors that provided a simple, non-destructive way to analyse the culture O_2 levels in the bioreactor chamber. The results clearly showed rapid consumption of oxygen which

could probably explain the slower proliferation rates observed in both static and loaded cultures in the multichamber bioreactor system. Utilization of cyclic compression for accelerated mineralization and osteogenesis has been validated successfully with the multichamber bioreactor system. Although we have been able to achieve accelerated maturation of grafts, cellular proliferation and uniform cellular distribution was not achieved by application of mechanical stimulation. Efficient tissue regeneration is also dependent on uniform cellular distributions on scaffolds [11]. This requires the use of dynamic fluid flow regimes to improve mass transport through scaffolds which is explored in combination with mechanical stimulation in the next chapter.

5.5 Conclusion

In summary, we evaluated the multi-chamber compression bioreactor system for its ability to induce osteogenic differentiation by application of physiological strains on 3D developing tissue grafts. We have demonstrated the potential of mechanical stimulation to upregulate osteogenic gene expression in premature engineered tissues. Further, we have shown increased production of mineralized matrix on the tissue constructs as a response to physiological loads within 2 weeks of bioreactor culture. In line with our hypothesis, the results from this study provide proof of the significant role played by bio-mimicking mechanical forces on the maturation of engineered tissue constructs. Following this, it would be important to develop a holistic strategy to produce effective bone tissue grafts with higher cell numbers and homogeneous cellular distribution, which in turn, could aid in promoting better mechanical response. As a next step, the use of dynamic fluid flow conditions was proposed, in combination with mechanical stimulation, to generate cellularized and mineralized bone grafts.

6. Combination of Cyclic Compression and Biaxial Rotation Bioreactor for Engineering Bone Grafts

6.1 Introduction

We have successfully validated the use of physiologically relevant cyclic compression for inducing accelerated mineralization of 3D tissue grafts in Chapter 5. Nonetheless, efficient tissue regeneration also depends on uniform cellular distributions on clinically sized scaffolds along with application of physiologically relevant mechanical stimuli [11]. It is well known that dynamic culture systems are necessary to enhance mass transport within critical sized grafts for uniform cellular distribution [58]. In addition, fluid flow is one of the key biomechanical forces sensed at a cellular level in native bone that triggers bone remodelling pathways [172]. During physiological loading, at a macroscopic level, bone tissue experiences complex strain patterns with varying proportions of compression, tension and torsion, and at a cellular level, they experience shear forces due to the interstitial fluid flow [190]. Understandably, several dynamic culture systems have focused on fluid flow systems in the past, especially perfusion bioreactors for engineering bone tissues [58]. Our group had demonstrated the use of a biaxial bioreactor for the generation of cellular scaffolds with superior cellular viabilities and enhanced osteogenic potential [24]. In a head-to-head comparison with other commercially available systems, Zhang et al. showed homogeneous cellular distribution and superior osteogenic induction in the scaffolds cultured in the biaxial bioreactor system [125]. In this chapter, we propose to develop and evaluate a multimodal

bioreactor system that combines the advantageous biaxial rotation with physiologically relevant cyclic compressive stimuli.

The multimodal bioreactor system can rotate in the biaxial mode and also simultaneously apply cyclic compression on scaffolds. The modified system allowed restricted rotation about the two axes due to the additional micromanipulator attached for mechanical loading. Hence we conducted a preliminary study to examine the effectiveness of partial biaxial rotation to generate highly cellularized tissue grafts under both non-induction and osteogenic induction media conditions. Having validated the advantage of this mode over static cultures for producing highly cellularized grafts, we performed a comparative study of the independent and combined effects of cyclic compression and biaxial rotation in the multimodal bioreactor system. The objective of this study was to mimic the mechanical microenvironment of native bone by application of compressive loading and the shear forces within the biaxial bioreactor system to increase the efficiency of *in vitro* bone regeneration. We also integrated sensors for dissolved oxygen and pH within the bioreactor system for real-time, non-invasive sensing. Mesenchymal Stem Cells (MSC) seeded Polycaprolactone- β Tricalcium Phosphate (PCL-TCP) scaffolds were cultured in the multimodal bioreactor for a period of 2 weeks under four different modes – static, cyclic compression, biaxial rotation and multimodal (combination of cyclic compression and biaxial rotation).

We hypothesized that the combination of cyclic mechanical stimulation and biaxial rotation will promote cellular proliferation, homogeneous distribution and osteogenic differentiation. Effects of the individual and combined bioreactor modes on cellular proliferation, stress fibre distribution, osteogenic gene expression and calcium deposition were analysed.

6.2 Design and setting up of multimodal bioreactor system

The multimodal bioreactor system consists of a biaxial rotation chamber (1 L) equipped with two shafts to culture the bone grafts inside a media reservoir (500 mL), gas-permeable tubings to allow media flow between the chamber and reservoir, a peristaltic pump for media perfusion and a micromanipulator to apply cyclic compression on scaffolds (Fig 6.1). The parameters for media perfusion, biaxial rotation and compression are controlled by an external controller unit. The bioreactor lid possesses two shafts to anchor the scaffolds and each shaft can hold up to 6 scaffolds. The bioreactor set up can be transferred to the incubator and maintained at optimum humidity conditions, 37°C and 5% CO₂.

Fig 6.1 The multimodal bioreactor system comprises a 1L chamber anchors the 3D scaffolds and can rotate about two axes simultaneously (x-axis and z-axis), the micromanipulators apply cyclic compressive stimuli to the scaffolds and peristaltic pump (not shown in the fig) allows media exchange between the chamber and the media reservoir.

6.3 Results

6.3.1 Optimization of media

Owing to the presence of a compression motor, bioreactor runs in the modified bioreactor were restricted to partial biaxial rotation (x-axis: 90° , z-axis: 90°). A preliminary study was conducted to characterize the role of restricted biaxial rotation on cellular proliferation and differentiation under both basal culture and osteogenic induction conditions.

6.3.1.1 Bioreactor culture without osteogenic induction factors

Partial biaxial rotation bioreactor culture in the absence of osteogenic induction factors (Dexamethasone + ascorbic acid + β -glycerophosphate) resulted in improved cellular distribution on the scaffold (Fig 6.2A). Quantitative results showed a 1.4-fold increase in the levels of cell numbers by the end of 2 weeks in comparison to static cultures (Fig 6.2B).

Fig 6.2 Cellular proliferation under biaxial rotation conditions, A. Increased cell numbers and improved cellular distribution on scaffolds can be observed on the grafts cultured in culture media under biaxial rotation in comparison to statically cultured grafts (Scale bar: 600 μ m)., B. Quantitative results showed that biaxial rotation under culture medium conditions led to generation of highly cellularized grafts in two weeks when compared with its static counterparts.

In the absence of osteogenic induction, biaxial rotation does not contribute to cellular differentiation as can be seen in the results from calcium assay (Fig 6.3)

Fig 6.3 Mineralization of grafts under biaxial rotation measured using calcium assay showing no significant differences in the levels of mineralization between static and biaxial rotation samples over a period of 2 weeks in the absence of osteogenic induction factors.

6.3.1.2 Bioreactor culture with osteogenic induction factors

Biaxial rotation bioreactor culture in the presence of osteogenic induction factors resulted in an anticipated increase in the cell numbers (Fig 6.4) and mineralization (Fig 6.5).

Fig 6.4 Cellular proliferation under biaxial rotation conditions, phase contrast images show cell-fibrin occupied pores of the scaffolds with mineral deposits (black). Under fluorescence, scaffolds can be clearly seen to be highly cellularized (live: green, dead: red) under biaxial rotation in the presence of osteogenic induction factors (Scale bar: 250 µm).

Fig 6.5 Mineralization of grafts under biaxial rotation, A. Calcium assay showing higher levels of mineralization in the first week of culture under biaxial rotation conditions in comparison to static cultures in the presence of osteogenic induction factors. B. Images of Von kossa staining of grafts harvested at the end of 2 weeks of biaxial rotation.

6.3.2 Effect of combination of cyclic compression and biaxial rotation

In this study, the bioreactor system was integrated with sensors to monitor culture conditions. Oxygen spot sensors (SP-PSt3, Presens) were adhered to the inner wall of the bioreactor chamber using silicone glue and left to dry overnight. Following sensor calibration as per the manual instructions, the chamber was sterilized along with all the components that come in contact with the media using steam sterilization (121°C, 30 min). The premature grafts were transferred to the anchor shaft in a sterile manner and separated by metallic spacers. The flow-through sterile pH sensor (FTC-SU-HP8, Presens) was placed in the media loop at a location that allowed easy access to the detector.

Fig 6.6 Multimodal bioreactor schematic showing the multimodal bioreactor system used in this study for generation of functional bone grafts integrated with sensors for monitoring O_2 and pH.

The different modes used in this study are listed in Table 6.1. Static mode means that bone grafts were cultured statically in the bioreactor chamber with only media perfusion using the peristaltic pump (Speed: 5 rpm). This acted as the baseline for all the modes. Cyclic compression would include compression at 0.22%, 1 Hz for 4 hours/day. Biaxial rotation would involve chamber rotation about two axes (x-axis and z-axis) at 5 rpm and an angle of rotation at 90°. The final mode would be the multimodal mode which is a combination of biaxial rotation and cyclic compression. The dissolved oxygen levels (%) in the chamber media were read out non-invasively from the four sensor spots using transmitters by halting the system twice a day. The pH of the media was

detected from the flow-through pH sensor twice a day in a non-invasive manner.

Table 6.1 Four different modes of bioreactor used with multimodal bioreactor system.

Bioreactor mode	Perfusion (5 rpm)	Biaxial rotation (X-axis: 5 rpm, Y-axis: 5 rpm)	Cyclic compression (0.22%, 1 Hz)
Static	✓		
Cyclic compression	✓		✓
Biaxial rotation	✓	✓	
Multimodal	✓	✓	✓

6.3.2.1 Non-invasive sensing of Dissolved Oxygen (DO) and pH

The culture parameters (pH and dissolved oxygen) were determined online in a non-invasive manner over the 2 weeks of bioreactor culture. The pH was maintained at an average of 7.2 over the entire culture period (Fig 6.7). The dissolved oxygen was maintained throughout the culture period under both static and multimodal conditions (Fig 6.7).

Fig 6.7 Non-invasive sensing of pH and DO in bioreactor. The plot depicts the maintenance of pH (left y-axis) and dissolved O₂ levels (right y-axis) over two weeks under static and multimodal bioreactor conditions measured using non-invasive sensing modalities.

6.3.2.2 Cellular viability and proliferation

The fluorescence images projected higher cell viability and cellular occupancy in the scaffolds under multimodal conditions in comparison to the scaffold cultured under static conditions (Fig 6.8).

Fig 6.8 Cellular viability under static and multimodal bioreactor modes. Fluorescence microscopy and phase contrast images of FDA/PI stained cells showing cell viability and distribution on day 7 (live cells: green, dead cells: red) under both planar and core sections (Scale bar: 250 μm).

In line with the qualitative analyses, results from the total protein assay and DNA quantification suggested that biaxial rotation mode led to highest cell numbers in the multimodal system (Fig 6.9). Within a week of bioreactor culture, cell numbers were found to be 2.3-fold ($p < 0.001$) and 1.8-fold ($p < 0.001$) higher in the biaxial rotation mode and multimodal mode in comparison to the static cultures respectively. In both the weeks, static cultures showed the lowest cell numbers as indicated by the dsDNA quantities.

Fig 6.9 Total protein content and cellular proliferation measured by Bradford protein assay and the total dsDNA quantities found in the lysates of tissues cultured under all the four different bioreactor modes respectively.

F-actin fibers in tissues cultured under static mode are small and lesser in numbers (Fig 6.10). Higher numbers and more prominent fibers were found in the tissues cultured in the multimodal bioreactor. By the second week, fibers were found to be more aligned in the multimodal bioreactor group.

Fig 6.10 F-actin distribution and alignment under static and multimodal modes. Tissues stained with rhodamine-phalloidin (red) to visualize fibre distribution and alignment of F-actin cultured under static and multimodal bioreactor conditions on weeks 1 and 2 (Scale bar: 50 µm).

6.3.2.3 Osteogenic gene expression

Differential expression of genes under different modes of the bioreactor was evaluated using RT-PCR (Fig 6.11). In the first week of bioreactor culture, COL1A1 was upregulated in the groups experiencing cyclic compressive stimuli (cyclic compression: 3x, $p<0.001$; multimodal: 1.9x, $p<0.01$) and ALP was found to be upregulated in individual stimulation groups (biaxial rotation and cyclic compression) in comparison to the static mode. By the end of second week, groups in the combined biaxial rotation and cyclic compression mode showed an upregulation of osteogenic gene expression including Runx2 (8.6x, $p<0.001$), ALP (3.2x, $p<0.001$), COL1A1 (2x, $p<0.001$), osteonectin (2.4x, $p<0.001$) and osteocalcin (10x, $p<0.001$) in comparison to static cultures.

Fig 6.11 Expression levels of genes that are associated with osteogenesis and cellular differentiation – Runx2, ALPL, TGF-B1, COL1A1, Osteonectin and Osteocalcin under the different modes of bioreactor assessed using RT-PCR (n=3, ** p<0.01, *** p<0.001).

6.3.2.4 Qualitative and quantitative analysis of mineralization

Alizarin red and von Kossa staining to visualize calcium and phosphate deposition respectively showed that, in the first 2 weeks of culture, there were visible differences in the levels of calcium and phosphate deposition between the respective modes of bioreactor culture (Fig 6.12, 6.13). The biaxial rotation group and multimodal group showed higher deposition and matrix filled pores in comparison to the poorly cellularized

grafts with empty pores in the static and cyclic compression groups. The side view of the grafts indicate a non-homogeneous distribution with more number of cells on just the top region of all four grafts and hence, higher deposition on the side of initial cell seeding. As expected, the biaxial rotation and multimodal groups exhibit higher deposition of matrix in comparison to the static and cyclic compression groups.

Fig 6.12 Qualitative staining for calcium and phosphate in the tissues: Week 1
Alizarin red staining for calcium shows the distribution of calcium deposits on the tissue as does the Von Kossa staining for the phosphates.

Fig 6.13 Qualitative staining for calcium and phosphate in the tissues: Week 2
Alizarin red staining for calcium shows the distribution of calcium deposits on the tissue as does the Von Kossa staining for the phosphates.

Acellular PCL-TCP scaffolds were cultured in the bioreactor in the four different modes along with cellular ones and the elution of calcium from the tricalcium phosphate component of the scaffold was measured using calcium assay for each of the modes after 1 and 2 weeks of bioreactor culture (Fig 6.14). Acellular scaffolds from first week of static culture prior to shifting in to the bioreactor system, served as the baseline.

Results show that by the first week, the TCP elution was different in each mode.

Fig 6.14 Elution of calcium from the TCP of scaffolds cultured under different modes in the multimodal bioreactor.

Fig 6.15 shows the normalized ALP activity and calcium deposition in the grafts under different bioreactor modes. Though a linear trend was not observed in the ALP enzymatic activity in the first week, an increased ALP activity in the groups with biaxial rotation was observed by week 2 ($p > 0.05$). A week of bioreactor culture resulted in a 1.9x ($p > 0.05$), 5.8x ($p < 0.001$), 5.7x ($p < 0.001$) higher calcium deposition in the cyclic compression mode, biaxial rotation mode and multimodal groups respectively in comparison to the static mode (Fig 6.15B). By the end of week 2, a saturation in the calcium levels in all the groups was noted.

Fig 6.15 Osteogenesis and mineralization under different bioreactor modes, A. Osteogenic differentiation measured by ALP activity of the tissues normalized to total protein levels on day 7 and 14 under different bioreactor modes, B. Mineralization levels of the tissues cultured in all the four modes of bioreactor assessed by evaluating the amount of calcium deposition on the grafts by the end of week 1 and week 2 (n=3, *** p<0.001).

6.3.2.5 Matrix deposition

The matrix deposition on the scaffolds was observed under SEM for all the four bioreactor modes (Fig 6.16). In line with the results from the qualitative analyses of mineralization the scaffolds under biaxial rotation and multimodal cultures showed enhanced deposition of matrix homogeneously on the scaffold struts and the pores (top view and side view). In the core of the scaffolds, little or no deposition of matrix was observed when they were cultured under static or cyclic compression modes. On the other hand, the scaffolds cultured under biaxial rotation and multimodal modes showed moderate matrix deposition.

Fig 6.16 Matrix deposition in the tissue grafts under different bioreactor modes, SEM images of the top view, side view and core views (at 30x, 500x magnifications) show matrix deposition on the scaffolds and the pores of the scaffolds under all four modes of bioreactor culture at the end of week 2.

6.4 Discussion

Recently, there has been an increasing interest in engineering functional tissues using physiological biomimicry [10]. Bioreactors have been proven to be effective platforms that provide biomimicking stimulation conditions for generation of 3D tissues. In addition, bioreactors also allow *in situ* sensing of culture parameters and evaluation of growing

tissues to obtain relevant information and ensure reproducibility of the studies. With respect to engineering bone tissues, mechanical stimuli and fluid flow have been shown to play important roles in promoting cellular proliferation and differentiation. Biaxial rotation bioreactor has been earlier shown to generate highly cellularized and mineralized tissue grafts [24]. Here, we modify the biaxial bioreactor system to apply physiologically relevant mechanical stimulus on MSC seeded 3D bone grafts in addition to culturing them under biaxial rotation conditions. The speeds of rotation about x-axis and z-axis were maintained based on previously optimized studies. The angle of rotation was kept at 90° to allow maximal movement with the additional motor fixed on the chamber that allows application of compressive stimuli. The perfusion rate was maintained across all the four groups (5 rpm) based on previous studies. The oxygen and pH sensors provided a simple, non-destructive way to measure the dissolved oxygen levels in the bioreactor chamber and pH of perfused media respectively. Media perfusion through the gas permeable tubings enabled maintenance of sustainable levels of oxygen for cell culture across the range of modes (static and multimodal) as can be observed from the results (Fig 6.7). No major differences were observed in the chamber oxygen levels between the groups. This suggests that the observed effects on cellular proliferation and differentiation were solely due to the fluid dynamics and/or mechanical stimulation in the respective groups.

Bioreactor experiments have been conducted in the past to study the effects of combination systems that have both fluid flow and mechanical stimulation components to induce cellular proliferation and/or differentiation of 3D premature tissue grafts [71, 78, 113, 127-131]. Few studies have compared the independent and combined effects of the perfusion flow and mechanical stimuli on cellular proliferation and differentiation [71, 127]. In our study, we have combined perfusion, cyclic compression and biaxial rotation for the first time for engineering bone tissues. Here, the perfusion group acted as the static control and the application of biaxial rotation and cyclic compression was hypothesized to stimulate proliferation and osteogenesis respectively. As expected, the biaxial rotation groups (with and without compression) maintained the highest cell numbers in both the weeks of bioreactor culture and no significant differences were observed in the total dsDNA quantities between the biaxial rotation and multimodal modes of bioreactor by the end of two weeks. In a similar study that analysed the independent effects of fluid perfusion and compressive mechanical stimulation, results showed that perfusion groups had a more dominating effect on proliferation in comparison to mechanical stimulation [127]. In the first week of culture, cyclic compression also seems to have induced cellular proliferation in our study (Fig 6.9). Witt et al reported a similar observation in their study where cyclic compression of MSCs on fibrin led to an increased oxygen transport that in turn possibly resulted in improved cellular proliferation [129].

With respect to osteogenic differentiation, combination of biaxial rotation and cyclic compression showed significant upregulation of osteogenic gene expression by the second week. Runx2 is a master regulator gene that is expressed early during osteoblastogenesis. In our study, Runx2 was found to be highly upregulated in the multimodal group in comparison to the other groups by the end of 2 weeks of culture. Jagodzinski et al had demonstrated a similar time-dependent upregulation of Runx2 mRNA in a study that compared effects of mechanical loading and perfusion on proliferation and differentiation of bone marrow stem cell seeded spongia discs [127]. Collagen1 (COL1A1) is another important marker of osteogenic differentiation of MSCs that has been found to be upregulated at gene and protein levels upon application of mechanical loading [69, 117, 120]. In our study, higher expressions of COL1A1 gene was found in the cyclic compression groups (cyclic compression and multimodal) in the first week of culture in comparison to the rest of the groups. These results possibly indicate potential role of physiologically relevant stimuli in a multimodal configuration in inducing differentiation of cells. Further, the upregulated expression of COL1A1 was sustained till the end of the 2 weeks of culture in multimodal conditions unlike the cyclic compression group where the expression levels dropped after day 7. The time-dependent differential gene expression in our study suggests the importance of identifying the timing of application of relevant stimuli.

Future studies, hence, can aim to understand the effects of timing of mechanical stimulation after achieving sufficient cell numbers in an attempt to mimic the developmental biology of humans, where no specific mechanical regulation of tissues is observed till cellular proliferation and organization is complete.

Qualitative analyses of mineralized matrix deposition using staining methods revealed an interesting trend in the distribution of the matrix under different modes of bioreactor culture. As expected, the cell distribution on the static and cyclic compression groups, were concentrated on the cell-seeding face of the scaffold. However, in the biaxial rotation groups, the deposition, though considerably better than the static groups, was not homogeneous and well-distributed throughout the scaffold as previously reported in biaxial rotation studies [24] (Fig 6.12, 6.13). This deviation from previously reported data could possibly be explained by the restricted rotation (partial biaxial rotation) in our study owing to the presence of compression motor that in turn leads to differential fluid dynamics and concomitant effects on tissue growth in scaffolds. Matrix deposition under SEM also showed a similar trend with higher deposits in the biaxial rotation and multimodal groups. Even as the multimodal culture demonstrated an upregulation of osteogenic gene expression and better matrix deposition, the results within the two week study did not reveal a significant anabolic effect on protein level expression and calcium deposition. Future studies with longer durations

could possibly reveal if the enhanced gene expressions are translated to phenotypic changes at later time points. Results from the elution of calcium from the TCP component in acellular scaffolds cultured under the different conditions, showed a decline by week 2. Zhang et al reported an increase in the elution of calcium with time in the biaxial rotation bioreactor culture [20]. In our study, most of the available TCP in the scaffold had eluted out and the trend was maintained across the different bioreactor modes by the end of study. These results are in line with the non-quantified calcium deposits observed in the media reservoir and the chamber (data not shown) for all the four modes of bioreactor culture. In this study, we have also demonstrated the potential advantages of integrating non-invasive sensing modalities with bioreactor culture to sense culture parameters. With respect to the stem cell differentiation, hypoxia has been shown to promote osteogenesis [191, 192]. This sensor integrated bioreactor system also provides a platform that allows real-time sensing of the oxygen levels for future studies that can analyse the effects of the hypoxic conditions combined with dynamic bioreactor conditions for osteogenic differentiation [17].

6.5 Conclusion

We have developed and evaluated a platform to engineer bone tissues using a multimodal bioreactor system that allows application of relevant compressive forces and an advantageous fluid flow regime. In line with our hypothesis, multimodal culture resulted in higher and rapid cellular proliferation and improved matrix deposition. The combined effects of optimal fluid flow conditions and cyclic compression led to osteogenic grafts marked by elevated expressions of osteogenic genes after 2-week culture. In summary, the independent and combined effects of physiologically relevant mechanical loading and biaxial rotation in the multimodal bioreactor was reported, and the potential advantages of using a combined system for generation and maintenance of bone grafts under physiologically relevant conditions was demonstrated.

7. Concluding Remarks and Future Outlook

The thesis began with an aim to study the role of cyclic mechanical forces in engineering bone tissues by undertaking hypothesis-driven research experiments. The developed multichamber bioreactor system enabled optimization of environmental parameters that demonstrated the superiority of physiologically relevant strains in inducing osteogenic differentiation. Cyclic compression with optimized strain values for a period of 4 weeks showed upregulation of osteogenic gene expression in the first week and elevated osteogenic enzyme activity by the end of two weeks in comparison to the static cultures. These results suggested the potential of cyclic compressive loading for generating mineralized tissues. With an aim to generate osteogenic grafts with homogeneous cellular distributions, the multimodal bioreactor system was used as a platform to study the independent and combined effects of fluid flow and cyclic mechanical stimulation on cellular proliferation and osteogenic differentiation of stem cells on 3D bone grafts. The bioreactor system was also integrated with sensors that allow real-time, non-invasive sensing of dissolved oxygen and pH in the bioreactor. Results from the study demonstrated the potential advantages of using the multimodal bioreactor system for generation of bone grafts with high cellularity and osteogenicity. In sum, these results provide proof of the importance of development of physiologically relevant bioreactor platforms for engineering functional bone tissues. There are a few limitations in the multimodal bioreactor study. Firstly, the design of multimodal system allowed only partial rotation of the chamber due to the presence of a

servo motor. This resulted in differential fluid dynamics that possibly led to non-homogeneous deposition of matrix that was observed across the length of the scaffold that was reflected in the results from qualitative analyses of mineralization. This also did not allow us to compare our results with previous studies with the biaxial set up owing to differences in the rotation angles. Looking forward, we are working towards modifying the motor size to allow complete rotation of the multimodal bioreactor chamber. Secondly, the cells used in the study were obtained from human fetal sources and hence not representative of clinically available cells. We propose the use of adult mesenchymal stem cells in future studies with multimodal platforms to extract clinically relevant responses.

Looking ahead, we would like to propose interesting and relevant ideas for future work with the multimodal bioreactor platform. The multimodal bioreactor system offers great scope for studies that could optimize the effect of timing and frequency of application of physiological compressive loading in a combination system which could reveal interesting insights into the biomechanics of *in vitro* tissue development. Firstly, long term studies of 4 weeks should be conducted to analyze multimodal effects on mineralization. Hutmacher had suggested dividing bone tissue engineering into six phases namely scaffold fabrication, cell seeding, growth of premature tissue under dynamic culture, growth of mature tissue under physiological loading, surgical implantation and

finally *in vivo* remodelling [8]. Hence, timing of application of mechanical forces will play a deciding factor in the cellular response and the multimodal platform can be effectively used to understand relevance of timing of application of mechanical stimulation in a combinational set up. In addition to bone tissue engineering, this bioreactor platform can be used to apply cyclic compressive stimuli along with biaxial rotation on 3D premature grafts for engineering other tissues as well. Tissues that experience compressive mechanical forces like cartilage, intervertebral disc, and teeth can also be engineered with this platform with some restrictions on the scaffold design.

Another scope for future studies will include the focus on generation of vascularized bone tissues. Previous studies have utilized dynamic bioreactor platforms to produce vascular structures in bone tissues [17]. Oxygen levels play an influential role in determining fate of stem cell environments and hypoxic conditions have been known to stimulate induction of vasculogenesis. With the non-invasive sensing integrated multimodal platform, future studies can analyze the influence of hypoxic conditions and mechanical forces to generate vascularized bone tissues from EPC/MSC constructs. The sensors could aid in evaluation of DO levels and the platform will enable understanding the role of hypoxic microenvironments in bioreactors for bone tissue engineering with real-time feedback. Adding a new dimension to the existing multimodal bioreactor, we propose the modification of the multimodal bioreactor to

allow application of electromagnetic stimuli for effective generation of bone grafts. To conduct these studies, electroactive scaffolds have already been developed by functionalizing PCL with poly(3,4ethylenedioxythiophene)(PEDOT) [193]. These scaffolds can function as effective platforms that will allow electrical stimulation of cells for bone tissue engineering. Last but not least, the multimodal bioreactor platform can be used in the future for development and maintenance of *in vitro* tissue models to understand biological mechanisms behind physiological conditions and disease progression [113, 194-199]. In addition to these studies, the bioreactor can act as controlled platform for drug testing as well [200-203]. Results from preliminary studies that were conducted in parallel with this thesis, have been presented in Chapter 8 – Annexe.

8. Annexe[#]

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Tissue engineering is made of three basic components – cells, scaffolds and cues [204]. Having evaluated mechanical stimulation as a biomimicking cue for bone tissue engineering, preliminary studies have been conducted in parallel to modify existing scaffold platforms, cellular sources and stimulatory cues to further advance our current understanding of bone tissue engineering. Firstly, we explore the modification of PCL based scaffolds using conducting polymers to make them electroactive. We also explore highly proliferative cellular sources for culturing on scaffolds. Further, we have conducted preliminary studies to explore electromagnetic cues to stimulate osteogenic differentiation of stem cells on scaffolds.

8.1 Scaffolds: Piezoelectric bone

Mechanical loading of bone has been associated with generation of electrical potentials which in turn have been hypothesized to play an important role in bone remodelling since the piezoelectric properties of bone were demonstrated in 1957 by Fukada and Yasuda [205]. Consequently, many *in vitro* and *in vivo* studies have been conducted to introduce exogenous electrical stimuli to emulate the physiological sequence of events leading up to obtaining adaptive response from bone. Bassett et al. reported that mechanical stress in mineralized tissues induces an electrical potential generated by collagen piezoelectricity: areas of bone under compression developed negative potentials, whereas those under tension developed positive potentials

[206, 207]. This was followed by introduction of electromagnetic field as a stimulatory tool to influence bone remodelling [208]. Having surveyed the potential of electrical stimulation in bone remodelling, we proposed combining regenerative properties of PCL based scaffolds with a conducting polymer to develop electroactive scaffolds that allow electrical stimulation of stem cells for bone tissue engineering. In a collaboration with Linköping University, we developed and characterized these electroactive scaffolds and the results have been published [193].

8.1.1 Electroactive scaffolds: Cytocompatibility

The PCL scaffolds were successfully functionalized with poly(3,4-ethylenedioxythiophene)(PEDOT), a conducting polymer using vapour-phase polymerization (Fig 8.1A). Two solutions containing either pyridine or the triblock copolymer were used to fabricate the PEDOT:Tos-coated scaffolds, denoted as Pyr-PEDOT:Tos or PPP-PEDOT:Tos, respectively. Colonization of the uncoated and coated scaffolds was determined using F-actin staining on weeks 1 and 2 (Fig 8.1B). 3D confocal microscopy images were captured for cells growing on the scaffolds struts. The cytocompatibility of the electroactive coating has been demonstrated using alamar blue assay (Fig 8.1C).

Fig 8.1 A. Photographs of the uncoated and coated scaffolds (a-c), B. F-actin staining of mesenchymal stem cell seeded on untreated and coated scaffolds. Z-stack images were taken for cells after 7 and 14 days of proliferation on different scaffolds surfaces. Scale bar: 50 μ m, C. Cellular metabolism on untreated (white bar), and Pyr-PEDOT:tos (squared bars) and PPP-PEDOT:Tos (striped bars) coated PCL scaffolds via AlamarBlue® assay ($p < 0.05$). n.s.: not significant difference [193].

8.1.2 Electroactive scaffolds: Osteogenesis

Further the osteoconductive potential of the coated scaffolds i.e. their ability to direct osteogenic differentiation of stem cells was evaluated. Assessment of alkaline phosphatase enzymatic activity revealed a similar trend on both the coated and uncoated scaffolds (8.2A). In addition, the levels of mineralization on the coated and uncoated scaffolds were also comparable over a period of 3 weeks, proving the potential of coated scaffolds to support osteogenesis of the stem cells (8.2B).

Fig 8.2 A. ALP activity of the stem cells under osteogenic conditions on the uncoated and coated scaffolds, B. Levels of calcium deposition in bone inducing medium on the uncoated and coated PCL scaffolds.

8.2 Cells: Neural crest stem cells

In a collaboration with Prof. Toh Yi-Chin, National University of Singapore, neural crest-derived MSCs (NC-MSC) were investigated as promising cellular sources for conducting bioreactor studies with macroporous scaffolds. Preliminary studies compared the proliferative capacity of these cells on macroporous PCL scaffolds in comparison to the commonly used human bone marrow-derived MSC (BM-MSC). Results demonstrated significantly higher cell numbers in the NC-MSC seeded scaffolds in both culture media and osteogenic media conditions (Fig 8.3). Higher proliferative capacities of these NC-MSCs suggest their utility in accelerated colonization of macroporous scaffolds. Future studies with bioreactor systems could evaluate these NC-MSC based premature grafts for rapid and efficient generation of functional bone grafts.

Fig 8.3 Cellular proliferation of NC-MSC and BM-MSC on PCL scaffolds for a period of 3 weeks.

8.3 Cues: Electromagnetic induction

Electromagnetic stimulation has also been reported to promote stem cell differentiation towards bone forming cells as well as induce mineral deposition [209]. In this study, we aimed to evaluate the effect of combined use of electroactive 3D macroporous scaffolds (hereafter referred to as coated scaffolds) and the electromagnetic stimuli (0.6 mT) to regulate *in vitro* bone formation. Results from the study showed that cellular viability (Fig 8.4A) and proliferation (Fig 8.4B) was not affected significantly between the coated and uncoated scaffolds (PCL scaffolds without conductive polymer coating) with and without electromagnetic stimulation.

Fig 8.4 A. Cellular viability (Scale bar: 250 μ m) and B. Proliferation of stem cells on uncoated and coated scaffolds in culture and bone medium conditions with and without electromagnetic stimulation.

With respect to matrix deposition, both the uncoated and coated scaffolds showed higher matrix deposition upon osteogenic differentiation under bone media conditions (Fig 8.5A). Interestingly, combination of electromagnetic stimulation and osteogenic induction factors in media promoted calcium deposition on both coated and non-coated scaffolds on the third week of stimulation in comparison to the control groups without stimulation ($p>0.05$) (Fig 8.5B).

Fig 8.5 Matrix deposition and mineralization of stem cells on uncoated and coated scaffolds in culture and bone medium conditions with and without electromagnetic stimulation.

Taken together, the results from the preliminary studies demonstrated the cytocompatibility and osteoconductivity of electroactive macroporous scaffolds. Further, a preliminary electromagnetic stimulation study has demonstrated positive effects on bone matrix deposition. Continued studies could enable better understanding of the role of a non-invasive electromagnetic field in inducing bone formation. For future studies, we propose to develop a multifunctional bioreactor in collaboration with a life science research company – Quintech Life Sciences, Singapore that

allows application of non-invasive, stimulatory electromagnetic stimuli within the multimodal bioreactor system to evaluate its potential effects on generation of bone grafts (Fig 8.6).

Fig 8.6 Proposed multifunctional bioreactor system that allows application of mechanical, electrical and magnetic stimulation on stem cells seeded on macroporous scaffolds.

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