SCAFFOLD BASED IN-VITRO OSTEODISTRACTION FOR BONE TISSUE ENGINEERING

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by

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THESIS

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DEDICATION

To my family and all friends I have met in this journey.

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–Always bear in mind that your own resolution to succeed is more important than any one thing- ***A. Lincoln***

ABSTRACT

Engineering of hard tissues such as bone is a challenge because of the limited ability of the body to heal large defects. Current treatment options, such as employing bone sections from the patient or a deceased donor have been utilized for some time now with various degrees of success. However, these methods still exhibit many limitations such as limited supply and the possibility of transmission of disease.

To address the many limitations of bone grafts, synthetic engineered substitutes have been developed and widely researched for nearly three decades. Generally termed scaffolds, these are matrices that serve the purpose of temporary cell structures to guide tissue regeneration. A similar area of research, sometimes referred as in-vivo tissue engineering, is distraction osteogenesis. This is a surgical procedure where the application of gradual traction to two segments of bone results in new tissue formation at their interface and it has been utilized for large bone defect reconstruction after trauma or disease.

This research focused on investigating the novel approach of scaffolds and distraction osteogenesis in synergy. A biocompatible material was seeded with cells induced to the bone forming phenotype and a simulated distraction study was performed to evaluate the ability of cells for in-vitro 3-D mineral deposition. Results of initial experiments carried out using a custom setup indicate that some degree of mineralization was achieved but further improvements are still required.

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CHAPTER 1
INTRODUCTION AND BACKGROUND FOR RESEARCH

1.1 TISSUE ENGINEERING

Engineering of tissues is a concept with roots in the mid to late 80's within the field of regenerative medicine. It stemmed from the need of a multidisciplinary field aiming at the development of living tissues for therapeutic applications. Formally, tissue engineering has been defined as the convergence of previous lines of work in the areas of clinical medicine, physical sciences as well as engineering and their synergistic application "to develop functional tissues that can maintain, restore, or improve damaged organs" [1] for addressing some health issues, particularly the deficit of

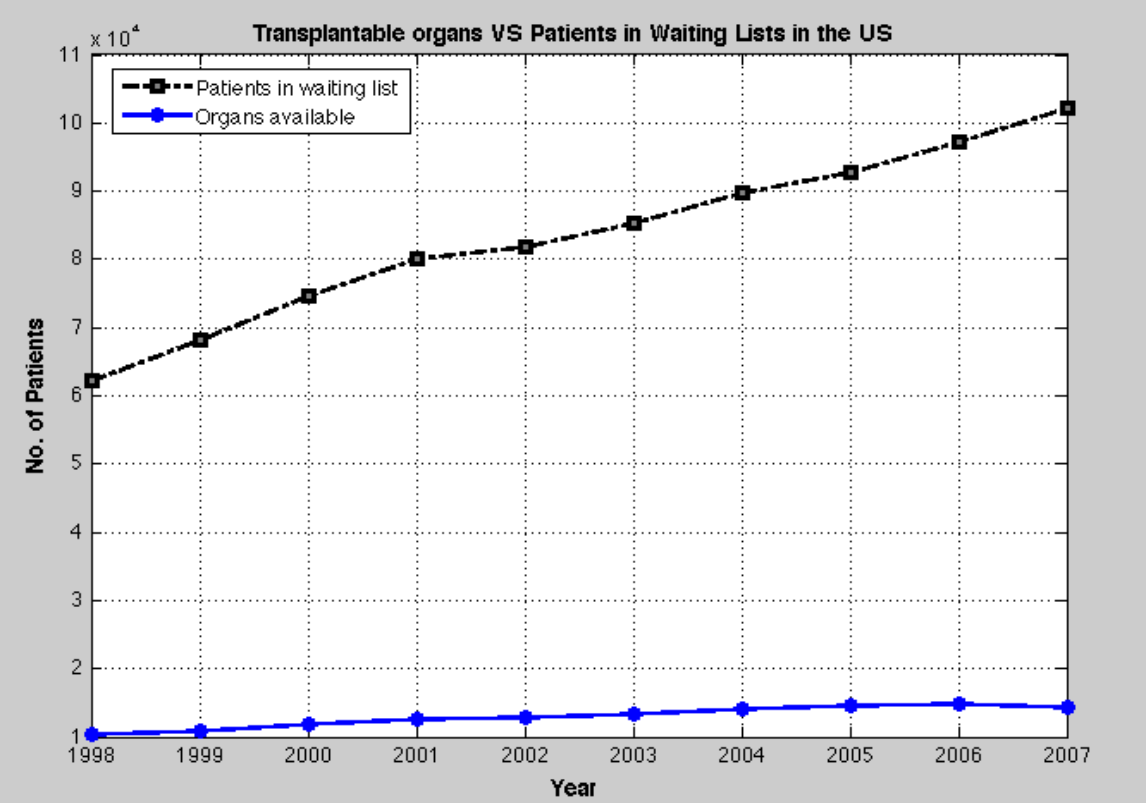

Figure 1 - Number of transplantable organs VS number of waiting patients by year for period 1998-2007

transplantable organs. According to data in the 2008 annual report provided by the Scientific Registry of Transplant Recipients (SRTR) and the Organ Procurement and Transplantation Network (OPTN), throughout the years there has been a marked and increasing gap of organs available for transplantation when compared to the number of awaiting recipients (refer to Figure 1) with at least one fifth of patients dying every year while waiting [2, 3]. As the number between the demand for transplantable organs continues to diverge from the number of organs available, tissue engineering could play a pivotal role providing means of meeting demand and advancing "the health and quality of life for millions of people worldwide" [4].

1.2 BONE TISSUE ENGINEERING

The lack of transplantable tissue takes importance regarding bone. With life expectancy rising in the coming decades, the population is projected to grow older with a consequential increase in musculoeskeletal conditions such as arthritis and osteoporosis as well as recurrence of injuries to bones, joints, ligaments and tendons because of trauma or disease [5, 6]. Research reveals that as much as "500,000 bone replacement procedures were performed in the United States in 2005" [7] and at least 4 million bone grafting surgeries worldwide in the treatment of musculoskeletal conditions in the same time frame with no signs of the tendency to revert [8]. Paired with the increased frequency of musculoeskeletal conditions, costs of treatment are also expected to multiply; in 2004 alone, both direct care expenses and indirect costs–such as

losses in productivity and premature death–were estimated at $849 billion dollars with projected figures to follow this uphill trend [9].

1.3 CURRENT TREATMENTS

Traditionally, musculoeskeletal conditions requiring transplant of bone tissue have been treated with varying degrees of success with the use of bone autografts and allografts. Bone autografts– the main source of which is the patient's iliac crest–have set the standard providing a datum from which to rate other grafting methods. But even though "autologous bone grafts have osteogenic, osteoconductive and osteoinductive properties" [10] providing bone progenitor cells with the ideal environment for triggering de novo tissue growth, they have also been associated with several widely documented complications among which infection, pelvis fracture and instability, gait disturbance, hematoma, abdominal pain, hernia formation, blood loss, tumor transplantation, nerve damage and donor site morbidity are commonly reported [11-13]. In addition, clinical amounts of bone tissue obtained from autologous grafts usually make this option unfeasible [14].

Bone allografts, on the other hand, virtually overcome the problem of limited graft material, and they cause neither pain nor morbidity since their origin is cadaveric bone. Nonetheless, there is a potential risk for bacterial and viral transmission to recipients. Contamination has been documented for viral diseases such as human immunodeficiency virus (HIV) and hepatitis B, bacteria such as *pseudomonas*, *serratia*

marcescens and *staphylococcus*, other diseases that trigger the autoimmune response and even signs of diseases without previous record [5, 15]. Rates for contagion oscillate around 5% but can escalate up to a 50% depending on such factors as the number of people in contact with the tissue after excision, aseptic technique in harvesting as well as quality control at varied stages of the tissue banking [16, 17]. And even though the risk for transmission can be reduced by the introduction of sterilization techniques such as ethylene oxide, gamma radiation and graft freezing, most processes significantly decrease the osteogenic capabilities and devitalize the natural properties of the graft providing yet another reason to disdain this option [5, 18].

1.4 SCAFFOLDS FOR TISSUE ENGINEERING OF BONE

The fact that current grafting techniques, despite their accomplishments, have been unable to solve the shortage of bone tissue for treatment of large bone losses due to illness, trauma or aging, has led to interest in the development of alternatives. One such alternative is the utilization of matrices or scaffolds to support the growth of new bone tissue.

1.4.1 Overview of Scaffold Requirements

The first mention for the application of resorbable matrices with seeded cells as a medium for transplantation is acknowledged to Langer and Vacanti in a paper published in 1988 [19]. Since then, substantial work has been carried out to find the optimal parameters of scaffolds for bone tissue engineering. Although the complete set

of mechanisms occurring in bone formation are not completely understood, several requirements of scaffolds have been identified that aid in the process. Among these needs are scaffold biocompatibility to lessen the potential of immunological or inflammatory response; scaffold material that is degradable [20, 21]; proper scaffold geometry to match defect site; high porosity in the range from 70 to 90% and with uniform distribution [22]; pore size from 150 to roughly 700 μm with high pore interconnectivity for nutrient perfusion [23, 24] ; high surface area with the appropriate chemistry to provide cell attachment; low to nonexistent toxicity to minimize necrosis of cells [25, 26], and the scaffold should provide temporary mechanical support to withstand loads upon implantation [27, 28].

1.4.2 Overview of Materials for Scaffold Construction

Multiple research studies have investigated the use of synthetic and naturally occurring polymers as well as metals and ceramics as scaffold materials for bone tissue engineering. For instance, Xuebin et al. [29] demonstrated the capabilities of type I collagen scaffolds in promoting osteoblasts adhesion, proliferation and differentiation and their potential for osteogenesis both in-vitro and in-vivo. Green [30] confirmed the presence of binding proteins in scaffolds created using marine sponge skeleton and their ability to support growth and differentiation of cultured osteoprogenitor cells. Composite scaffolds using polymeric materials and ceramics such as polycaprolactone-calcium phosphate (PCL-CaP), PCL-calcite (PCL-$CaCO_3$), PCL-hydroxyapatite (PCL-HA) or chitin-nanosilica have been shown to be biocompatible [31] and able to support

bone cell replication, function and viability [20, 25, 32]. Metals like titanium have also been employed; one study using TiH_2 powder and the sacrificed polymeric sponge method produced highly interconnected porous and bioactive scaffold with average porosity of 75% and pore size of 100 to 600 μm to mineralize in simulated body fluid plasma [33]. Nonetheless, most materials investigated fall in the synthetic polymer category. Poly (L-lactic acid) (PLLA), PCL, polyglycolic acid (PGA), poly(D-L-lactic acid) (PLDA) and poly(D,L-lactide-co-glycolide) (PLG) scaffolds have been studied for musculoskeletal tissue engineering applications [34-39].

1.4.3 Scaffold Fabrication Techniques

Several techniques have been used in the fabrication of scaffolds for bone tissue engineering, many of which have been properly reviewed elsewhere [18, 40, 41]. Conventional techniques such as salt leaching, solvent casting, fiber bonding, phase separation and gas foaming have been employed to produce porous scaffolds. Nevertheless, most of these methods lack the ability to create highly interconnected porous 3-D structures, fail at producing uniform pore size, usually leave solvent residues, and in the case of salt leaching, limit the construction to small thickness membranes or wafers [36, 40]. In trying to circumvent many of these limitations, particularly to achieve control on scaffold inner microstructure [42], solid free form fabrication (SFF) techniques were introduced in scaffold fabrication. Three-dimensional printing (3D-P), selective laser sintering (SLS), fuse deposition modeling (FDM) and

stereolithography (SLA) have been successfully utilized to fabricate scaffolds for tissue engineering applications [43-51].

Although some success has been achieved, scaffolds created employing all previously described methods exhibit properties that are still far off from ideal and prevent their widespread utilization in tissue engineering applications. Accurate resolution in all building directions, material over cure, range of materials that can be used due to processing conditions and toxicity of employed materials are issues that still need further improvement [41, 42, 52].

1.5 CELL MECHANOTRANSDUCTION

The term mechanotransduction was coined to refer to the ability of cells to respond to different mechanical stimuli such as pressure, fluid shear, stress, and strain present in their in-vivo environment. This is particularly true for bone cells as the combination of such events trigger some of the steps involved in the process of in-vivo bone remodeling by giving cells cues and signals that guide their behavior. As research suggests [53-57], there is a variety of mechanical signals such as fluid flow, hydrostatic pressure and substrate deformation that are actively involved in regulating the function and activity of bone remodeling cells.

Studies have been performed to improve the understanding of the effects that such mechanical conditions have on bone cells with the aim of better mimicking these factors on further studies and improving bioreactor designs. The osteogenic potential of

mechanical forces on cells has been commonly described. One research study using polyurethane (PU) scaffolds seeded with rapid mineralizing osteoblasts demonstrated a cascade of events occurring when scaffolds were subjected to total 5% 1 Hz cyclic bouts of compressive strain [53]. Increased extracellular matrix (ECM) production with more uniform distribution, higher mineralization, increased collagen type 1 (Coll-I) and calcium contents, as well as a minimum twofold increase in osteoblast mRNA markers such as osteopontin (OPN) and osteocalcin (OCN) were reported. In another study using HA ceramic scaffolds subjected to mechanical compression regimes, the up regulation of Coll-I and fibronectin as well as increased production of vascular endothelial growth factor (VEGF) protein was confirmed [54]. Research carried out by Rath et. al. employing PCL scaffolds that were subjected to levels of 10% 0.5 Hz mechanical compression, correlated up-regulation of both Coll-I and of the hydrolase enzyme alkaline phosphatase (ALP), important during matrix deposition [55].

Other studies using fluid flow perfusion bioreactors to induce shear stress at the interior of seeded scaffolds have found analogous results to direct mechanical compression. The team lead by Bancroft [56], demonstrated that compared with static culture controls, fluid shear "dramatically improved the distribution of extracellular matrix" through scaffolds made of titanium, observing the highest deposition and uniformity at peak levels of flow rate. Their findings also suggested that calcium content was proportional to the rate of flow used. Josef et al. compared calcium phosphate scaffolds subjected to mechanical stimulation by strain and shear flow to

static cultured scaffolds and quantified a 2.5 fold increase in the signaling molecule prostaglandin E_2 (PGE_2)–believed to be involved at various stages of osteoblast differentiation–in the group subjected to shear flow and as much as 80% more PGE_2 release in strained scaffolds [57].

Even though no comprehensive study has yet elucidated every parameter, current research points to the fact that mechanical stimulation provides many of the signals bone remodeling cells receive that regulate their behavior, activity and function.

1.6 CELL CULTURE

Cell culture refers to the growth of cells obtained from animal or plant tissue in an artificial environment favorable to maintain their function. Cells might be obtained by disaggregation of tissue by mechanical or enzymatic action before culture [58]. A major advantage of cell culture is that this process is commonly carried out inside an incubator where controlled conditions on the physiochemical environment are achieved. Control of the pH, osmotic pressure, oxygen (O_2) and carbon dioxide (CO_2) levels and ambient temperature are precisely monitored. Likewise, periodic supplement of the growth medium allows control on the physiological environment, which provides cells with essential nutrients such as amino acids, carbohydrates, vitamins and minerals, growth factors and hormones to lead their growth and differentiation [58]. Antibiotics and fungicides are also utilized to maintain the health of the culture by preventing bacterial and fungi infection or contamination. During the culture period,

medium must be regularly changed to replenish the set of nutrients cells are provided. Also, observations must be made from time to time to assess the general health of the culture.

As a primary culture reaches confluence, cells need to be sub-cultured or passaged into a new container with fresh medium to allow continued expansion of cells. Sub-culture involves detachment of adherent cells to generate a single-cell suspension that could later be re-seeded at reduced concentrations and give rise to new cell cultures. Nonetheless, differentiation potential reduces as the number of cell passages increases and thus, the number of sub-cultures is limited depending on cell type [59]. For the experiments carried out in this research, first passage bone marrow stromal cells (BMSC) were utilized. As can be seen on the next page in Figure 2, cells appear scattered in a T-25 flask after 24 hours of culture. Then, at 96 hours of culture they had reached confluence. At this point, cells need to be detached from the culture surface by enzyme action–trypsin is usually utilized for this process–and seeded in new recipients to continue expansion. The space required for cells to continue growing is also a factor that depends on the cell type cultured. Some cells will only grow as long as there is free surface for them to attach while othercell types–such is the case of osteoblasts–might start forming multi-layer agglomerations.

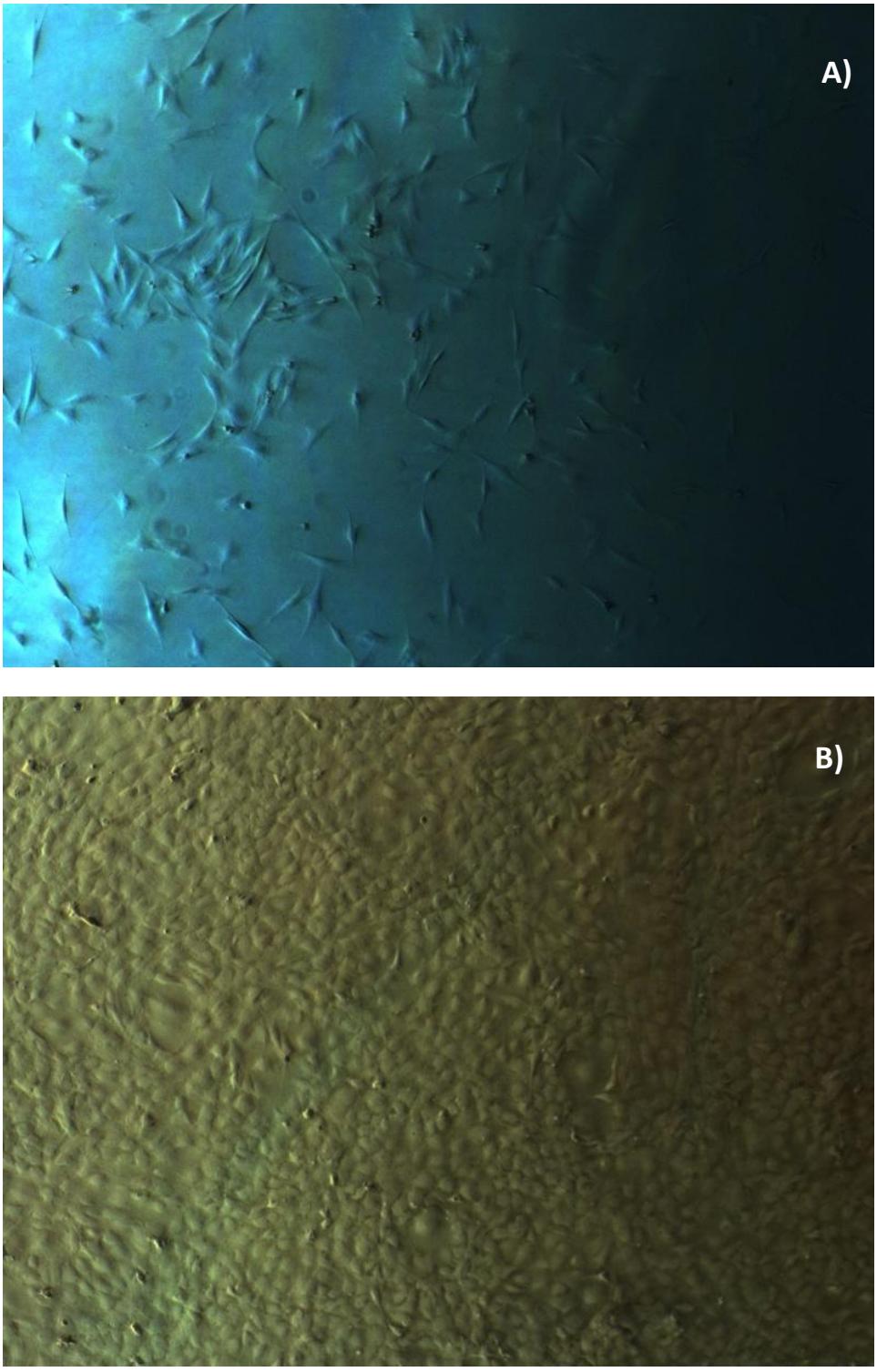

Figure 2 - A) Cells cultured after 24 hours. B) After 96 hours in culture, BMSCs reached confluence.

1.6.1 Bone Marrow Stromal Cells (BMSC)

The bone marrow stroma contains different cell types among which endothelial cells, adipocytes, smooth muscle cells, osteoblasts and stromal fibroblasts are encountered. Even though extensive expansion is needed for a relevant count of cells to be obtained, BMSC can be easily isolated by aspirating bone marrow, most commonly from the iliac crest or femur, and subsequently expanded in-vitro [60, 61].

Experimental evidence suggests that BMSC have a tremendous potential for differentiation into multiple cell lineages making them candidates in prospective clinical therapies [60, 62]. For instance, extended periods of in-vitro culture have demonstrated the capacity of BMSC to form various tissues like cartilage, fibrous tissue, bone, and muscle, indicating their ability to differentiate into various mesenchymal cell lineages [62]. In 2000, Woodbury et al. demonstrated that BMSC even have the capacity to evolve into phenotypes of non-mesenchymal cell types by inducing differentiation of rodent and human BMSC into neuron cells [63].

Aside from their use in treatment of musculoskeletal diseases, BMSC are also believed to be useful in several applications like pre-vascularization of implanted synthetic grafts, as carriers for cell and gene therapy, in the treatment of neurological diseases and for treating conditions of organs such as the heart, lungs, kidney and liver [61, 62, 64].

1.6.2 Medium for Differentiation into Osteoblast Phenotype

The culture of BMSC can be induced towards the osteoblast phenotype. Cues such as dexamethasone, ascorbic acid and beta-glycerophosphate have been shown to cause osteogenic induction of cultured BMSC.

Dexamethasone (Dex) is a synthetic glucocorticoid in the steroid drug family that is commonly used as anti-inflammatory and immunosuppressant [65, 66]. Research indicates that in BMSC cultures, glucocorticoids like Dex have a potent osteogenic differentiation effect. One study demonstrated that continuous Dex treatment of precursor cell lines induced differentiation into bone forming osteoblasts at early days of culture as measured by increases in mineralization and alkaline phosphatase (ALP) activity [67]. Moreover, enhanced in-vitro mineralization has been observed in cultures of human BMSC with Dex along beta-glycerophosphate (β-GP) and ascorbic acid (AA) [68]. A team in Japan led by Mikami [66], exposed osteoblast-like cells to various concentrations of Dex in-vitro and for all effective solutions, they observed accelerated rates of mineralization compared to control cultures.

Ascorbic acid is also identified as a cofactor in cell cultures by intervening in collagen production and promoting proliferation of various mesenchyme-derived cell types. Concentrations as low as 250 μM added to cell cultures are shown to positively modulate proliferation of BMSC while maintaining their differentiation capacity [69]. Similarly, in cultures of human osteoblast-like cells with ascorbic acid 2-phosphate

(Asc2-P) supplemented medium, significant growth stimulation was achieved and the ability of cells to deposit 3-D tissue-like material confirmed [70].

Another biochemical factor utilized as osteogenic inductor in culture medium is beta-glycerophosphate (β-GP), which is added to promote mineral deposition. Coelho and Fernandes [71] cultured human osteoblast-like cells in Dex only, AA only or β-GP only medium, in medium with both AA and β-GP, and in medium with all three reagents. They found that when all three ingredients were present, they collaborated to the positive differentiation and growth of cells. They observed that AA or Dex alone did not influence calcium phosphate deposition–as indicated by von Kossa and Alizarin red staining–but that the presence of β-GP, alone or in combination with the other supplements did, suggesting its central role in mineralization of formed tissue in-vitro [71]. Corroborating the role of this compound on mineralization, Fratzl-Zelman et al. [72] indicated that the absence of β-GP in 4-week old cultures of pre-osteoblastic cells lacked signs of mineralized material.

1.7 DISTRACTION OSTEOGENESIS

Distraction osteogenesis is an orthopedic technique based on the application of mechanical traction between two segments of bone in order to induce new tissue formation at their interface. In 1905, professor Codivilla first described this breakthrough method of applying the traction force directly into the skeletal system and its successful implementation in lengthening lower limbs from 3 to 8 cm in several

patients [73]. However, the procedure did not gain clinical relevance until the work of Dr. Ilizarov [74] who in 1951 formulated the Law of Tension-Stress after noting that "gradual distraction on living tissues created stresses that stimulated and maintained active regeneration" [74] which also led him to the development of a distraction apparatus bearing his name. From then on, distraction devices have been designed and employed in limb lengthening and reconstruction as well as treatment of other conditions such as axis correction and craniofacial malformations [75-77].

The procedure has been successfully employed–despite the risk for complications such as osteomyelitis, fracture and infection–in reconstruction of large defects of long bones in human patients affected by trauma or congenital diseases [78-80]. Similarly, clinical studies have been carried out to correct facial asymmetry on patients with both automatic and manually operated devices with results of over 10 mm of new bone tissue formed [81, 82]. Other studies in animals have investigated different distractor designs and rates of application also with good clinical outcomes [83, 84] demonstrating the potential for bone formation in-vivo.

Bone transport is the basic principle of distraction osteogenesis, which consists of the application of an incremental pull to set apart two bone segments after a larger section is osteotomized (cut in two). The continuous application of traction on one segment of bone, while maintaining a stable fixation on the site, drives cells towards deposition of a new layer of soft tissue that precedes mineralization and ultimately actual bone formation.

Protocols utilized in clinical practice and/or animal models, allocate a recovery or latency period of several days to one week, after the osteotomy is performed, to allow the proper callus or soft tissue to develop. Then, incremental pulls in the range of 0.5 to 1 mm every 24 or 48 hours are applied increasing the volume of soft tissue formed. This pattern is continued until the desired length of new bone has been achieved. To finalize, the patient undergoes a consolidation period, in the scale of weeks, in order for the bone segments to fuse, mineralize and become dense prior to full weight bearing [78-80, 83-86]. The sequence of images shown in Figure 3, taken from [87], depicts the process of distraction osteogenesis in a femur rat model. Radiographs a) through c) were taken at days zero, 10 and 21 after the osteotomy respectively. The next two radiographs were taken 7 and 21 days after distraction and the last after pins removal and bone consolidation.

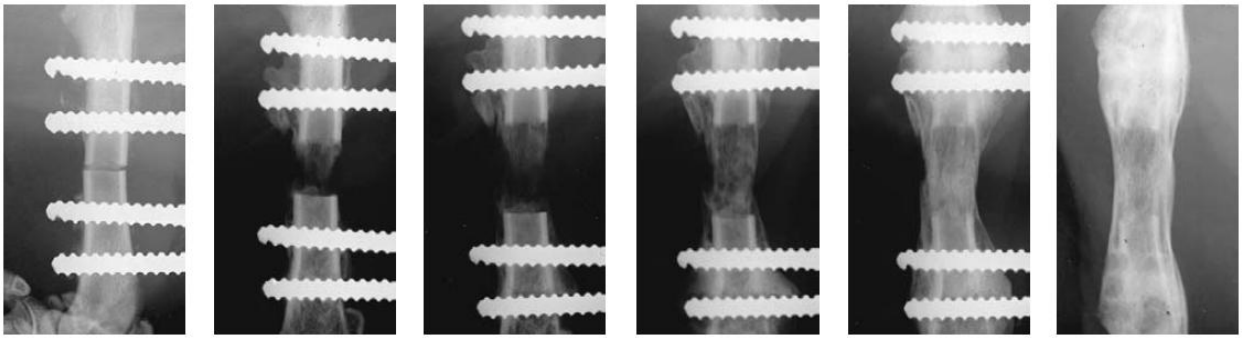

Figure 3 – Process of Distraction Osteogenesis in a white rat femur model. Reproduced from [87].

CHAPTER 2
RESEARCH AIMS AND HYPOTHESIS OF THE STUDY

2.1 INTRODUCTION

This research will focus on investigating the results of the synergistic application of both osteoblast seeded scaffolds and simulated in-vitro osteodistraction. Based on the literature reviewed in the previous chapter, a hypothesis is formulated indicating that three-dimensional tissue formation at the interface of two pre-seeded matrices–whether natural graft or synthetic material–might be possible and strongly influenced when subjected to the in-vitro distraction regime. The fact that clinical outcomes of distraction osteogenesis have been positive for numerous documented in-vivo cases, allows the conclusion that new tissue formation might also be possible in-vitro using the proper cell culture and growing conditions.

Despite the many efforts taken in trying to mimic the conditions in-vivo, it will be impossible to achieve all signaling and mechanisms occurring in the human body when bone is remodeling and hence, actual bone formation in-vitro is not very feasible under the setup to be utilized. Nonetheless, it is possible for this experiment to demonstrate the ability of osteoblast cells to construct mineralized 3-D structures that closely resemble mineralized tissue. Also, this model should provide a better understanding of the conditions and factors that cells undergo while the process of bone formation occurs in order to further improve bioreactor design and culture techniques. Furthermore, if positive results are obtained, future iterations of this setup

could become implemented in the culturing and growing of safe and fully biocompatible human tissue.

2.2 AIMS OF THE EXPERIMENT

- Design a platform for parallel testing of samples in a simulated in-vitro distraction osteogenesis.
- Study the effect that a simulated distraction regime has on PCL scaffolds seeded with BMSC.
- Study the potential of BMSC to deposit and mineralize 3-D tissue in clinically relevant amounts.

2.3 HYPOTHESIS TO TEST

- The in-vitro simulated distraction osteogenesis regime might drive cells towards increased deposition of mineralized ECM that could allow growth of relatively large 3-D constructs closely resembling bone tissue.

In order to test the feasibility of the hypothesis formulated, a plan of action was devised. This plan consisted on performing a mechanical procedure that resembled distraction osteogenesis in an in-vitro model. The distraction regime was carried out on PCL scaffolds fabricated via salt leaching technique, which had been previously seeded with BMSC induced into the osteogeblast lineage. After the distraction procedure was finalized, the Alizarin red marker for calcium was added into the scaffolds to assess, in a qualitative manner, if mineralization had occurred. The process flow shown in Figure

4 depicts the major steps of the culturing process and the distraction regime. Methodology and experiments are described in detail in the following chapters.

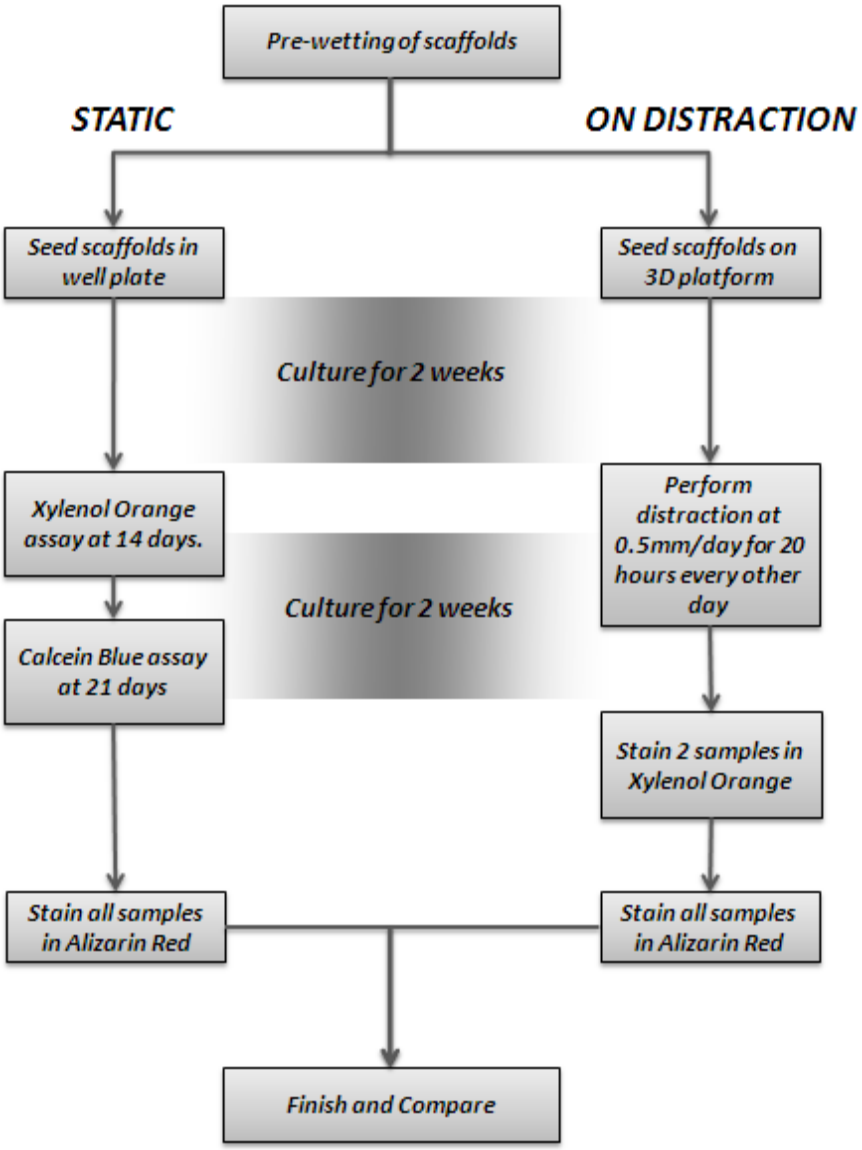

Figure 4-Process flow of the main steps in the Experiment

2.4 THESIS OUTLINE

This thesis is organized in 5 chapters. As presented already, Chapter 1 provides background and an overall literature review on the current state of tissue engineering as well as the concept of distraction osteogenesis. This chapter provides figures that support the claim for engineering of tissues and general information on the medical procedure that is attempted to replicate in-vitro. Chapter 2 provides the hypothesis that will be tested as well as a process flow depicting the major steps in the experiment setup. Chapter 3 will discuss information on the design and development of a platform suitable for in-vitro testing focusing on the needs of common culture procedures and properties necessary for cell growth. Chapter 4 continues with the description of the qualitative experimentation on cell culture, both in the proposed regime and on the static culture utilized for control. Finally, Chapter 5 gives the overall findings of the experiment and provides reference to upcoming work that can be addressed for improving the results of further experiments.

CHAPTER 3
EXPERIMENTAL SETUP FOR IN-VITRO DISTRACTION OSTEOGENESIS

3.1 INTRODUCTION

This chapter presents information on the materials selected for scaffold fabrication and details on their construction. Also, based on the requirements for cell culture, the implemented setup for in-vitro distraction osteogenesis is presented along with the process for designing and building of a proper tool for in-vitro experimentation. Later, Chapter 4 will present details on the culturing experiments and assays utilized for assessing mineralization.

3.2 SETUP FOR IN-VITRO DISTRACTION OSTEOGENESIS

The culture of cells requires a sterile environment for cells to properly grow and maintain function. On the in-vitro distraction regime, there was a need to seed two scaffolds, bring them into contact at their seeded surfaces, culture for a period of time and progressively increase the gap separating the scaffolds' contact surfaces. With this purpose in mind, a platform had to be designed that permitted the implementation of this simulated regime while maintaining the appropriate cell culture conditions.

3.2.1 Incubation Conditions and Application of Linear Displacement

For the in-vitro distraction regime, application of precise and continuous movement was needed. For this reason, a BOSE ElectroForce BioDynamic® mechanical tester was fitted inside an Excella ECO-170 incubator. This setup would allow

controlling the distraction regime while maintaining culture conditions of 37 ˚C temperature and 5% CO_2. The factory setup of the ElectroForce BioDynamic® was slightly modified leaving in place the main grips to which a platform for experimentation–described in detail later–would be attached. An actuator connected to a transducer provided the linear displacement required. The force-measuring or load cell was detached to properly fit the ElectroForce BioDynamic® inside the incubator.

3.2.2 Win-Test 4.1 Software

The proprietary software WinTest® version 4.1 was utilized for controlling the ElectroForce BioDynamic® using a PC running Windows® XP. According to the manufacturer, this software comprises an integrated solution for PCI control and data acquisition within an easy-to-use and intuitive interface–the main window of the application is shown in Figure 5–with real time readings. Among the many options this software provides is the ability to interface with the used test instrument to apply varied load and displacement patterns to specimens allowing study of their material properties such as fatigue.

By selecting displacement as input feedback, the linear actuator can be driven into various waveforms including sinusoidal, ramp, square and triangle. For the purposes of this experiment, the ramp waveform was utilized. This pattern generates downward motion of the lower grip from one position to another. The rate of displacement, measured in millimeters per second (mm/sec), can be defined according to each experiment's needs. A factory lower limit on this rate is set at 6.5E-6 mm/sec,

making very precise displacement possible. This fact was used to the advantage of this experiment since fine motion of the system, in the micrometer range, was required to simulate distraction. Using the capabilities of this hardware-software combination, a schedule was prepared to carry out the simulated distraction regime as described in the in-vitro experimentation sections of Chapter 4.

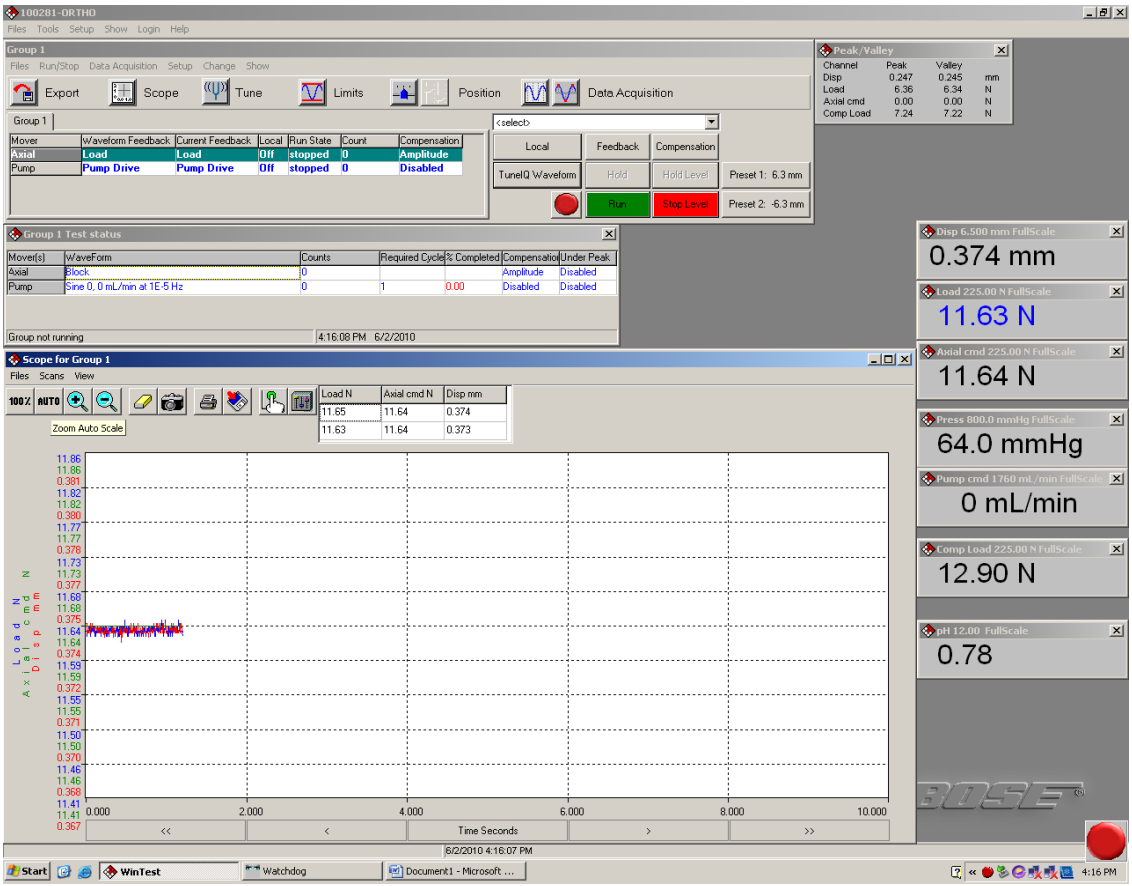

Figure 5 - Main interface of the WinTest® 4.1 software.

3.2.3 Design of Platform for In-vitro Osteodistraction

Because the main requirement in cell culture experiments is the ability to maintain sterile conditions, a device was designed and constructed to offer this degree of hygiene while still providing a platform for the linear displacement to be applied. A modular design was implemented that consisted of several parts presented below in Table 1.

Table 1 - List of parts for the modular design of the platform.

Part Name	*Quantity*
Vial Cap	**4**
Upper support platform	**1**
Inner pistons	**4**
Vial holders	**4**
Connecting rods	**4**
Lower support platform	**4**
Moving platform	**1**

As can be seen in the computer aided design (CAD) model of Figure 6, the vials containing the pre-fabricated scaffolds attached into holders. The holders in turn, coupled to the moving platform, by means of 4 connecting rods, which finally assembled to the ElectroForce BioDynamic® lower grip. Atop each vial, a cap with two orifices was placed to provide means of periodically changing the medium during culture. These vial caps were connected to the main support structure which held statically from the top as it fastened to the upper grip using a compression nut. The caps also supported a piston that dropped into the vial, each one holding a porous scaffold. The main structure consisted of both upper and lower support bases with guiding

sleeves to maintain the vials in position. The support bases were fixed to one another by 3 rods. Also, 4 screws, serving as guides, were attached to the moving platform leaving one nut per screw free to move. This was done to hold the position of the platform, and hence of the gap separating scaffolds, between periods of distraction and latency. All parts were constructed using an available 3-D systems' uPrint 3-D printer–which utilizes ABS plastic–and attached together using nuts and # 6-32 screws of various lengths. The CAD was modeled in Pro-E version 4.0 and it is shown below in Figure 6.

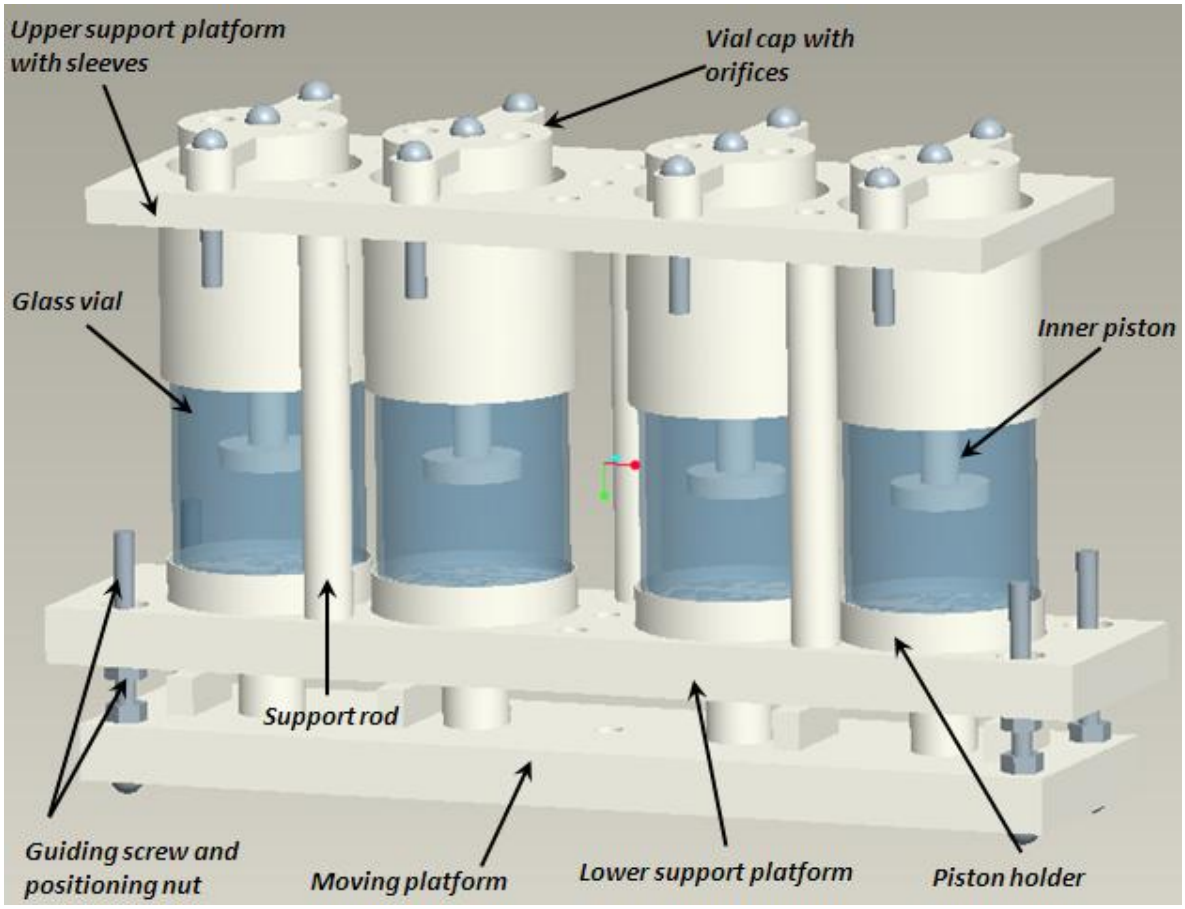

Figure 6 - CAD model of the platform for simulated distraction

3.3 SCAFFOLD FABRICATION

For this study, scaffolds needed to fulfill the two basic requirements of biocompatibility, to allow cell attachment, and low degradation rates to maintain its shape during the length of the experiment. Internal porosity was not of utmost importance since cell penetration and survival at the interior of the scaffold was not the focus of this study. Rather, seeded cells were needed to attach into the upper surface of the scaffolds to facilitate interaction and function at the interface of two samples. In this fashion, continuous cell deposition of layers of ECM might be achieved along with the formation of mineralized tissue. After some research, Poly-ε-caprolactone was selected as the scaffold fabrication material due to its characteristics, and salt leaching was the employed method since it is an easy and inexpensive technique. The fabrication steps are explained next.

3.3.1 Poly-ε-Caprolactone (PCL)

Poly-ε-caprolactone is an aliphatic polyester that has been termed safe for use in human experiments. Due to the many properties it exhibits, PCL has been used in various applications like fabrication of scaffolds for tissue engineering, adhesives and food packaging [36, 88-90]. It has also been termed safe for medical applications, such as drug delivery devices, by the FDA [91, 92]. Furthermore its mechanical properties such as tensile strength and Young's modulus–a set of properties is summarized in Table 2–along with its capacity to slowly biodegrade, make PCL a choice in implantable devices and other medical applications [93]. The PCL utilized for this experiment was obtained

from Sigma-Aldrich in the form of small pellets and had a stated average molecular weight (Mw) of 80,000 Daltons.

Table 2 - Properties of PCL. Reproduced from [93]

Properties	*Range*
Number average molecular weight (Mn/g mol-1)	**530-630,000**
Density (ρ/ g cm-3)	**1.071-1.200**
Glass transition temperature (Tg/˚C)	**(-65)-(-60)**
Melting temperature (Tm/˚C)	**56-65**
Decomposition temperature (/˚C)	**350**
Inherent viscosity (ηinh/cm3 g-1)	**100-130**
Intrinsic viscosity (η/cm3 g-1)	**0.9**
Tensile strength (σ/ Mpa)	**4-785**
Young modulus (*E*/GPa)	**0.21-0.44**
Elongation at break (ε/ %)	**20-1000**

3.3.2 PCL Degradation Study

As recalled from the literature reviewed, one of the requisites of scaffolds is their ability to maintain their shape from the time of implantation up to the required length of time tissue takes to regenerate. Similarly, for this experiment, the shape of scaffolds needed to remain with minimal modification to keep the most possible area of contact during a simulated latency period in the experiment. Furthermore, since degradation of the polymer also affects the pH of the medium, making sure the polymer degraded slowly was also required to preserve the proper medium acidity and hence its positive effect on cells.

In general, the higher the molecular weight of the polymer, the higher the degradation time. This was the reason to utilize a polymer with a relatively high

molecular weight. The properties of the PCL utilized were confirmed by performing a gel permeation chromatography (GPC) analysis as follows. First, 0.25 grams of PCL were dissolved using tetrahydrofuran as solvent. Then, 200 µL of this solution were injected in a Viscotek TDA 302 Triple Detector Array. This apparatus measures and compares the refractive index of the polymer relative to that of the solvent. As can be seen in the graph of Figure 7, the software generates a plot of the refraction index versus the volume analyzed. Then, it computes the area below the curve to provide a measure of the molecular weight of the compound analyzed in units of Daltons (Da). The results of this study confirmed that the molecular weight (Mw) was relatively close to the value of 80 kilodaltons (kDa) expressed by the manufacturer which confirmed that degradation should be minimal for this material. Table 3 reproduced from the information of the experiment, lists the properties found for the polymer.

To further support the analysis, three sample scaffolds (porous type) were analyzed for weight changes for a period of 3 weeks. The scaffolds were incubated at 37 °C and 5% CO_2 in phosphate buffer saline (PBS) since it approximates the pH of the medium used in the cell culture experiments. The initial weights were recorded and then each scaffold submerged in excess PBS. At days 7, 14 and 21, their weights were again recorded after drying inside an oven for one hour. The weight of scaffolds remained virtually unchanged during the time of culture further indicating the slow degradation rate of the PCL utilized, as shown in Figure 8.

Table 3 - Results of GPC Analysis.

Multi-Detectos Homopolymers: Results	
Peak RV - (ml)	4.833
Mn - (Daltons)	18315
Mw - (Daltons)	74545
Mz - (Daltons)	2.18E+08
Mp - (Daltons)	23931
Mw/Mn	4.070
Percent above Mw: 0	0.000
Percent below Mw: 0	10.108
IV - (dl/g)	1.0571
Rh - (nm)	6.980
Rg - (nm	No calc
Wt Fr (peak)	1.000
Mark-Houwink a	0.800
Mark-Houwink logK	-3.419
Brances	0.000
Branch Freq.	0.000
RI Area - (mvml)	69.5
UV Area - (mvml)	0
RALS Area - (mvml)	44.79
LALS Area - (mvml)	28.84
DP Area - (mvml)	1214.99

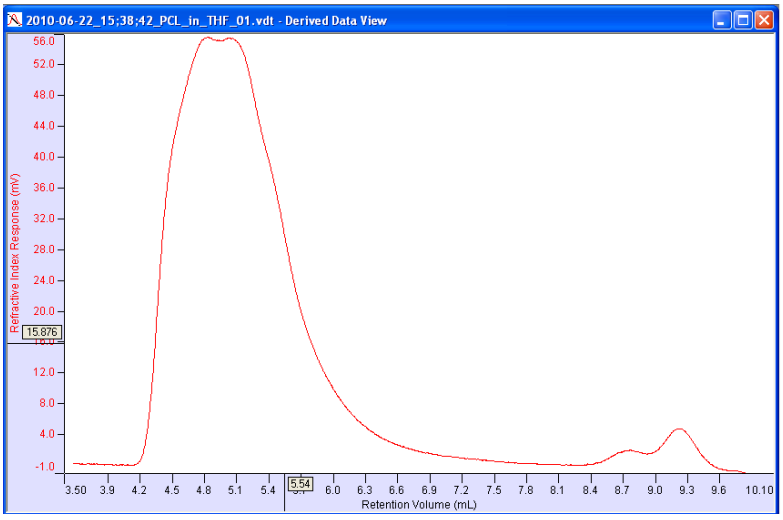

Figure 7 - Curve of refractive Index VS volume analyzed generated by the GPC Analysis.

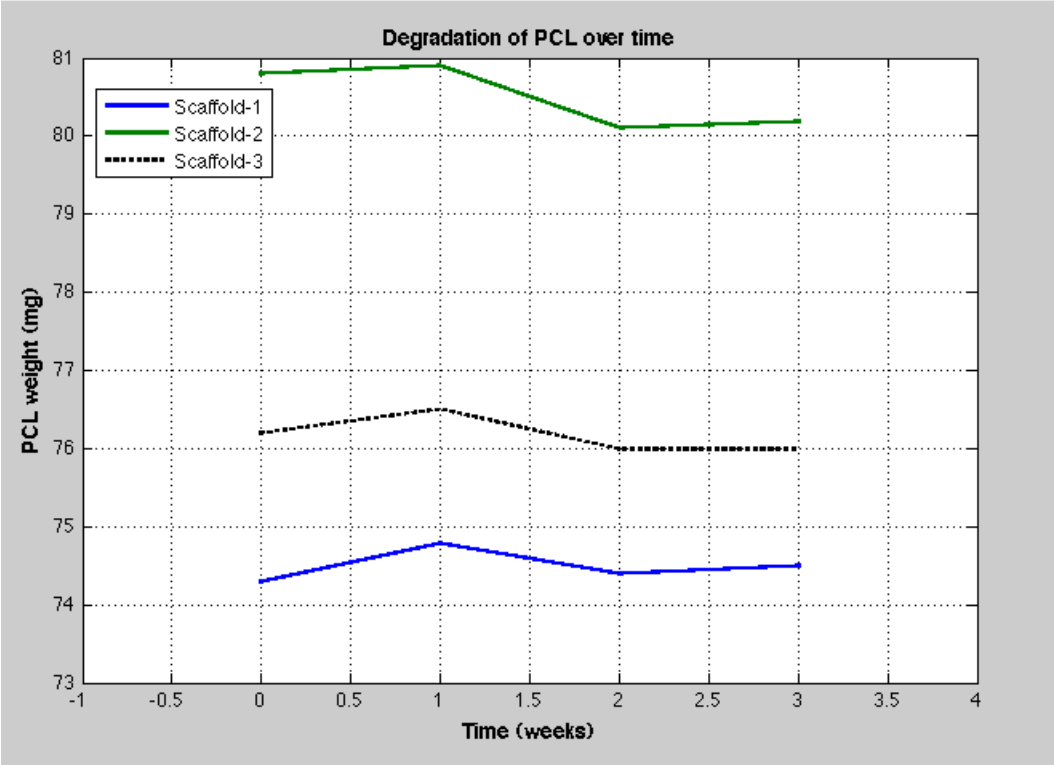

Figure 8 - Degradation behavior of PCL scaffolds.

3.3.3 Salt Leaching

Scaffolds were fabricated utilizing the simple and affordable technique of salt leaching (SL). This method produced scaffolds that were appropriate for culturing of cells, even though they exhibited a non-uniform pore distribution. Two variations of the SL technique were utilized based on two types of scaffolds needed.

One scaffold type was directly cast to the glass vial to create a solid bond hence keeping the scaffold in place during simulated distraction. This was achieved by pouring four grams of PCL pellets and melting the material by heat conduction for approximately 30 minutes. After the PCL had melted into a clear and viscous substance,

0.25 grams of sodium chloride salt (NaCl), sieved to a particle size of about 300 μm, were added per vial to act as pore generators (porogens) at the uppermost surface of the PCL. As the vials cooled, the excess salt was removed and once the PCL had completely solidified, the vials and their contents were submerged in ultrapure water for 72 hours with water changes taking place roughly every 8 hours. After the salt dissolved, it resulted in a substrate adhered to the vial with a highly porous upper surface. Figure 9 A) depicts the process utilized in the fabrication of this type of scaffold (vial scaffold).

The second type of scaffold (porous scaffold) was fabricated using a more traditional technique following a slight modification to the method presented by [36]. As depicted in Figure 9 B), scaffolds were fabricated by dissolving 1 g of PCL on 10 mL of chloroform inside a chemical hood. The PCL pellets and chloroform were manually stirred in a beaker for 45 minutes until a uniform viscous mixture developed which had a concentration of 10 % (w/v). Next, 7 g of NaCl sieved particles were added to achieve a PCL to salt weight ratio of 7:1. At this point, the slurry that formed was poured into pre-fabricated plastic molds of 10 mm diameter and 5 mm thickness that were covered with aluminum foil for easy detachment. Then, molds were left for over 24 hours under the chemical hood at which point the PCL had solidified, entrapping the salt particles, and chloroform evaporated. Next, scaffolds were detached from the molds and submerged in ultrapure water for over 72 hours for the salt to dissolve. After this time, the scaffolds appeared floating over the water serving as an indicator that salt had completely dissolved.

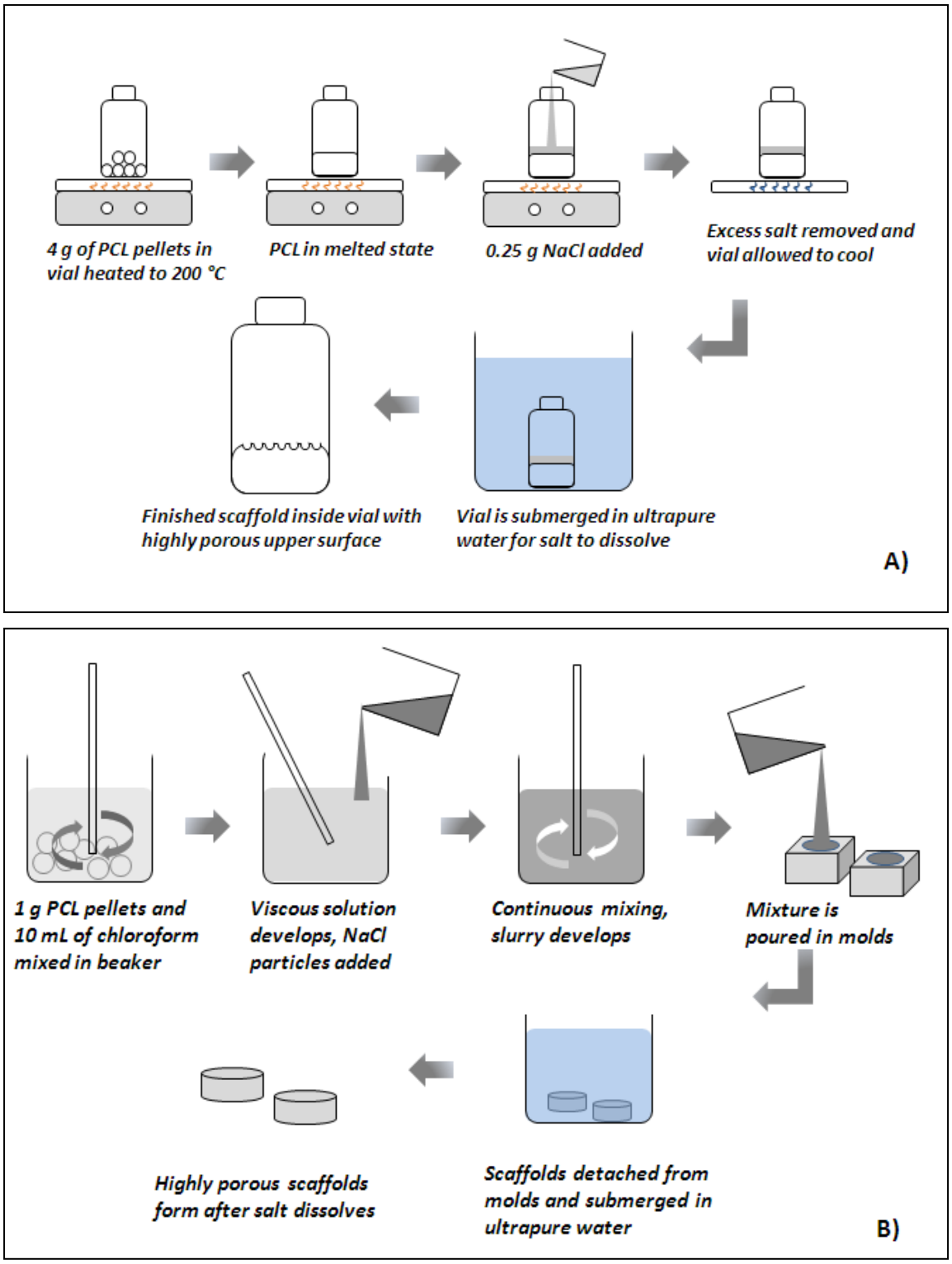

Figure 9 - Process for scaffolds fabrication. A) For scaffolds attached to the vial. B) For porous scaffolds to be fixed to the inner piston.

3.4 SCAFFOLD CHARACTERIZATION

3.4.1 Microscopy Observations

After the scaffolds were washed in distilled water for over 72 hours, one sample of each type was removed and analyzed at its outer surface by using light microscopy. This was done to assess that scaffolds exhibited the proper morphology, suitable for cell culture experiments, in the exposed or external surfaces. Observations were made using a Carl Zeiss Axiovert 40 CFL microscope. The examination indicated the presence of a PCL matrix with interconnected voids or pores with a size of roughly 300 μm, the same as the sieved salt particles. This porous morphology was observed at the surfaces of both types of scaffolds fabricated. For the observations of the melted PCL scaffold, the vial was broken to expose the porous surface. The images of Figure 10 depict the observed surfaces indicating the size–as measured using the microscope software–of the pores in the appropriate range.

3.4.2 μ-CT Scanning of Porous Scaffolds

Followed by observations under the microscope, a more comprehensive visualization of the scaffolds was required to have a description of their internal architecture. With this purpose in mind, a sample scaffold was left drying inside an oven for 45 minutes and then scanned using a SCANCO Micro-40CT, μ-CT scanner. Using the scanner's standard resolution, the process took around one hour and it provided high-resolution images of the scaffold's structure. The output was given as 320 slides with a thickness of 16 μm that were saved as a sequence of TIFF images.

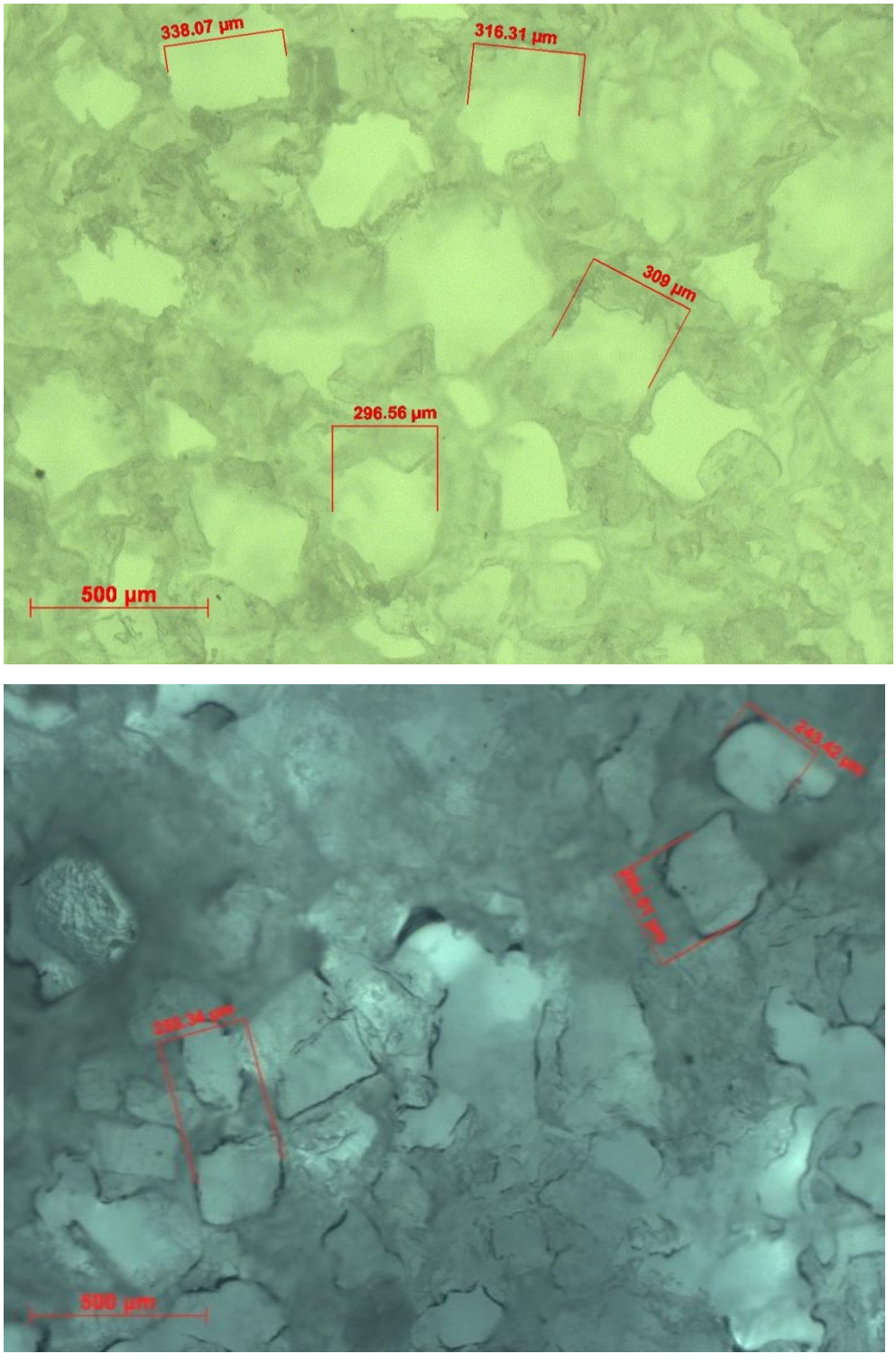

Figure 10 - Microscopy observation of scaffolds. The upper image corresponds to the porous surface of melt scaffolds. The lower image shows surface of porous salt leached scaffolds.

The internal porosity of the scaffold, as mentioned before, was not truly important for this study. Nonetheless, a CAD reconstruction of the scanned images was performed solely to have a better description of the porosity and internal architecture of porous scaffolds. This was performed using the software Mimics® version 14.0 as described next.

3.4.3 Scaffold 3-D Reconstruction and Porosity Analysis

Mimics® provided the means of selecting, in a slide by slide fashion, the material of interest to reconstruct based on the sequence of TIFF images. Once the set of images had been imported, the brightness and contrast in the working environment were adjusted. Then, upper and lower threshold values were chosen to select a uniform type of material throughout all slides. All regions in each slide falling within the threshold values were selected although manual editing of each layer was needed in some regions. The upper and lower threshold values utilized were -849 to -638 units. The thresholding process is shown in Figure 11–two upper and lower left images–where the green color indicates the scaffold material (PCL) selected for reconstruction as seen from the top, right and posterior views.

Once the sections of PCL material had been selected in each slide, the 3-D reconstruction was done in a relatively simple way just by selecting the quality of the construct. For the means of this experiment, the capabilities of the computing equipment restricted quality to medium setting. Nonetheless, this resolution permitted good observations of the inner morphology of the scaffolds while allowing a porosity

analysis to be carried out. The 3D reconstruction of the scaffold is shown in the lower right image of Figure 11.

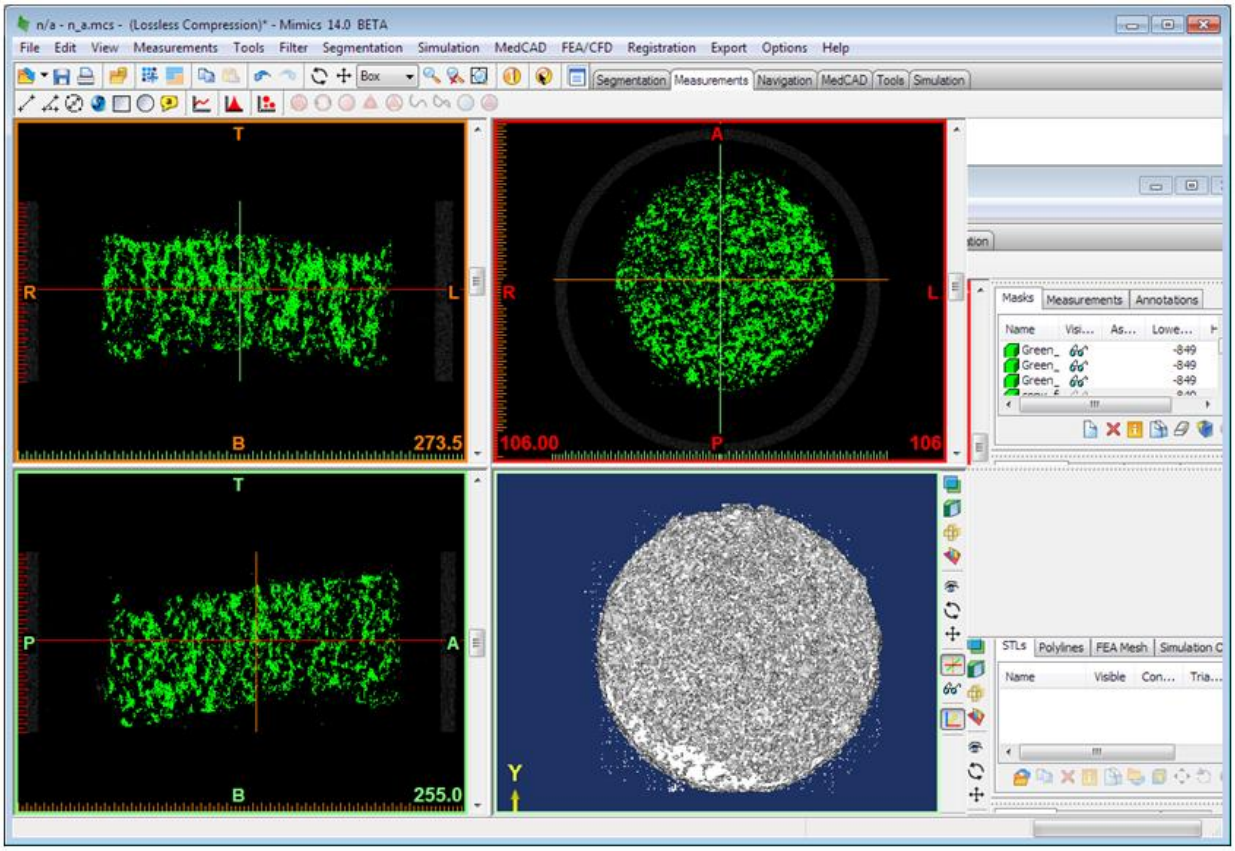

Figure 11 - Process of "thresholding" and 3-D reconstruction using Mimics 14.1

Using a built-in pore measurement function in Mimics®, it was possible to calculate the porosity in sixteen samples taken out of the reconstructed scaffold model. The reason for this was due to restricted memory and processing power of the computer utilized which prevented making the analysis on the whole scaffold. Hence, sixteen cubes of the same size (2.5 mm^3) were selected randomly throughout the scaffold–upper image of Figure 12–for analysis. The cubic sections were analyzed using

this function and the averages calculated yielding mean porosity of 45.8%, with standard deviation of 7.08, and 100% average pore interconnectivity. These values were taken as representative for the whole scaffold structure and for all porous scaffolds fabricated. Table 4 summarizes the results of the pore analysis.

Table 4 - Porosity analysis on fabricated scaffolds.

3D Section	*Porosity (%)*	*Pore Interconnectivity (%)*
Cube-1	**45.41**	**100**
Cube-2	**34.54**	**100**
Cube-3	**38.19**	**100**
Cube-4	**42.78**	**100**
Cube-5	**49.78**	**100**
Cube-6	**52.41**	**100**
Cube-7	**49.46**	**100**
Cube-8	**53.98**	**100**
Cube-9	**41.63**	**100**
Cube-10	**30.29**	**100**
Cube-11	**46.57**	**100**
Cube-12	**55.6**	**100**
Cube-13	**50.41**	**100**
Cube-14	**42.58**	**100**
Cube-15	**50.41**	**100**
Cube-16	**49.11**	**100**
Average	**45.82**	**100.00**
Std Deviation	**7.08**	**0.00**

In a close-up view at the 3-D reconstruction–shown below in Figure 12–of a section of the whole scaffold, the inner morphology can be appreciated more clearly. This image demonstrates there are pores of various sizes less than 300 μm. Also, the pores appear highly interconnected in accord with the calculations made using

Mimics®. Some voids in the PCL matrix with the shape of salt crystals, are visible in this cut through plane and indicated by dark arrows.

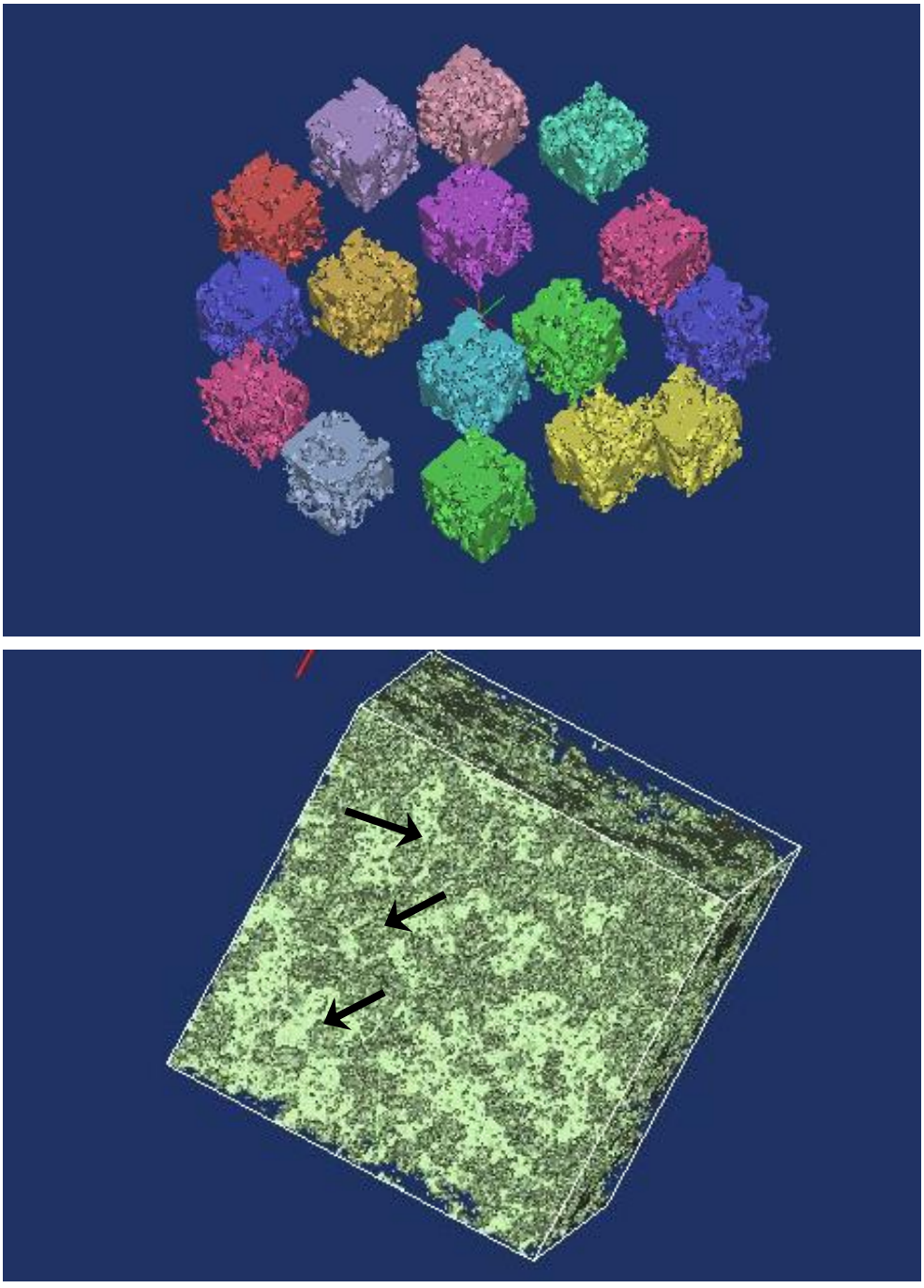

Figure 12 - Upper: 16 cubic sections selected for pore analysis. Lower: close-up view of inner microstructure where pores can be identified (black arrows).

3.5 CONCLUSION

This chapter presented the methodology for the design and implementation of a setup for testing the hypothesis of in-vitro osteodistraction. Using tools available, it was possible to design and assemble a platform that permitted similar conditions of cell culture while also providing means for implementing an in-vitro distraction regime. This platform was designed to complement into an apparatus that provided control of linear movement to simulate distraction (gap increase). Upon testing of this system, it was seen that indeed the gap could be increased very smoothly over long periods of time. Also, chemical and physiological conditions were achieved by fitting the whole setup at the interior of an incubator. Moreover, two different types of scaffolds, to be utilized in cell seeding experiments, were produced following the simple technique of salt leaching. Microscopy observations and μ-CT reconstruction as well as a GPC analysis of the PCL utilized for their construction, indicated scaffolds exhibited properties suitable for cell culture experiments.

CHAPTER 4
METHODOLOGY FOR CELL CULTURE EXPERIMENTS

4.1 INTRODUCTION

Three cell culture experiments were carried out each with a purpose in mind. First, one cell culture experiment was performed to assess the biocompatibility of PCL and its ability to support growth and function of BMSC. In this experiment, maturation and viability of BMSC was evaluated by inspecting for calcium deposits. Both second and third experiments were carried out as the in-vitro osteodistraction model and its corresponding static control. BMSC utilized were harvested from the bone marrow extracted from white rat femurs as described by [94]. Two medium preparations, one containing dexamethasone (+Dex) and the other without it (-Dex), were prepared according to a recipe borrowed from [94]–refer to Appendix 1–and utilized for cell culture. The experiments are described in detail in the following sections.

4.2 CULTURE TO EVALUATE SCAFFOLD MATERIAL BIOCOMPATIBILITY

In this experiment, minimum essential medium without dexamethasone (α-MEM –Dex) was utilized. BMSC cryogenically preserved in a vial at a concentration of 10^6 cells were reanimated following a standard protocol as follows. First, the vial was set under running warm water until the solution completely liquefied. Then the cell suspension was aspirated using a pipette and deposited into a T-25 flask for culturing. Four mL of –Dex medium, warmed to 37 °C, were added to the flask to provide

nutrients and left incubating overnight. After 24 hours, surviving cells were observed under the microscope and the medium was changed to allow continued cell growth until they achieved confluence. Once cells had reached that level of growth, trypsin was added to the flask with subsequent incubation for 5 minutes for cells to detach. Followed by two rinses in phosphate buffer saline (PBS) and the addition of 5 mL of new medium into the flask, the whole cell suspension was aspirated and seeded into five scaffolds (1 mL per scaffold). This process was accomplished by depositing the solution drop by drop into the upper surface of the scaffolds. The scaffolds had been previously fixed to the bottom of a well plate by using autoclaved silicone. Then, the well plate was put inside an incubator at 37 °C and 5% CO_2. The culture medium was changed every two days at a volume of 1 mL per well. Under these conditions, scaffolds were cultured for a period of 24 days.

At various days through the culture period, cells were observed to qualitatively evaluate if the PCL scaffolds had an effect on cell viability. Also, the xylenol orange (XO) assay for calcium deposition was performed to observe if cells had reached their mature phenotype and continued function.

4.2.1 Results of Material Biocompatibility Experiment

Seeded cells that did not adhere to scaffolds were observed at the surface of the well plate. Images taken at various points of time during culture, demonstrate that the PCL had little effect on the cells as they continued growing until reaching confluence. As can be appreciated in the images in Figure 13 next page, taken using both the 5x and

10x microscope objectives, cells remained alive in the presence of the scaffold material and a very particular behavior was observed in which cells formed larger agglomerations next to the scaffold wall proving the biocompatibility of the material.

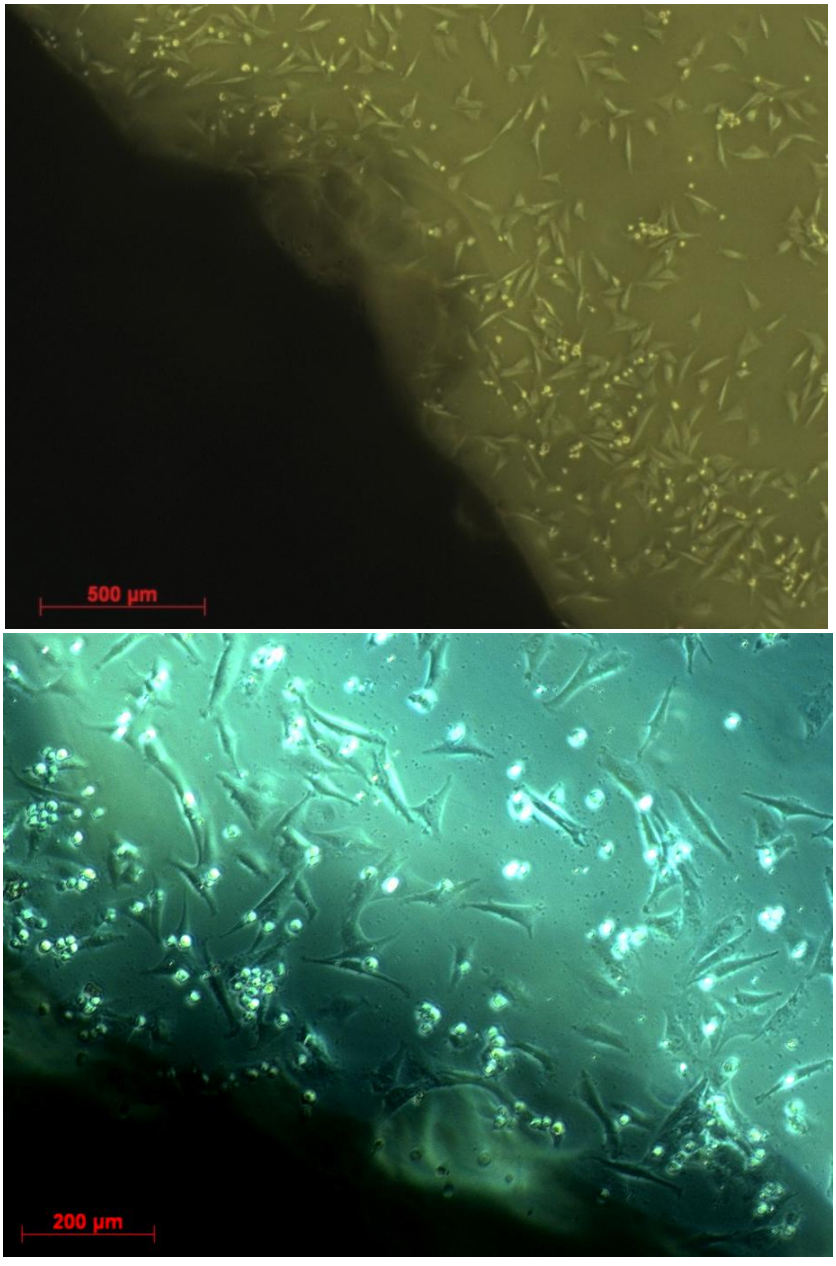

Figure 13 - Live cells next to scaffold material at 24 and 72 hours after seeding respectively. The dark regions correspond to the scaffold wall. Photographs taken using the 5X and 10X objectives, respectively. In the pictures, some cells appear already attached–elongated bodies–while others still float in the medium resembling small dots.

Employing only optical microscopy, it was not possible to observe cells adhered into the scaffold since the PCL did not offer a good contrast for cells to be seen. For this reason, after 18 days of culture, the XO marker was utilized to have an indication of whether cells had continued to grow and differentiate in the presence of scaffolds. XO is a fluorochrome that binds to calcium deposits and fluoresces with a heavily red-orange coloration when observed under a microscope at specific wavelengths of 440/570 and 610 nanometers [95]. The indicator has been successfully utilized in determining calcium nodules in in-vitro cell cultures as well as in-vivo studies using small (rat, rabbit, chicken) and large (sheep, dogs) animal models [96, 97]. XO offers an advantage over other fluorochromes such as Von-Kossa and Alizarin Red since it can be used without the need to end the culture (fixation of cells) as it does not have deleterious effects on cells [95]. Since this study evaluated continued cell function past 18 days of culture, XO was a suitable indicator of osteoblast phenotypic activity of cultured BMSC.

In this assay, the XO was added to the seeded scaffolds on days 18 and 24 of culture. On both days, medium was changed and 1 μL of XO was added per milliliter of medium using the same concentration (20 μM) presented by [95]. Then, the cells were further incubated overnight and observed on the following day after the old medium was replaced by fresh one. Again, observations were carried out using a Carl Zeiss Axiovert 40 CFL microscope which was fed with fluorescent light by using a HBO 50 mercury lamp. All pictures were obtained using a Carl Zeiss AxioCam MRC attached to the microscope using the 5x objective.

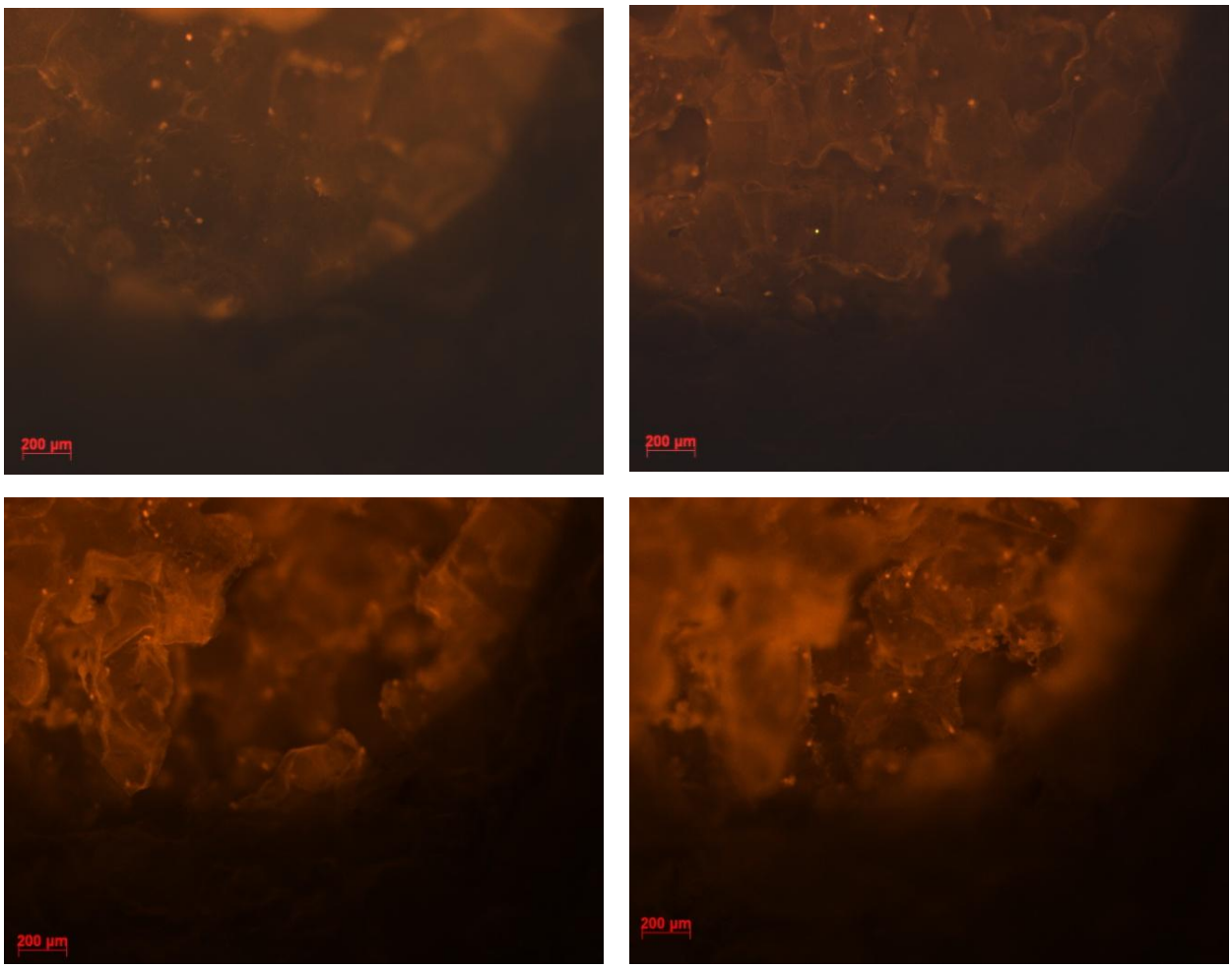

Figure 14 - Xylenol Orange assay for Calcium deposition. A and B taken at days 18 and 24 respectively. C and D show the same area, on day 24, photographed by adjusting the focus to two different depths. Calcium nodules are present indicating cells had penetrated and continued mineralizing.

In this experiment, the pictures indicated that even though cells had been cultured with medium lacking dexamethasone (-Dex), nonetheless the osteoblast phenotype had been reached since some calcified nodules were present within the structure of the scaffold as can be seen in images A) and B) of Figure 14. The results also demonstrated that the fabrication process yielded scaffolds that were biocompatible as confirmed by its ability to support continued cell function and growth. Moreover,

photographs taken after day 24 showed that cells had penetrated and formed calcium into varied layers within the structure of the scaffold. This can be seen in serial images C) and D) of Figure 14. These two images depict a single section of the scaffold photographed two times by adjusting the focus to a different depth for each photograph. Calcium nodules (orange dots) are observed on both layers shot indicating cells had matured into osteoblast and had attached to internal surfaces of the scaffold.

4.3 SIMULATED OSTEODISTRACTION EXPERIMENT

For this experiment, cells were seeded directly in the scaffold that had been created inside the vial by melting and salt leaching. Prior to this step, several measures were taken to guarantee proper hygiene and to enhance the chances for cell survival and attachment. After seeding, the porous scaffold was set into contact with the seeded surface and cells were incubated in this configuration for two weeks. A proper static control with both 3-D scaffolds and static surface culture was used as comparisons to the results obtained from this experiment. The following sections describe the methodology followed.

4.3.1 Scaffold Washing and Pre-wetting

As a first step, scaffolds were rinsed using distilled water and two concentrations of alcohol, 70% and 95% respectively. The three washes were done for ten minutes in each solution. Next, the scaffolds were put inside an oven at 40 °C for roughly one hour which ensured that most water, absorbed by scaffolds during salt leaching and

washing, had evaporated. Then, for a period of approximately four hours, scaffolds were pre-wetted by submerging them in +Dex culture medium to allow proteins and other substances that promote cell adhesion to coat the surface of the scaffold hence improving the ability of cells to attach. At this point, scaffolds were ready for cell seeding and culture started. Figure 15 shows a photograph of the porous and the vial scaffolds at the interior of the oven.

Figure 15 - Porous scaffolds drying inside oven at 40° Celsius.

4.3.2 Sterilization Precautions

Established guidelines such as cleaning all surfaces with 70% ethanol and UV radiation were followed to guarantee sterile conditions inside the biological hood prior to using it. Similarly, the pieces of the platform constructed for testing were put inside the biological hood and treated for some time with UV light. This process was done for over five days to make sure most surfaces were free of any bacteria or microbes that

could contaminate the experiment at the moment of culture. Care was also exercised to clean the platform with 70% ethanol prior to seeding the cells and every time medium changes took place. Glass vials were autoclaved prior to use. Similarly, after rinsing with the varied alcohol concentrations and with ultrapure water, scaffolds were handled carefully to prevent contamination.

4.3.3 Cell Seeding

Using the most sterile condition possible, all materials were brought inside the biological hood to start the culture. The porous scaffolds were glued to the inner pistons of the platform using silicone. Then, cells were seeded directly on top of the surface of the scaffold affixed to the vial at a concentration of 10^6 cells per sample. The fact that such a huge concentration of cells was utilized corresponded to efforts to boost the chances of ECM deposition and mineralization. The cell suspension occupied 1 mL and 2 more mL of warmed +Dex media were added for a final volume of 3 mL per vial. After seeding the cells, the scaffolds fixed to the pistons were brought to their initial positions in contact with the seeded surface of the vial scaffold. All other parts of the platform were assembled and the setup was housed inside the incubator. Serving as a latency period, the culture under these stationary conditions was carried out for 2 weeks prior to simulating distraction. Regular changes of media were carried out every two days. The platform with the seeded scaffolds can be seen in the upper image of Figure 16. The red liquid corresponds to the +Dex medium utilized.

Another culture was also started in a well plate where three scaffolds were glued each to one well. Then, scaffolds were seeded by depositing the cell solution in a drop by drop fashion. An empty well was also seeded with cells at the same concentration. To have means of comparison, the total volume of media was set also to 3 mL per each individual well. The static culture control is shown in the well plate in the lower image of Figure 16.

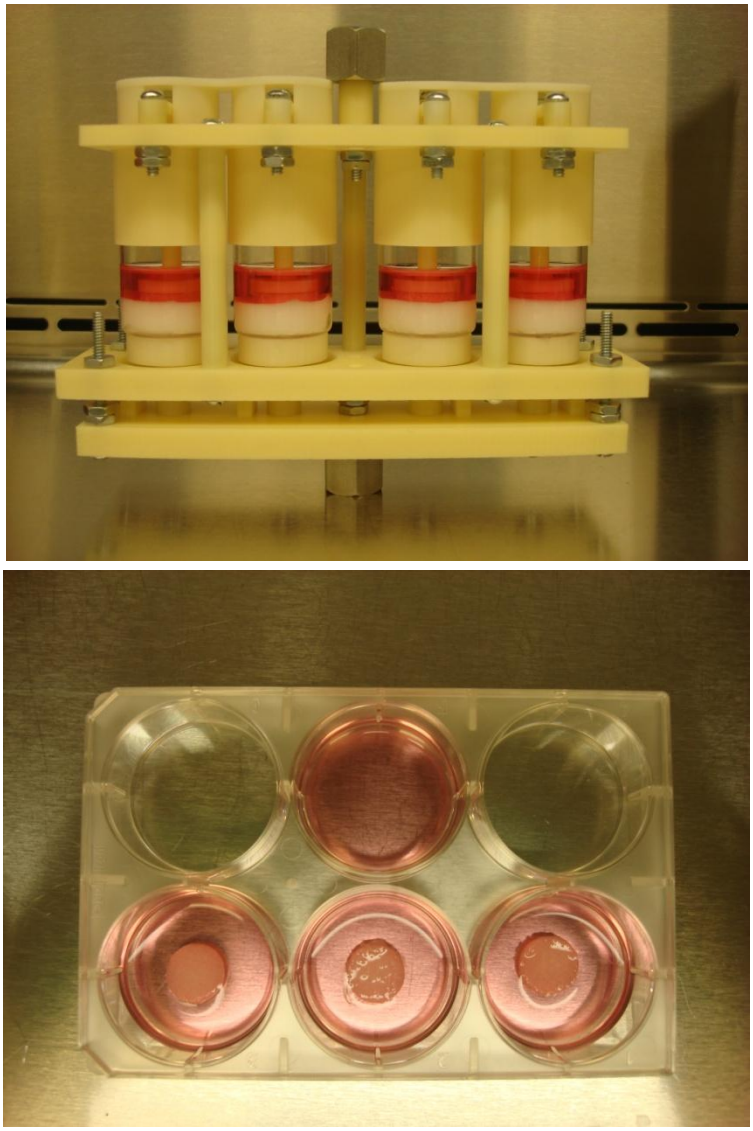

Figure 16 - Upper: Platform for in-vitro osteodistraction. Lower: Static culture of scaffolds to serve as control.

4.3.4 Implementation of Distraction Steps

At the moment the cells had reached the 14th day of culture, the latency period proposed had been finalized and it was time for the distraction osteogenesis experiment to begin. The rate of displacement in the proposed regime needed to take into account parameters from both the biological and medical perspectives to approximate better in-vivo conditions. The biological view indicates that mature osteoblast, among other mammalian cell types, need to reside no further than 250 microns from a blood vessel to obtain nutrients and exchange metabolic waste for their survival [98]. On the other hand, medical literature reviewed on distraction osteogenesis protocols point to displacement rates in the range from half to one millimeter per day [78-80] with better consolidation of bone tissue when continuous displacement, rather than periodic, was implemented on in-vivo animal studies [99]. With this in mind, it was decided to utilize an intermediate value of 0.5 mm per distraction step with continuous traction over a period of 20 hours followed by a rest phase of 24 hours. In deciding this parameter, a heavier weight was assigned to medical protocols since it was assumed that medium would permeate into the gap and thus fulfilling the requisite of providing nutrients and means for metabolic exchange to cells.

After attaching the platform to the ElectroForce BioDynamic® grips, WinTest 4.1 software was used to impose a ramp curve at a rate of 6.94×10^{-6} mm/sec which would translate to a time of 20 hours to complete half a millimeter. Under incubation conditions, the experiment was allowed to run overnight and data captured every

second for each 20 hour cycle. This setup was run four times for a total of 2 mm gap increase overall with 24 hour rest periods in between. The graph in Figure 17 shows the plot of distance versus time for one of the cycles carried out with measurements condensed to one data point every ten minutes passed. The graph demonstrates the linear behavior, due to the constant rate, imposed by the linear actuator on the displacement.

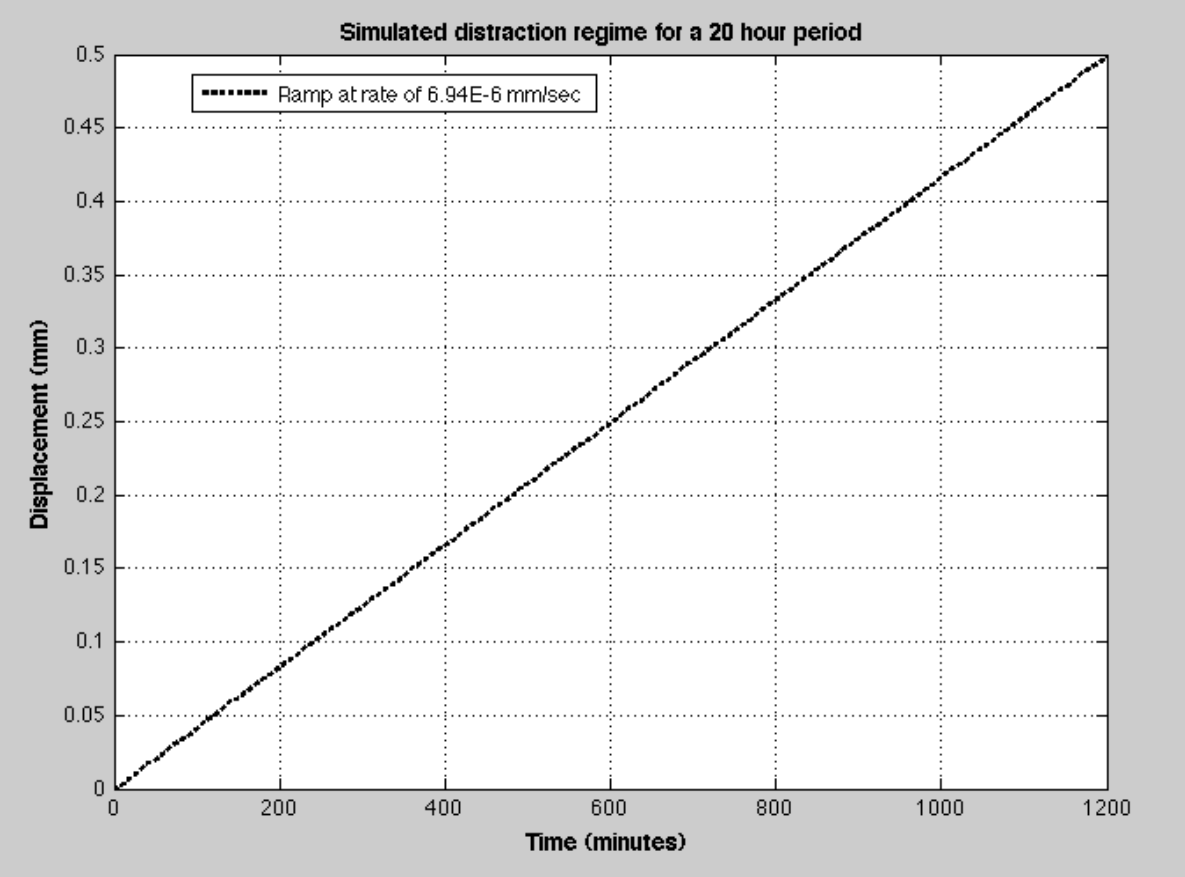

Figure 17 - Plot of one cycle of simulated distraction. The plot shows information for a running period of 20 hours with measurements taken in cycles of 10 minutes.

After the distraction phase had been performed, the culture was allowed to rest in a simulated consolidation stage for ten days. On day 9 of the consolidation phase,

some scaffolds (vial and porous) were stained with xylenol orange for later observations using microscopy. Moreover, on the last day of consolidation, an alizarin red staining was performed on all samples to evaluate the level of mineralization and to corroborate by visual inspection if indeed tissue had formed at the interface of scaffolds. The methodology followed during this process is described in detail in the following section.

4.4 ASSESSMENT OF MINERALIZATION

4.4.1 Xylenol Orange Assay

One day prior to finalizing the experiment, 3 mL of the XO fluorochrome were added to half the vials where scaffolds had been cultured. All samples were incubated overnight and observed the following day. To observe the surface of the porous scaffolds, they were detached from the inner pistons and placed in a well-plate with each in one well. Then medium was added and observations made under the microscope. The other two samples that were not stained served as controls.

When the process of removing the inner pistons was carried out, it was seen that no significant tissue had developed in the gap separating the scaffolds. Nonetheless, when observed under the microscope, it was possible to identify some calcified nodules present in the lower and lateral surfaces of porous scaffolds. Nodules of what appears as calcified material are shown in the series of images depicted in Figure 18. To obtain contrast, images of unstained control scaffolds are also included where marked differences in fluorescence can be appreciated.

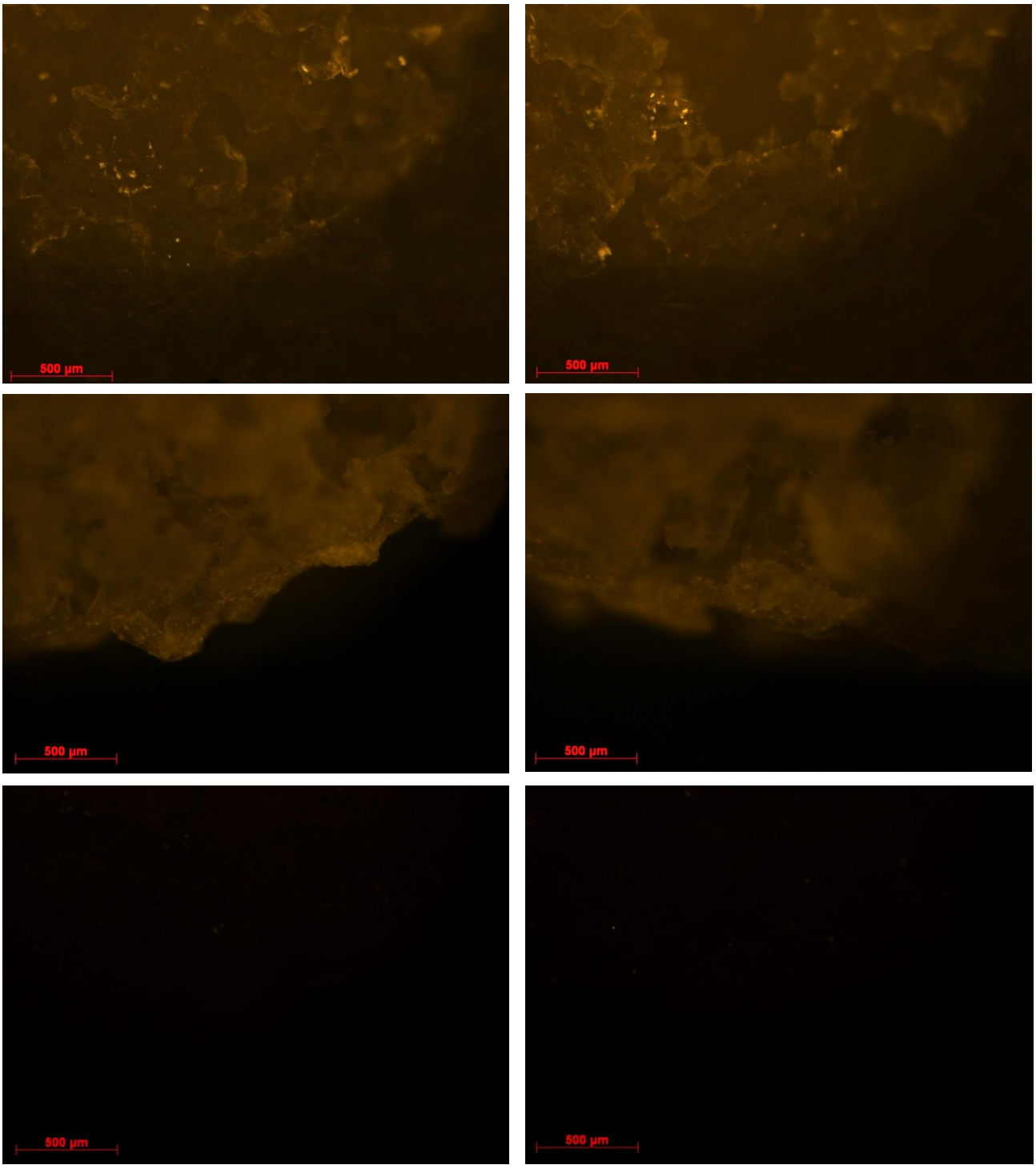

Figure 18 - Inner scaffolds observed for mineral tissue formation. The two images on the top correspond to the two samples stained with XO. Middle section images are photographs of the boundary of stained scaffolds. The bottom images are the controls without XO. Mineral nodules (fluorescent orange dots) are readily observed in stained samples.

Even though no large quantities of mineralization were detected, the photographs demonstrate to some extent that indeed cells that were initially cultured in the substrate of the vial scaffolds, migrated to the surface of the porous scaffolds, attached and mineralized with concentrated mineralization in the lower surface and around the circumference of porous scaffolds.

4.4.2 Alizarin Red Staining

Alizarin red (AR) is a dye that has been employed to detect calcium formation in static culture and in 3-D seeded scaffolds with bone forming cells. For this experiment, staining with AR would allow for the observation of mineralized tissue with no special equipment. A recipe for the preparation of an alizarin red solution (ARS) was borrowed from [53] and is included in Appendix 1.

After observations were made using XO fluorescence, the vials were removed from the platform and their contents stained with ARS following a method presented by [100]. First, the scaffolds were washed three times with phosphate buffer saline (PBS) and fixed with 10% (v/v) formalin inside a chemical fume hood at room temperature for 15 minutes. Then, they were washed two times with ultrapure water prior to staining with 1 mL of ARS. Scaffolds were incubated at room temperature for 20 minutes and afterwards, the excess dye removed. Finally, the samples were washed several times with ultrapure water with gentle shaking and left at an angle for excess water to drip. After that, the samples could be observed for signs of any stained

mineralized tissue. Porous scaffolds, which hung from the inner pistons, were also stained using the same method to seek any traces of calcium at their surfaces.

Visual inspection of the stained samples indicated that no tissue developed in the gap separating the scaffolds. As a matter of fact, after looking at pictures of the contents of the vials, it was seen that the majority of staining occurred around the space where porous scaffolds were resting during the simulated latency phase. As shown in the picture below in Figure 19, the dye concentrated outwards to the wall of the glass vial with no heavy staining in its center. Similarly, images of stained porous scaffolds were also obtained and they indicated that most of the alizarin red dye had attached to the lower surface and in the wall comprising their circumferential surface. This was in agreement with the observations made of mineralized nodules by XO. Figure 19 also includes photographs of the stained porous scaffolds in a well plate.

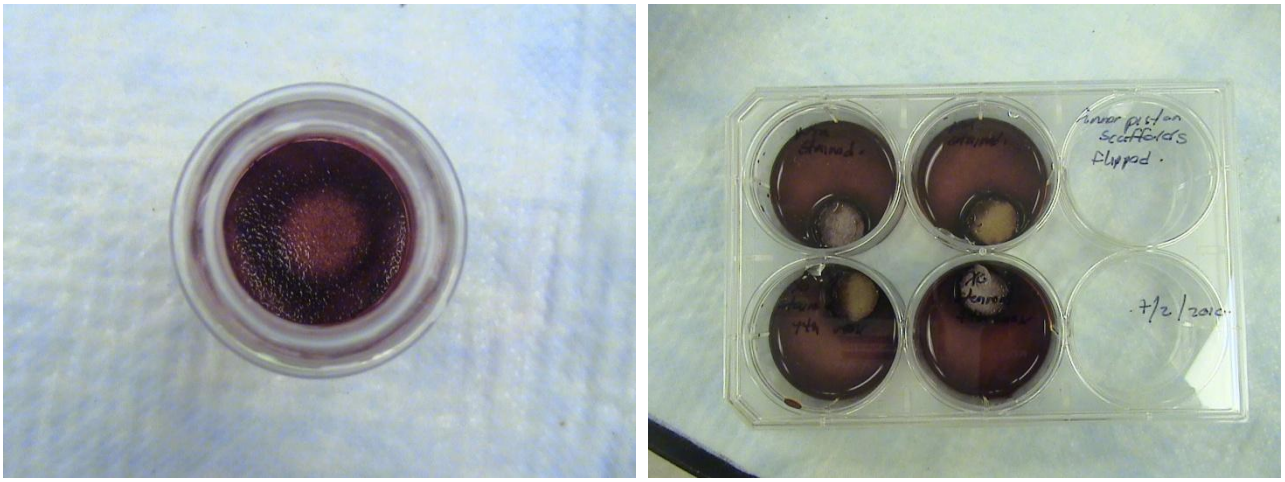

Figure 19 - Pictures of scaffolds stained with Alizarin Red solution.

4.4.3 Fluorochrome Staining of Static Controls

Similar to the experiment for biocompatibility of the material, the static controls were also subjected to two fluorochrome analyses. This was done to have a means for comparing mineralization of scaffolds in static conditions to scaffolds in the distraction regime. Hence, the well plate containing the seeded scaffolds and a static culture of cells were stained using the fluorochromes xylenol orange and calcein blue (CB) at days 14 and 21 post-confluence respectively. In this fashion, calcium deposition by cells could be observed at two points in time with the ability to distinguish old mineralization from new. At day 14, XO was added as described previously. Pictures were taken the next day using microscopy. At day 21, the CB assay was performed following more or less the same procedure; First, old culture medium was aspirated and replaced with fresh medium. Then, 1.5 μL CB per mL of medium were added utilizing the concentration described by [95]. Observations were made also on the following day.

The images taken on days 14 and 21 revealed that cells had indeed created some mineralized nodules in what appears to be a soft tissue material or ECM. Figure 20 reveals this effect concentrated at the boundary of wells. Moreover, since the fluorescence effect remains even when fluorochromes are combined, it was possible to identify old mineral material from new. This was done on day 21 by changing the filter through which observations were made. The following images correspond to the same area photographed utilizing only microscopy, then the XO filter and the CB filter afterwards. Mineralization of this soft-tissue-like material is evident.

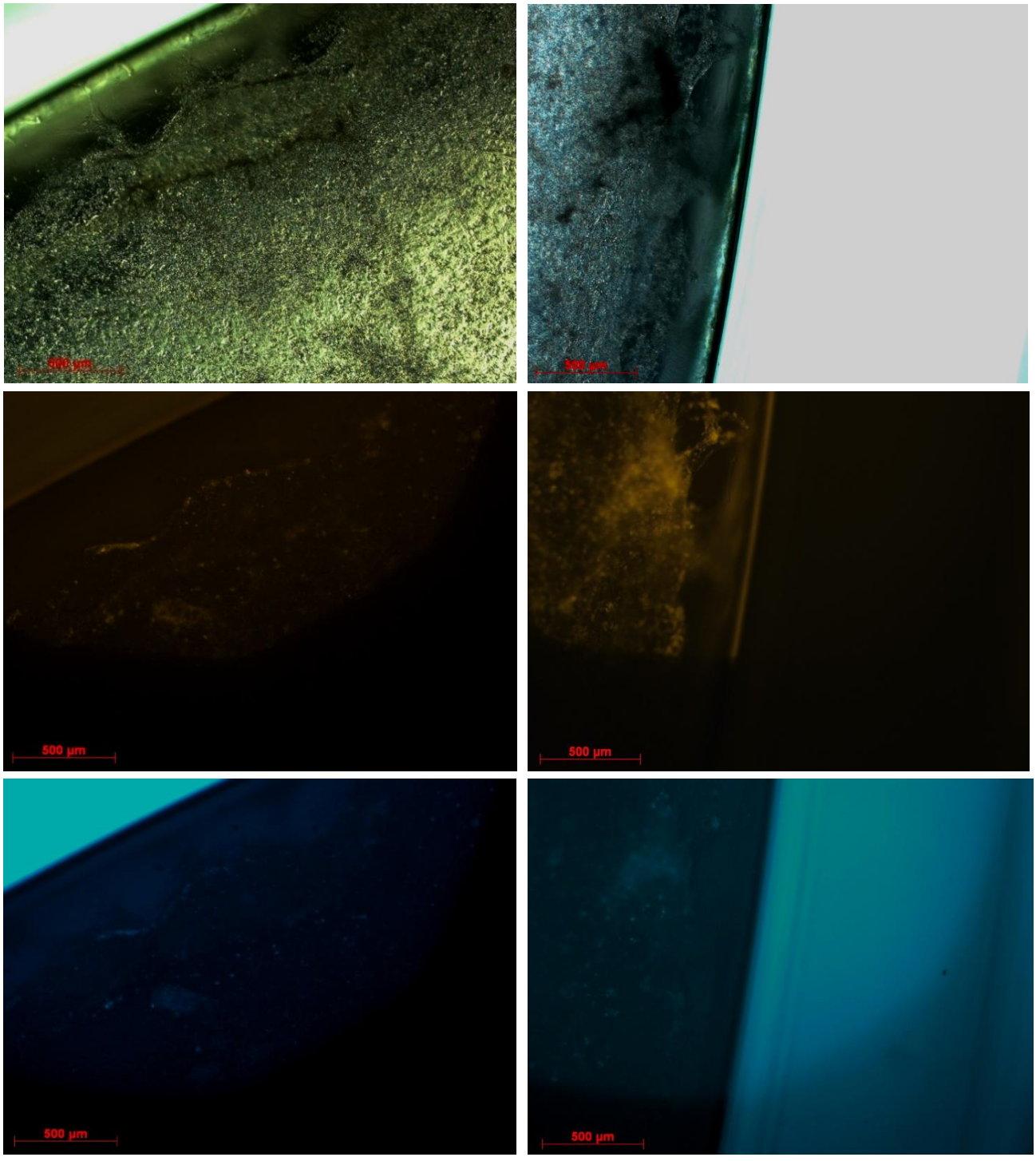

Figure 20 - Photographs of static cultured scaffolds. Top pictures show what appears to be soft tissue or ECM at the boundary of the well. The middle and bottom images, taken in the same area as the top ones, point to mineralization of tissue as seen by the XO and CB filters respectively.

CHAPTER 5
OVERALL CONCLUSIONS AND RECOMMENDATIONS

5.1 IMPLEMENTED SETUP

This thesis focused on the development of a suitable device to replicate, in an in-vitro setup, the surgical procedure of distraction osteogenesis with the aim of growing mineralized tissue in-vitro. The setup was planned for meeting all requirements of cell culture and medicine and it proved to be appropriate for testing the formulated hypothesis of in-vitro mineralized tissue growing. The platform constructed performed correctly, allowing hygienic culture of cells as well as the application of linear movement mimicking the steps of distraction osteogenesis. Further iterations on the design of this device should consider several recommendations listed in section 5.3.

5.2 CONCLUSION OF IN-VITRO OSTEODISTRACTION

The experimentation carried out with BMSC resulted in no evident mineralization of tissue in the structure and quantity expected. In experimental results, seeded bone marrow stromal cells did not attach in the center of the lower vial scaffolds since a void was present in this zone where porous scaffolds were in contact with the surfaces of the vial scaffolds. Cells instead, attached to the exposed surfaces (circumference and bottom) of the porous scaffolds and appeared to mineralize small nodules in this environment. The mineralized material mostly corresponds to alkaline phosphatase produced by cells. These results suggest that indeed there was some

activity at the contact surfaces of scaffolds resulting in, at minimal, the migration of cells from one scaffold to another.

From the photographs obtained of the static cultured controls, cells were observed to form a relatively thick layer of soft tissue similar to ECM within the wells containing them. Furthermore, the images of mineralized tissue with XO and CB markers in Figure 20 corroborate the fact that cells are able to continuously mineralize portions of tissue during various weeks of culture. These observations point to the feasibility of obtaining a similar behavior under the distraction regime if different substrates, rather than PCL are utilized as matrices to culture cells. Nonetheless, using the current experimental setup, the expected behavior was not observed and tissue did not develop in 3-D structures of clinically relevant amounts.

5.3 RECOMMENDATIONS FOR FUTURE WORK

Even though the experiment did not result in the formation of large quantities of mineralized tissue, this setup has tremendous room for improvement. Some of the recommendations for continuing this research are described next, grouped according to particular areas identified.

5.3.1 Materials for Scaffold

This is one of the most important aspects that could be refined in future iterations of this experiment. We strongly believe that other various materials should be researched as scaffold materials under a similar setup. Of particular interest should be

exploring the utilization of naturally occurring polymers like collagen or bone tissue that has been treated with enzymes to wash away cells leaving a demineralized matrix.

5.3.2 Culture of Cells

On the static culture of cells, it was seen that they started to produce ECM and continued mineralization of the same at about 18 days of culture. The fact that cells did not form tissue at the interface of scaffolds might be in part to the lack of proper time to allow tissue to first grow. As recalled from the experiment, right after cell seeding in the vial scaffold, the porous scaffold was set in contact. Thus, after seeding the cells, a period for ECM to be deposited might be required prior to bringing scaffolds into contact. This inherently will require extending the period of culture.

Furthermore, the effect of culturing other cell types could also be investigated. For the cell culture experiments of this research, BMSC were induced to the osteoblast phenotype which might impact the rate at which cells are able to mineralize. If other cell types such as fully mature osteoblast are utilized, rates of mineralization can be greater enhancing the probability for success.

5.3.3 Culture Conditions

The setup utilized took into account many of the traditional factors utilized in culture of cells, namely incubation temperature, humidity and levels of CO_2. However, future work should also concentrate on investigating the effects of the introduction of other variables or factors to the setup. For instance, rather than solely increasing the gap between scaffolds, a cyclic compression-tension regime which literally squeezes the

scaffolds might improve the activity of cells and hence of mineral deposition. Of similar interest should be the investigation of the effects that induced shear stress has on cells. This could be achieved by re-designing the device to incorporate a perfusion bioreactor system.

5.3.4 Material for Platform

The platform designed and constructed for experimentation had a good performance in terms of maintaining the general hygiene of cells and providing means of simulating the increase in the distraction gap. However, at the conclusion of the present experiment, slight deformation–not affecting the results of this research–was observed on some of the ABS parts such as the support bases. This might be the result of long exposure to the temperature inside the incubator. For this reason, other stronger materials, like aluminum or stainless steel, should be utilized to achieve more dimensional control particularly if extended periods of culture are planned.

APPENDIX 1 – CULTURE MEDIA PREPARATION

MINIMUM ESSENTIAL MEDIUM (A-MEM) (SIGMA M0664-1L)

- The contents of the bottle are added to ~900 mL of pure water and continuous stirring using a magnetic stirring bar rotating at around 200 rpm. This should form a well mixed suspension
- 2.2 g/L of sodium bicarbonate to a pH of 7.0. Final volume can be up to 1 L using pure water. The medium is stored in a bottle and refrigerated to 4 °C

SUPPLEMENTED MEDIA (15% FBS) (α-MEM +DEX MEDIUM)

-Ingredients-

- 340 mL of culture media
- 0.864 grams of Sodium-β-glycerophosphate (Sigma G9891- 100G)
- 0.02 g L-ascorbic acid (Sigma A4544 – 25G)
- 60 mL of FBS
- 0.04 mL of Dexamethasone (97%) (Sigma D4902 – 100mg)
- 0.4 mL of sterile antibiotic stock
- 0.48 mL Sterile fungizone

-Preparation-

- The 340 mL of culture media are poured into a bottle
- After adding the Sodium-β-glycerophosphate, and L-ascorbic acid, the liquid mixture is filtered into the bottle using autoclaved bottle-top filter
- The remaining ingredients are added to the bottle. Then, the solution is mixed well by swirling making sure formation of bubbles is kept to a minimum
- The media is ready and it needs to be stored at 4 °C

*** NOTE: Preparation of –Dex medium required the same steps and ingredients except dexamethasone.**

ANTIBIOTIC STOCK

-Ingredients-

- 10mL Normal saline (0.85%)
- 1.0g penicillin-G (sigma P-3032 100mg/mL)
- 0.5g gentamicin sulfate (Sigma G-1264)

-Preparation-

- Filter sterilize with syringe and a 0.2 micron filter.
- Store at 4°C if used immediately
- Store at -20°C to preserve efficacy for later use

FUNGIZONE

- Sterile fungizone (VWR fungizone SV30078.01)
- Store at -20°C after aliquating in plastic amber micro-centrifuge tubes

PREPARATION OF FLUOROCHROME SOLUTION XYLENOL ORANGE

-Ingredients-

- 71.662 mg of Xylenol Orange
- 5 mL distilled water

-Preparation-

- Filter sterilize the solution using a 0.2 micron syringe filter
- Store at room temperature

PREPARATION OF ALIZARIN RED SOLUTION

-Ingredients-

- Alizarin Red powder (Sigma 122777-25G)
- Distilled water
- Ammonium Hydroxide

-Preparation-

- Using 10 mL of distilled water, 40 m-mol of AR powder were added thus forming a 40mM solution.
- Then, drops of ammonium hydroxide were carefully added while measuring the pH of the solution.
- After some mixing, and once the pH stabilized around 4.1, the alizarin red solution was stored at room temperature until use.

APPENDIX 2 – SAMPLE DATA FROM DISTRACTION REGIME

Time (s)	*Displacement (mm)*
0	***0.000***
60	***0.025***
120	***0.050***
180	***0.075***
240	***0.100***
300	***0.124***
360	***0.150***
420	***0.175***
480	***0.200***
540	***0.225***
600	***0.249***
660	***0.275***
720	***0.300***
780	***0.325***
840	***0.350***
900	***0.375***
960	***0.400***
1020	***0.425***
1080	***0.449***
1140	***0.475***
1200	***0.499***

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