THE EFFECTS OF OSTEOGENESIS ON THE MECHANICAL PROPERTIES OF A 3-D POLY LACTIC ACID SCAFFOLD

A Thesis Submitted to the Department of

Materials Science and Engineering

African University of Science and Technology

in partial fulfillment of the requirements for the degree of

Master of Materials Science and Engineering.

OGBONNIA CHINAZA ESTHER

2021

CERTIFICATION PAGE

This is to certify that the thesis titled “The Effects of Osteogenesis on the Mechanical Properties of a 3-D Poly Lactic Acid Scaffold”

submitted to the school of postgraduate studies, African University of Science and Technology (AUST), Abuja, Nigeria for the award of the Master's degree is a record of original research carried out by

Ogbonnia, Chinaza Esther in the Department of Materials Science and Engineering.

SIGNATURE PAGE

THE EFFECTS OF OSTEOGENESIS ON THE MECHANICAL PROPERTIES OF A 3-D POLY LACTIC ACID SCAFFOLD

By

Ogbonnia Chinaza Esther

A THESIS APPROVED BY THE MATERIALS SCIENCE AND ENGINEERING DEPARTMENT

RECOMMENDED: ----------

Dr. Shola Odusanya

Prof. Wole Soboyejo

Prof. Peter Onwualu

APPROVED: ----------

Chief Academic Officer

June, 2021

Date

COPYRIGHT PAGE

ABSTRACT

Poly-Lactic Acid is used widely in biomedical applications due to its good biocompatibility (i.e. biological and chemical inertness) and biodegradability. The area of tissue Engineering has been explored for the regeneration of human organs and tissues. This study explores the structure and the mechanical properties of 3D PLA scaffolds that are cultured with human osteoblasts for bone regeneration. The initial stages of cell spreading and interactions are studied along with the mechanical properties of the scaffolds before and after cell spreading and interactions. The effects of simulated body fluid are also studied to provide insights into the potential degradation that can occur during exposure to human body fluids. The implications of the work are discussed for the integration of 3D PLA scaffolds during the initial stages of bone regeneration and repair.

KEYWORDS: Poly-Lactic Acid scaffolds, degradation mechanisms, human osteoblasts, cell/surface interactions, bone tissue regeneration.

ACKNOWLEDGEMENTS

I give thanks to God almighty for giving me the opportunity to complete this program and I sincerely appreciate the guidance and encouragement of my supervisors Prof. Wole Soboyejo and Dr. Shola Odusanya throughout the course of my thesis.

I am truly grateful to Dr Ali Salifu, for his assistance with the experimental work. Special thanks and acknowledgements are also due to Madam Theresa Ezenwafor for her timeless support and care, and my senior colleagues and advisors, Dr Tido Stanislas, and Mrs Josephine Opara for their assistance.

Special thanks to the HOD of Materials Science and Engineering (MSE) at AUST, Prof. Peter Onwualu, for being a patient and accommodating father. I would also like to thank my colleagues in the MSE department at AUST, most especially Kaosarat Ayodamola, Jamael Ajah, and Darius Woyen, for their support and encouragement.

Finally, I would like to appreciate my dear husband for his patience and understanding during the period that I spent working on this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BTE- Bone Tissue Engineering

SBF- Simulated Body Fluid

TE- Tissue Engineering

PLA- Poly-Lactic Acid

PLGA- Poly (lactic-co-glycolic acid)

HA- Hyaluronic Acid

Tg- Glass Temperature

SEM- Scanning Electron Microscopy

CaP- Calcium Phosphate

PLL- Poly-L-Lysine

PCL- Poly(caprolactone)

PLLA- Poly (L-Lactic Acid)

CHAPTER ONE

1.1 Background and Introduction

Bone is a dynamic and highly vascularized tissue that continues to remodel throughout the lifetime of an individual(Stevens, 2008). It plays an integral role in locomotion, ensures the skeleton has adequate load-bearing capacity, and acts as a protective casing for the delicate internal organs of the body(Stevens, 2008). The worldwide incidence of bone disorders and conditions has trended steeply upward and is expected to double by 2022, especially in populations where aging is coupled with increased obesity and limited physical activity(Amini, 2013).

On the other hand, bone tissue engineering is concerned with creating implantable bone alternatives for life-threatening skeletal defects that cannot heal on their own. These defects are common clinical situations in orthopedics and craniofacial surgery, for the treatment of bone loss due to accidents, trauma, infection, and tumor resection(Bambole & Yakhmi, 2016). Engineered bone tissue has been viewed as a potential alternative to the conventional use of bone grafts (Amini, 2013). Skeletal defects are found to be one of the most frequent, devastating, and costly problems in human health care(Langer & Vacanti, 1993.). To circumvent these problems, the innovative therapeutic approach of the 21st century is focusing on bio artificial bones using a multidisciplinary and complementary approach with the long-term goal of achieving better treatments. For the past decades, Nigeria has witnessed a surge in the number of road accidents especially those involving motorbikes and bone fractures(Nantulya et al., 2010). There is, therefore, the potential to explore the use of tissue engineering in the repair of bone fractures other bone conditions.

Hence, in this study, we explore the initial stages of bone regeneration from porous 3-D printed Poly-Lactic Acid (PLA) structures that are seeded with human osteoblasts. The initial stages of cell spreading and integration are studied using a combination of light microscopy, confocal microscopy and scanning

electron microscopy. The scaffold degradation mechanisms are also elucidated in the presence of simulated body fluids. The study also examines the changes in mechanical properties due to cell spreading and exposure to simulated body fluid. The implications of the results are then discussed for the development of PLA scaffolds for bone regeneration and repair.

1.2 Problem Statement

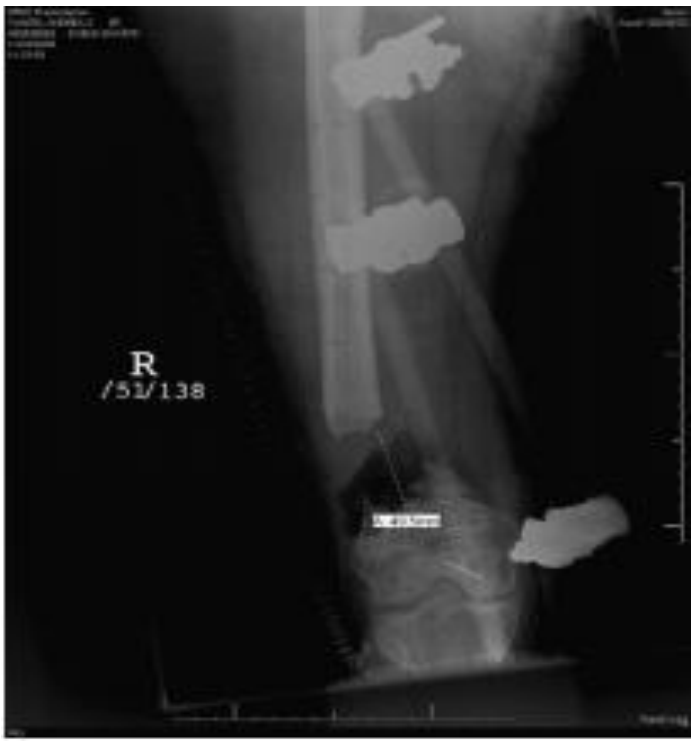


Fig. 1 showing a nounion as a result of trauma

The replacement of injured bones or fracture repair were/are done with autografts and allografts (Grabowski & Cornett, 2013). These grafts provide the necessary mechanical and structural support during bone healing (Jayatissa & Bhaduri, 2019). Autograft bone tissue is taken from the healthy bone of same patient and implanted at the fractured site (Tripathi et al., 2011). This autograft contains viable cells, including new forming bone cells, it also supports the stability of the fractured site.

However, the harvest site of autograft is subjected to injury due to the removal of the graft, and the patient suffers donor site morbidity such as blood loss, infection risk, and scar formation (Jayatissa & Bhaduri, 2019). Allograft is gotten from a corpse and it supports mechanical stability at the defect site and allows for cell attachment and cell function for surrounding cells (Boyce et al., 1999). The two clinical disadvantages of the allograft are immunologic misalliance and risk of transmission of virus-related diseases.

Also, Ti-6Al-4V is often used because of its compatibility and ability to enhance bone remodelling, including its favorable properties of high strength, rigidity, fracture toughness. However, such implants have limited expected lifetimes of about 10 years. They are also non-biodegradable, unlike biopolymers such as PLA, PGA, PCA. However, further work is needed to improve the mechanical properties of the scaffolds in ways that could enhance their capacity to support load-bearing functions, especially at bone defect sites(Salifu et al., 2020).

Human	Tensile strength (MPa)	Compressive strength (MPa)	Flexural strength (MPa)	Elastic modulus (GPa)	Porosity (%)
Cortical Bone	50-151	100-150	135-193	10-20	5-10
Trabecular bone	1-5	2-12	10-20	0.1-5	50-90

Table 1. Showing some mechanical properties of the human bone

1.3 Objective of the Study

In the course of this study, the goals are:

1. To design and print a 3D degradable PLA Scaffolds for the regeneration of bone tissue from human osteoblasts (bone cells).
2. To study the degradation mechanisms of PLA scaffolds in Simulated Body Fluid and its impact on the mechanical properties of the scaffolds.
3. To study the initial stages of human osteoblast spreading and proliferation on PLA scaffolds, and their effects on the mechanical properties of the scaffolds.

1.4 Scope of Work

The outline of this thesis is as follows:

The first chapter presents the background of bone tissue engineering and their potential applications. It also presents the problem statement, followed by aims and objectives then the scope of work. Chapter two presents a review of prior work on bone tissue engineering, especially as it concerns 3D printed biocompatible and biodegradable polymers. The materials and methods are presented in chapter three, showing the procedures from the design of scaffold to printing down to characterization. The mean pore sizes and pore geometries of the scaffolds are also considered as they control cell growth and cell adhesion. Nanoindentation and tensile testing techniques are also used to determine the mechanical properties of scaffold surfaces whereas mechanical stimulation with infusion bioreactor are applied for the equitable distribution of osteoblasts on the PLA scaffold. Chapter four presents the in-depth discussion of results obtained from SEM, fluorescence microscopy, tensile tests and degradation studies. The implications of the results are also discussed for the initial stages of bone regeneration. The salient conclusions arising from this work are then presented in Chapter 5, along with some suggestions for future work.

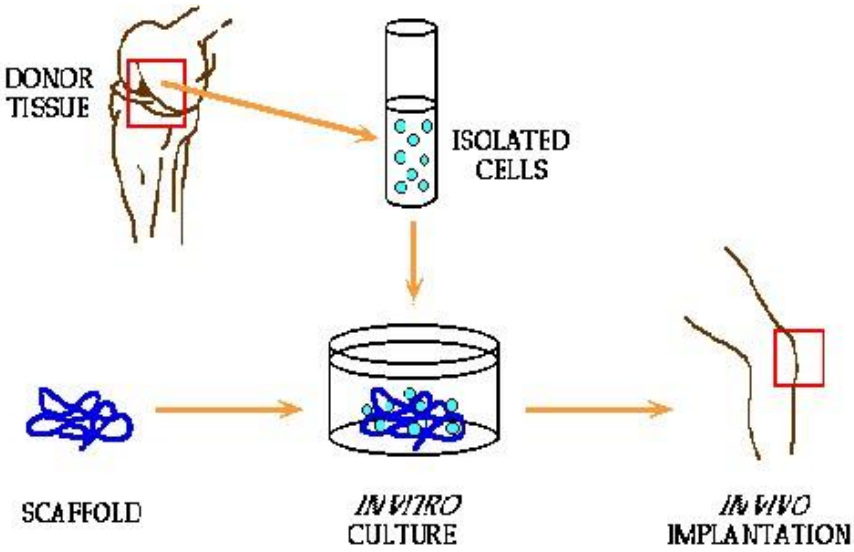


Fig 2. Schematic diagram of the different phases in Tissue Engineering, from scaffold fabrication and cell isolation to in vivo implantation

CHAPTER TWO

2.1 Literature Review

2.2 Introduction

This chapter presents a review of related prior work on: bone loss and defects; the structure of bone; osteoblasts; the mechanical properties of bone; biomaterials and tissue engineering/regeneration.

2.3 Review on the Causes of Bone loss and defects

The term bone loss can refer to either structural defects and regions of missing bone caused by external factors or true defects and structural loss within existing bone(Wiese & Pape, 2010). Bone diseases may also result in bone loss in most cases. Example of this disease is malignancy (primary bone loss). For secondary bone loss, we have metastatic disease. Both primary and secondary bone disease incur damages to the bone structurally, nutrition wise and metabolically. Nevertheless, the most common cause of bone loss is known to be trauma.

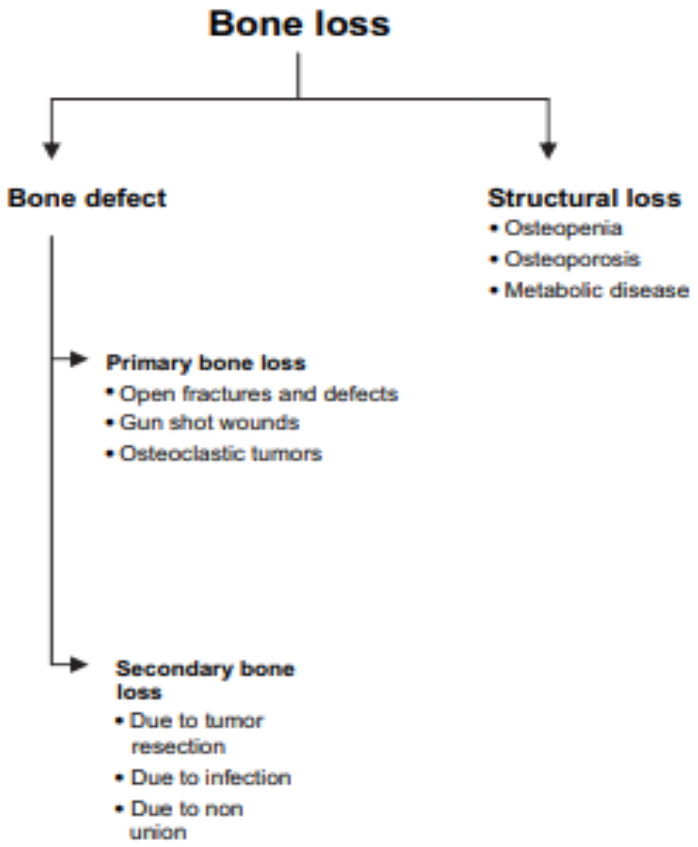


Fig. 3 showing types of bone loss(Wiese & Pape, 2010)**.**

2.4 Nature of Bone

Bone is majorly made up of collagen protein, calcium phosphate, and calcium carbonate, the proteins are woven into flexible framework while the minerals add strength and harden the framework. Therefore, we can say that calcium and protein combine to give the bone flexibility (makes it able to withstand stress and further protects it from breaking) and strength. In as much as bone is strong because of calcium, it also acts as a store house for same calcium. In fact, more than 99% of the body's calcium mineral is contained in the bones and teeth while the remaining 1% is in the blood.

Even though it is mostly made of protein and minerals, bone is a living, growing tissue. Throughout a person's lifetime, old bone is broken down (this process is known as resorption) and new bone is added to the skeleton (called formation). Also, when more bone is broken down than is added to the skeleton, bone loss occurs.

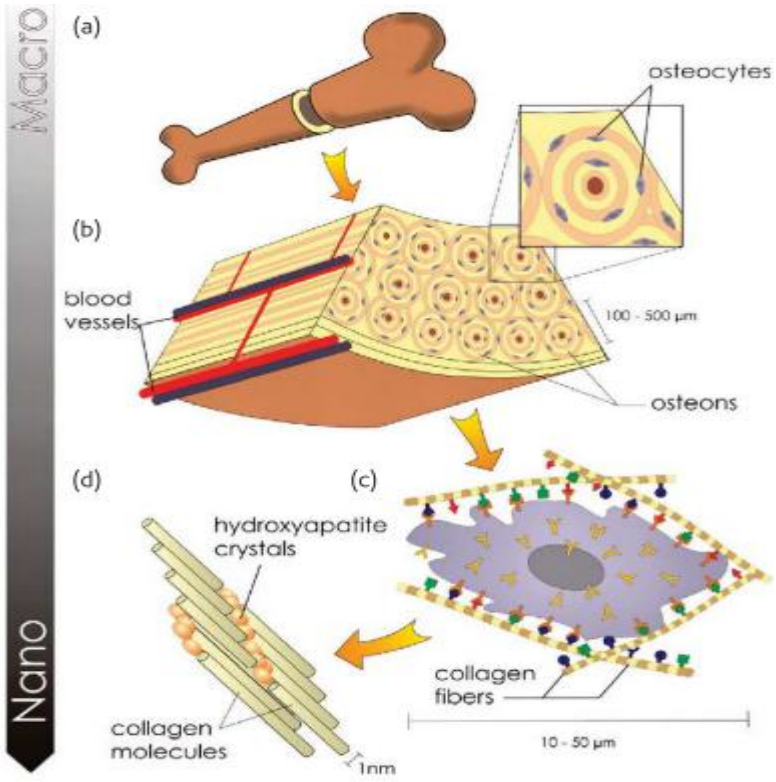


Fig. 4 Hierarchical organization of bone over different length scales. Bone has a strong calcified outer compact layer (a), which comprises many cylindrical Haversian systems, or osteons (b). The resident cells are coated in a forest of cell membrane receptors that respond to specific binding sites (c) and the well-defined nanoarchitecture of the surrounding extracellular matrix (Stevens, 2008)

2.5 Osteoblasts

Bone cells are of three known types which are osteoblasts, osteocytes and osteoclasts. Osteoblasts are the migratory cells that initially propagate around a structure(Redmond, 2015), which once attached and properly stimulated, they begin to form osteocytes under suitable structures. Osteocytes substitute bone in a process known as osteocytic osteolysis. Osteoclasts are born directly from mesenchymal stem cells in the bone marrow and regarded as a progenitor cell. They absorb bone while osteoblasts generate bone.

Bone Cells and Their Interactions During Bone Remodeling

Bone remodeling is partially caused by a need for calcium mineral in the extracellular fluid but it also takes place in response to mechanical stresses on the bone tissue and this can be achieved through the use of 3 types of bone cell that have been mentioned earlier. Communications among the cells Cytokines act as communication molecules between cells and are part of extracellular signaling network that wheels every function. This is a notable factor in TE, although cell types may have common origin, they often have very dissimilar functions which depend on balance in the body.

2.6 Mechanical Properties of Human Bone

1. **Cortical Bone:** At the scale of 1–10 mm, bone tissue can be characterized into two types: cortical bone (also known as compact bone or dense bone) and trabecular bone (also known as cancellous bone or spongy bone). The differences between these two types of bone tissue can be comprised largely on the basis of porosity: Cortical bone has a porosity of 5% to 15%, whereas the porosity of trabecular bone ranges from 40% to 95%. Cortical bone is found in the diaphysis of long bones and in the form of a thin shell surrounding the trabecular compartment in the metaphyses and epiphyses(Morgan et al., 2018).

Longitudinal direction	
Elastic modulus (MPa)	17,900 ± 3,900[b] 18,160 ± 1,880[c]
Poisson's ratio	0.62 ± 0.26[b]
Tensile yield stress (MPa)	71.56 ± 10.19[c,f]
Tensile yield strain (%)	0.67 ± 0.04[c,f]
Tensile ultimate stress (MPa)	135 ± 15.6[b] 92.95 ± 10.07[c]
Tensile ultimate strain (%)	1.9 ± 0.6[c]
Compressive yield stress (MPa)	115.06 ± 16.36[c,f]
Compressive yield strain (%)	0.98 ± 0.09[c,f]
Compressive ultimate stress (MPa)	205 ± 17.3[b] 153.59 ± 21.63[c]
Compressive ultimate strain (%)	1.3 ± 0.3[c]
Shear modulus (MPa)	3,300 ± 400[c] 6,070 ± 570[c]
Shear yield stress (MPa)	40.95 ± 5.16[c,f]
Shear yield strain (%)	0.87 ± 0.04[c,f]
Shear ultimate stress (MPa)	65 ± 4.0[b] 46.31 ± 5.82[c]

Table 2. showing the mechanical properties of human bone in longitudinal direction(Morgan et al., 2018).

2. **Trabecular Bone:** The trabecular bone transfers mechanical loads from the articular surface to the cortical bone. The mechanical properties of this trabecular bone differ and are significantly higher in density, elastic modulus, and ultimate compressive strength. Ultimate strains in trabecular bone ranges from 1.0% to 2.5% and they appear to be isotropic. The yield strains range from 0.70% to 0.77% in compression and 0.65% to 0.71% in tension, while the elastic modulus ranges from 10-20 GPa.

2.7 Scaffolds

Scaffolds are three-dimensional (3D), biocompatible, and porous structures, which can mimic the extracellular matrix (ECM) properties including mechanical support, cellular activity, and protein production through biochemical and mechanical interactions (Jayatissa & Bhaduri, 2019). These porous

scaffolds play a central role in our modern regenerative medicine and tissue engineering as it offers a controlled biophysical and biochemical environment that can direct cellular behavior and function. In general, scaffold gives a tentative three-dimensional (3D) support for initial cell attachment and to control their functionality, thereby guiding complex multicellular processes of tissue formation and regeneration (Lutolf & Hubbell, 2005). The design of the scaffolds is done in such a way that maximum adhesion can be achieved along with rapid growth within the scaffold to allow diffusion of cells throughout while allowing the cells access to the medium, additionally, good surface area allows the rapid propagation and proliferation of osteoblasts. The mechanical properties of scaffold composite structures are strongly influenced by the morphologic properties such as shape, size and arrangement of the structure therefore having knowledge of these factors is crucial in the accurate predictions of bone's mechanical properties (Sultana, 2018).

Primarily, the scaffold should be biocompatible, biodegradable and have desired surface properties for cell adhesion, migration and normal functionality. Secondly, since human tissues have selective mechanical properties ranging from soft (brain, about 0.5 kPa), to moderately stiff (skin and muscles, around 10 kPa) and stiff (precalcified bone, >30 kPa), then the scaffold construct must have desired mechanical strength and porosity to be able to fit in to the surrounding tissue(Anderson et al., 2009). Also, the scaffold should be the size or area of the defect site.

Moreover, it is proven that controlled delivery of biological signals from porous scaffolds, such as small drug molecule, growth factors and cytokines in vitro or in vivo, is crucial in the support and enhancement of tissue morphogenesis, viability and functionality, therefore, the design should consider the physico-chemical properties of scaffold materials so that they can release desired biomolecule to guide and regulate biological responses of the cells into specific tissue (Mohammadi et al., 2018).

2.8 Biomaterials

1. **Osteoinductive Materials**: Osteoinductive also known as smart biomaterials are able to

incite ectopic bone formation. They do this by instructing its environment in vivo to generate

bone(Blokhuis & Arts, 2011). Osteoinductive materials hold great ability for regeneration of bone tissues. This family of biomaterial include natural and synthetic (e.g. hydroxyapatite) ceramics, calcium phosphate composites (e.g. polylactic-coglycolic acid) having osteoinductive properties. Some other studies have explained osteoinduction by the various forms of CaP-based biomaterials especially in the

form of sintered ceramics(Experimental & Ripamonti, 1991), coral derived ceramics and coatings(Pollick et al., 1995). Recently, some other ceramics like alumina ceramic, polymer/ceramic composites e.g. PLGA/HA are considered osteoinductive(Khan et al., 2004).

However, it is important to note that other material properties play a crucial role in osteoinduction, besides the chemical composition of the biomaterial, which may include porosity of the biomaterial implant and its surface properties, such as nano/micro topography(Amini, 2013). Significantly, osteoinductivity level is also dependent on the interspecies variation and explained by two existing theories although without concluding evidence. One of the theories are based on the biomaterial surface features that take in and introduce osteoinductive factors to the environment cells. The other theory is that the CaP–based materials release calcium and phosphate ions, which later stimulate stem cell differentiation into osteoblasts(Ftipamonti, 1996).

2. **Hybrid Materials**: A number of synthetic and natural polymers, as well as ceramics

have been developed and identified as biomaterials for BTE. Bonescaffolding are done using biomaterials which must have basic properties (physical, chemical and biological) though it is of great difficulty to have any biomaterial that will be up to standard, satisfying all the above properties.

In recent times, advanced biomaterial class such as co-polymers, polymer-polymer blends or polymer-ceramic composites having improved functionalities are considered as they are more useful in the scaffolding of bones and related applications.

a. **Co-polymers**: These are biomaterials derived from two or more monomers. For example, polylactide-co-glycolic acid (PLGA) are co-polymer systems derived from poly lactide and polyglycolide. polylactide displays a glass transition temperature (Tg) above room temperature with a long degradation time, but polyglycolide displays Tg below room temperature and a shorter degradation time. PLGA-PCL, PLGA copolymerized with PLL, and PLA- co-polymerized PCL are examples of other co-polymers that are effective(Ulery et al., 2011).
b. **Polymer–polymer blends:** These biomaterials involves mixture of two polymers(Krogman et al., 2009). Here the aim is to achieve a miscible system with improved properties and polymers with necessary intermolecular interaction(Krogman et al., 2007). PLGA biomaterials generate acidic byproducts upon degradation, and this has been a great problem since the long-term tissue exposure to acidic products may result in tissue necrosis and implant failure(Lakshmi et al., 2003). Moreover, polyphosphazenes release neutral or basic products in degradation(Deng et al., 2008). Therefore, PLGA has been mixed with a wide variety of polyphosphazenes to achieve novel biomaterials with near-neutral degradation products(Ambrosio et al., 2002).

c. **Polymer-ceramic composites:** Bone is a composite of hydroxyapatite crystals (HA) and organic collagen fibers, so, composite materials are good candidates for bone tissue engineering(Athanasiou et al., 2000). Polymer and ceramics composites capitalize on the strengths of each of the components and have shown great success in the regeneration of bones greater than achievements gotten from individual usage(Kim et al., 2006). Composites of HA and various polymers, including poly(lactic acid) (PLA),(G. Wei & Ma, 2009) PLGA, gelatin, chitosan,(Zhang et al., 2003) and collagen (C. V. M. Rodrigues et al., 2003) are fabricated successfully and have shown enhanced bone formation in vitro and/or in vivo. These materials are considered to stimulate the development, precipitation, and deposition of CaP from simulated body fluid (SBF) that will give rise to enhanced bone-matrix interface strength(Krogman et al., 2009). In addition, porous poly(L-lactic acid) (PLLA)/HA composite scaffolds have shown to possess higher osteoconductivity properties and to enhance osteoblastic cell survival and proliferation when compared to pure PLLA scaffolds in their 6 weeks of cultivation in-vitro. Upon implantation, the addition of HA to natural polymer scaffolds has been shown to improve the bioactivity and mechanical properties compared to polymer control scaffolds(Ho et al., 2006). Accelerated bone generation with composite PLA/HA scaffolds in a rat femur defect model have been observed as compared to pure PLA scaffolds(Higashi & Yamamuro, 1986). In all, polymer/HA composites show osteoconductive superiority to their pure polymer counterparts.

3**. Advanced Hydrogels:** These biomaterials have exceptional biocompatibility and necessary

physical characteristics and have been in use for tissue engineering. Not only that hydrogels serve as matrices for tissue engineering as well as in regenerative medicine, they also act as an extracellular matrix topography(K. Y. Lee & Mooney, 2001) and in delivering necessary bioactive agents that promote tissue regeneration. Recently, some scaffolds self-assemblers known as self-assembling peptides have gained recognition for their biodegradability and biocompatibility(International & Cell, 2007). These perform like the natural extracellular matrix as they are easily produced biologically and chemically and are usually used as starter materials.

4. Immuno-modulatory Biomaterials: immune-modulatory biomaterials as their name implies are materials that are used to modulate immune system favorably for improved bone repair and regeneration(Mountziaris et al., 2011).

2.9 Biodegradable Scaffolds

1. **Scaffold Mechanical Integrity, Structure, and Mechanotransduction**: A good scaffold for bone tissue engineering should be able to provide a non-permanent mechanical integrity at the bone defect area

till the bone can be repaired or till it gets regenerated, regaining its biomechanical function. It is also important to ensure the mechanical properties of the scaffold for BTE rhymes with that of the surrounding bone of the patient so as to be regarded as functional(Butler & Goldstein, 2016). Also, the mechanical strength of the scaffold has an effect on the mechanotransduction of the seeded osteoblasts on the scaffold thereby playing a special role in repairing and remodeling.

It has been projected that, generally, the structural biomechanics of the BTE scaffold is linked to the osteoconductive properties of the scaffold, while mechanotransduction is linked to its potential osteoinductive properties(Taylor & Pioletti, 2010). Biomechanical stimuli of cells due to the scaffold distortion largely powers osteoinduction (i.e., bone ingrowth from the host).

2. **Scaffold Porosity**: The porosity of a scaffold is a major element of the properties of a scaffold as regards osteoconductivity. The size, volume and other pore structures plays a major role in the proper cell seeding, growth, proliferation, vascularization and organization of choice cells(Anagnostou et al., 2005).

However, some uncertainty remains regarding the ideal porosity and pore size for a 3-D bone scaffold, there are some suggestions that scaffolds designed with small pore sizes (i.e., <200 μm) show in vitro and in vivo osteoblast survival and bone formation only on the periphery, this is because of low rate of oxygen and nutrient diffusion all over the scaffolds(Karageorgiou & Ã, 2005). On the contrary, scaffolds with a mean pore size of 300 μm display increased osteoblast proliferation and differentiation throughout the entire scaffold, due to high rate of oxygen and nutrient diffusion all over the scaffolds(Osteogenesis, 1997).

However, as porosity and mean pore sizes increase, mechanical strength is incised; determination of a

balance between mechanical strength and porosity is vital. This study shows that, by producing scaffolds with maximum pore size, it is possible to maintain oxygen tension and pH levels inside a scaffold that are almost comparable to the values measured on the scaffold peripheral. Such scaffolds, called oxygen tension-controlled matrices, have been proven to support cell proliferation and mineralization throughout the scaffold structure (i.e., fully osteoconductive) in vitro and in static culture, and they may have the potential to repair large-scale or critical-size bone defects in vivo(Hou et al., 2003).

2.10 Poly-Lactic Acid (PLA)

This is a polyester which degrades within the human body to form lactic acid, a naturally occurring chemical which is easily removed from the body. Similar materials are polyglycolic acid (PGA) and polycaprolactone (PCL) with their degradation mechanism similar to that of PLA, but they exhibit

respectively a faster and a slower rate of degradation compared to PLA(Gregor et al., 2017). These polymers exhibit hydrophobicity but high mechanical strength and structural integrity. Their hydrophobic nature inhibits their biocompatibility, making them less effective for in vivo use as tissue scaffolding(N. Rodrigues, 2016). In a bid to fix this biocompatibility, researches have been done to combine these hydrophobic materials with hydrophilic and more biocompatible hydrogels. These hydrogels have a superior biocompatibility, but they lack the structural integrity of PLA, PCL, and PGA. These two different materials are then combined to create a synergistic relationship which produces a more biocompatible tissue scaffolding(Bosworth et al., 2012). Polylactic acid (PLA) has been extremely useful and used to do a lot of work, but there are still issues relating immune system responses to lactic acid during its degradation in vivo as well as its low modulus, nevertheless, these can be overcome by adding components such as HA (hyaluronic acid) as well as additional composite materials(Hutmacher, 2000).

2.11 Physiochemical Properties of PLA

PLA was first synthesized in 1932 by Carothers (DuPont), and its patenting occurred in 1954, as a higher molecular weight was attained. Its monomer is the lactic acid molecule, and its esterification leads to the existence of stereoisomers, such as poly (L-lactide) (PLLA), poly (D-lactide) (PDLA), and poly (DL-lactide) (PDLLA). The eco-friendly characteristics of PLA, in terms of renewability, recyclability, non-toxicity and compostability, make it very promising in the perspective of green chemistry applications, whereas its cytocompatibility and the biocompatibility of its degradation products make it attractive as material for biomedical and drug delivery applications. Depending on these characteristics, PLA may be amorphous or semi-crystalline and its crystallinity may depend on intrinsic chemical-physical properties, such as stereochemistry, or preparation conditions, such as thermal history(Elsawy et al., 2017). PLA tends to be crystalline when the amount of PLLA is higher than 90%; otherwise, it tends to be amorphous. The content of PLLA affects even melting temperature (Tm) and glass transition temperature (Tg), as well as mechanical properties. Elastic modulus proved to increase from less than 1–3.5 to 2.7–4.1 GPa as a function of L-lactide content, whereas tensile strength was found to vary from 20– 50 to 60–80 MPa. Notably, elongation at break of PLA seems to be scarcely affected by stereoisomer content and equal to 2–10%. Physical characteristics such as density, heat capacity, and mechanical and rheological properties of PLA are dependent on its transition temperatures(Scaffaro et al., 2020).

2.12 Hydrolysis of PLA

The degradation properties of a scaffold are of essential importance for biomaterial selection and design especially for the long-term success of a tissue engineered construct. Polymers have been shown to degrade mainly by simple hydrolysis of the ester bond into acidic monomers, which can be removed from

the body by normal metabolic pathways. Other factors that affect degradation include hydrophobicity and molecular weight. The chain cleavage reaction during the hydrolytic degradation of PLA proceeds preferentially in amorphous regions, which leads to an increase in the polymer crystallinity and it, has also been noted that the crystallinity of PLA tends to increase as it degrades. In aqueous solutions, the hydrolytic degradation of PLA proceeds via random cleavage of the ester bond, which is controlled by four basic parameters: the rate constant, the amount of absorbed water, the diffusion coefficient of chain fragments within the polymer, and the solubility of degradation products. In general, the hydrolytic degradation of PLA-based solid polymer matrices can proceed through under two different mechanisms: (i) surface or heterogeneous reactions and (ii) bulk or homogeneous erosion. Hydrolytic rate was dependent on the molecular weight of the oligomer along with pH and temperature of the media.

2.13 Cell Adhesion

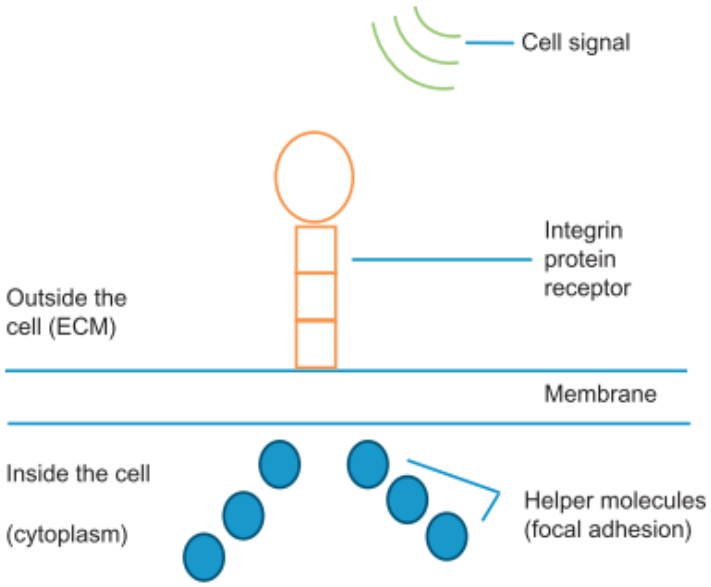


Fig. 5 Schematic diagram of integrin protein and cell adhesion process. (Sultana, 2018)

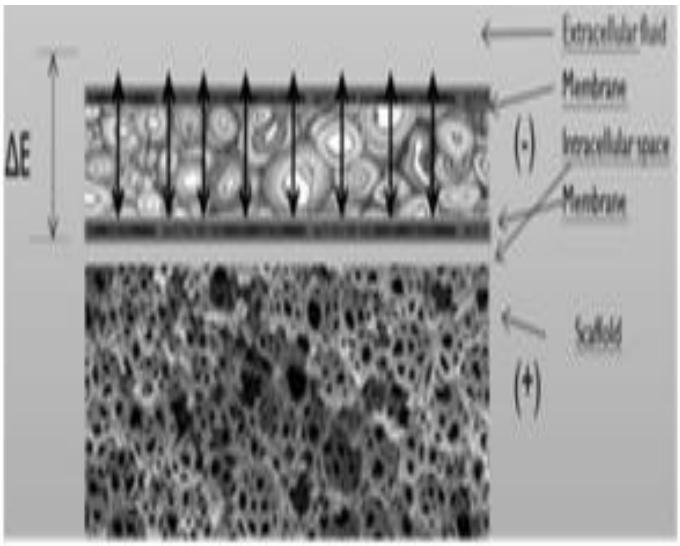


Fig. 6 showing mechanism of cell adhesion (Duran et al., 2012)

The typical values for cell adhesion of mammalian osteoblast cells range from 0.2 kPa to 20 kPa (Redmond, 2015). Cell adhesion as regards 3-D scaffold are of important consideration; if the adhesion forces are great, the cell growth will be slower, but if the adhesion forces are weak, the cells will be detached and there will be no growth (Anderson et al., 2009). The behavior of cells on a scaffold are influenced by the chemistry, architecture and surface topography of the scaffold, whereas the interaction is by focal adhesion. Protein adsorption occurs, likewise, cell adhesion receptor (integrin) is used to provide signaling to the cells, thus mediating cell (Yildirim et al., 2010) **.** Subsequently, cell adhesion enables the release of active compounds that give signals for cell proliferation and differentiation(Goddard & Hotchkiss, 2007). Vitality of a cell can be determined through its adhesive ability.

Another important consideration is the hydrophobicity or hydrophilicity of the scaffold surface. This property effects cell responses and can be determined by the contact angle measurement. An angle above 90 degrees is considered hydrophobic, and that below 90 degrees is considered hydrophilic. It has been proven overtime that cells thrive better on scaffolds with hydrophilic surfaces, they facilitate better cell adhesion and proliferation (J. Wei et al., 2009)**.**

2.14 Degradation Studies

Biodegradability is one of the required scaffold functions for bone tissue engineering, and it is influenced by the mechanical micro-environment after implantation into the body(Shui et al., 2019). PLA degrade by simple hydrolysis of the ester bond into acidic monomers, which can be removed from the body by normal metabolic pathways. Factors that affect degradation include hydrophobicity, molecular weight, chemical structure, porosity, surface/volume ratio, degradation temperature and pH of the medium. The rate of hydrolysis is known to increase with degradation temperature which would potentially make the correlation between short-term effects at high temperature and long-term effects at physiological temperature achievable (Felfel et al., 2016). The main aim of accelerated degradation studies is to achieve degradation profiles within a shorter period of time and descriptive to what is obtained at standard degradation conditions (Ferreira et al., 2017). Accelerated degradation at elevated temperature allows effective fast screening rather than long term studies in other to save research funds and time (Pietrzak et al., 2003). The rate of degradation might affect cellular interaction including cell proliferation, tissue synthesis and host response (Babensee et al., 1998). Details of the potential effects of the acidic by products on the 3D cell culture or upon in vivo host response remain understudied.

2.15 Simulated Body Fluid

SBF is a solution with an ion content that is close to that of a human blood plasma. Simulated body fluid (SBF) has been widely used for bioactivity assessment of biomaterials(J. T. Y. Lee et al., 2011). In 1991, Kokubo et al. (1991) projected that the major requirement for an artificial material to bond to living bone is the formation of bone-like apatite on its surface when implanted in the living body, and this in vivo apatite formation can be mimicked in a simulated body fluid (SBF) with its ion concentrations nearly equal to that of human blood plasma. They also suggested that the in vivo bone bioactivity of a material can be predicted from the apatite formation on its surface in SBF.

Ion	Concentration/mol m^3	
	SBF	Human Blood Plasma
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	147.8	103.0
HCO_3^-	4.2	27.0
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5

Table* 3. *Nominal ion concentrations of SBF in comparison with those in human blood plasma(Ã & Takadama, 2006)

CHAPTER THREE

3.1 MATERIAL AND EXPERIMENTAL METHODS

3.2 Introduction

This chapter presents the various materials that were used in the course of this work.

3.3 Fabrication of porous Poly-Lactic Acid Scaffold

First, a commercial low cost 3-D filament printer (Lulzbot TAZ made in North Dakota, USA) was used for printing the scaffold of specified length, width and height using a non-water dissolvable polymer, Poly-Lactic Acid (PLA) filament with grey color pigment.
The square-shaped scaffold design was done using a computer aided design (CAD) software package (Fusion Autodesk 360). The 3D scaffold CAD models is then saved in a .stl format, converted into G-code files to enable the Lulzbot TAZ to proceed for printing the scaffold.
During the printing process, a mobile nozzle (x- and y-axis control) extrudes a heated polymer filament which then solidifies once it is deposited. Subsequently, the z-axis control is lowered and the extrusion process is repeated so that the following layers are placed on top of one another forming a 3D object. The printing infill density was constant to achieve scaffolds with same porosity. Below (table 4) is the 3D printing parameters and settings used for printing PLA scaffold.

3D Printing Parameters	Settings
Layer Diameter	2.85 mm
Nozzle temperature	195^{o}C -200^{o}C
Density	1.25 g/cm^{3}
Printing Speed	40-90 mm/s

Table 4: 3-D Printing parameters as specified by the manufacturers

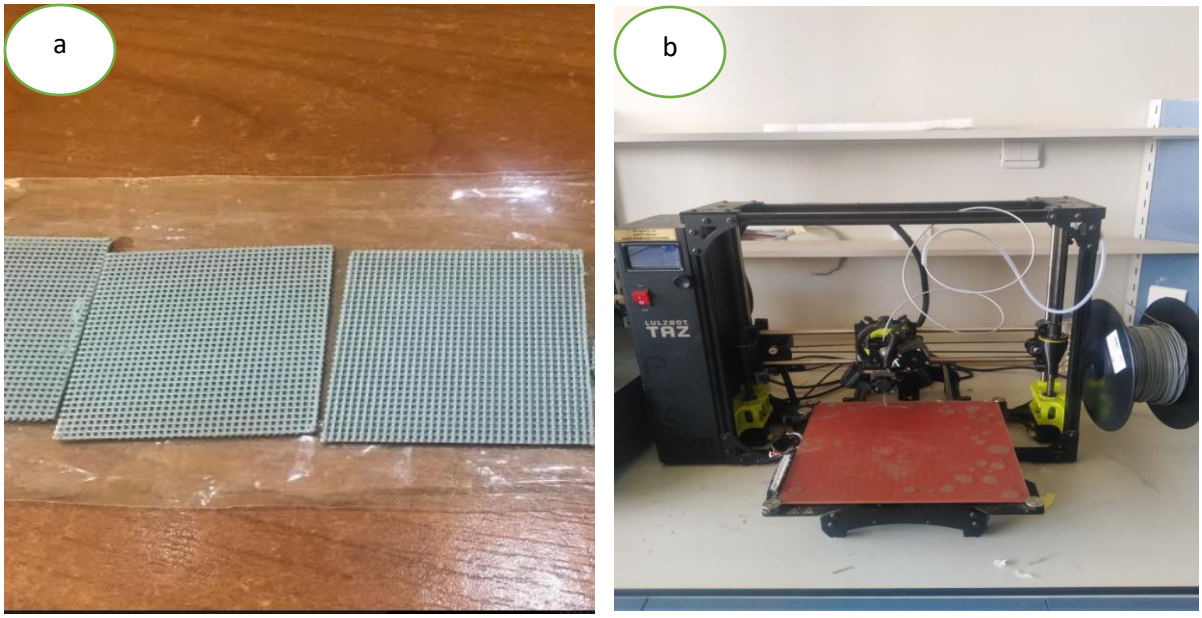


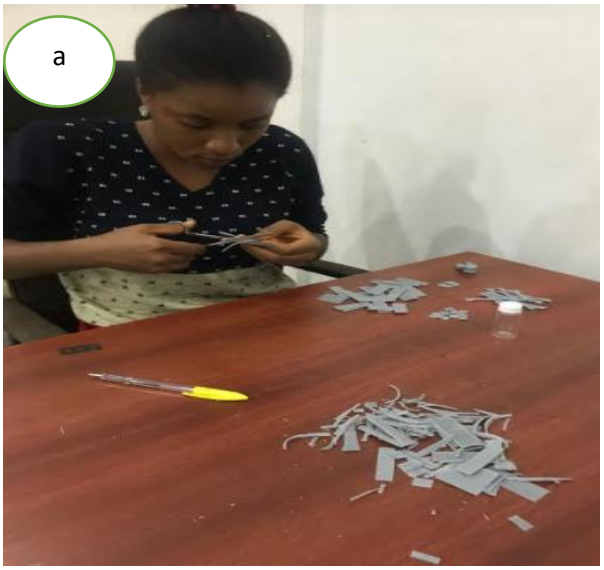
Fig. 7 : a) 3-D printed PLA scaffold (b) Lulzbot printing machine

3.4 Degradation Studies

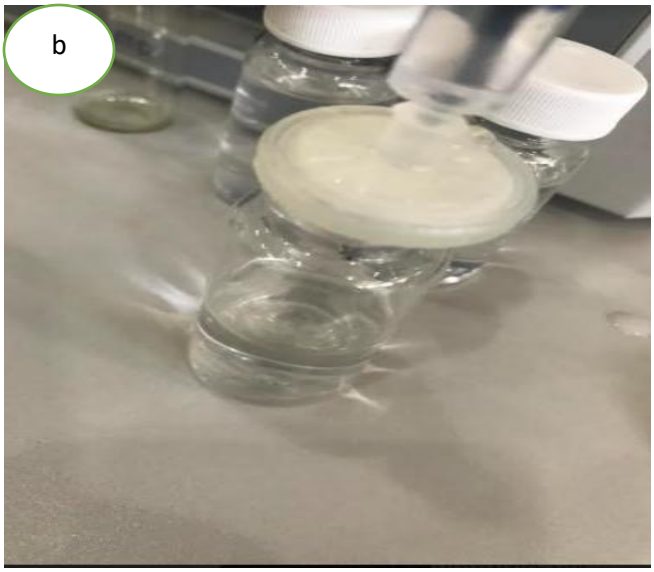
Here, the scaffolds were first cut into dog-bone shapes using a pair of scissors for ease of study (tensile test). Thereafter, a syringe strainer was used to sieve some Simulated Body Fluids (SBF) to remove unwanted micro and macro particles then placed into sample containers.

The dog-bone shaped scaffold samples were then immersed into SBF at a ratio of about 1: 250 and labelled for easy use, after which they are carefully placed in a warm water bath at a constant temperature of 37.5^{o}C.

a



b



c


1 2
3 4
WK 7 SEM

d


CLEAN MONTHLY
IMPORTANT USER INFORMATION
AUST/PH/009

e


37.5
ON
OFF
isotemp

Figs. 8 : (a) process of cutting the PLA scaffold into dogbone shape for tensile tests (b) use of micro filter to remove unwanted particles from the SBF (c) dogbone shaped scaffols immersed in filtered SBF (d) water bath with the samples (e) shows the temperature at which the degradation studies were carried out

3.5 Characterization of Scaffolds

3.5.1 Scanning Electron Microscopy (SEM) Imaging

Scanning Electron Microscope (SEM, ZEISS EVO LS10 USA) was used to investigate the structural morphology and microstructure of the printed PLA scaffold. Before the SEM analysis, scaffold sample was air dried overnight then coated with gold (sputtering) to make it conducting. The scaffold was then analyzed via SEM imaging at an accelerating voltage of 12 kV. The SEM micrographs were used to characterize the pore sizes.

3.5.2 Mechanical Testing

Universal Testing Machine (UTM) designed and built by Instron, a division of Illinois Tool work (ITW) of USA with maximum capacity of 5000N/500N was used to evaluate the tensile mechanical properties of the developed scaffolds (with and without osteoblast laden at various stages) cut into dog bone shapes at a of speed of 1 mm/min and a preloading of 0.5N was applied. The stress-strain curves were then used to determine Young's modulus and tensile strength measurements.

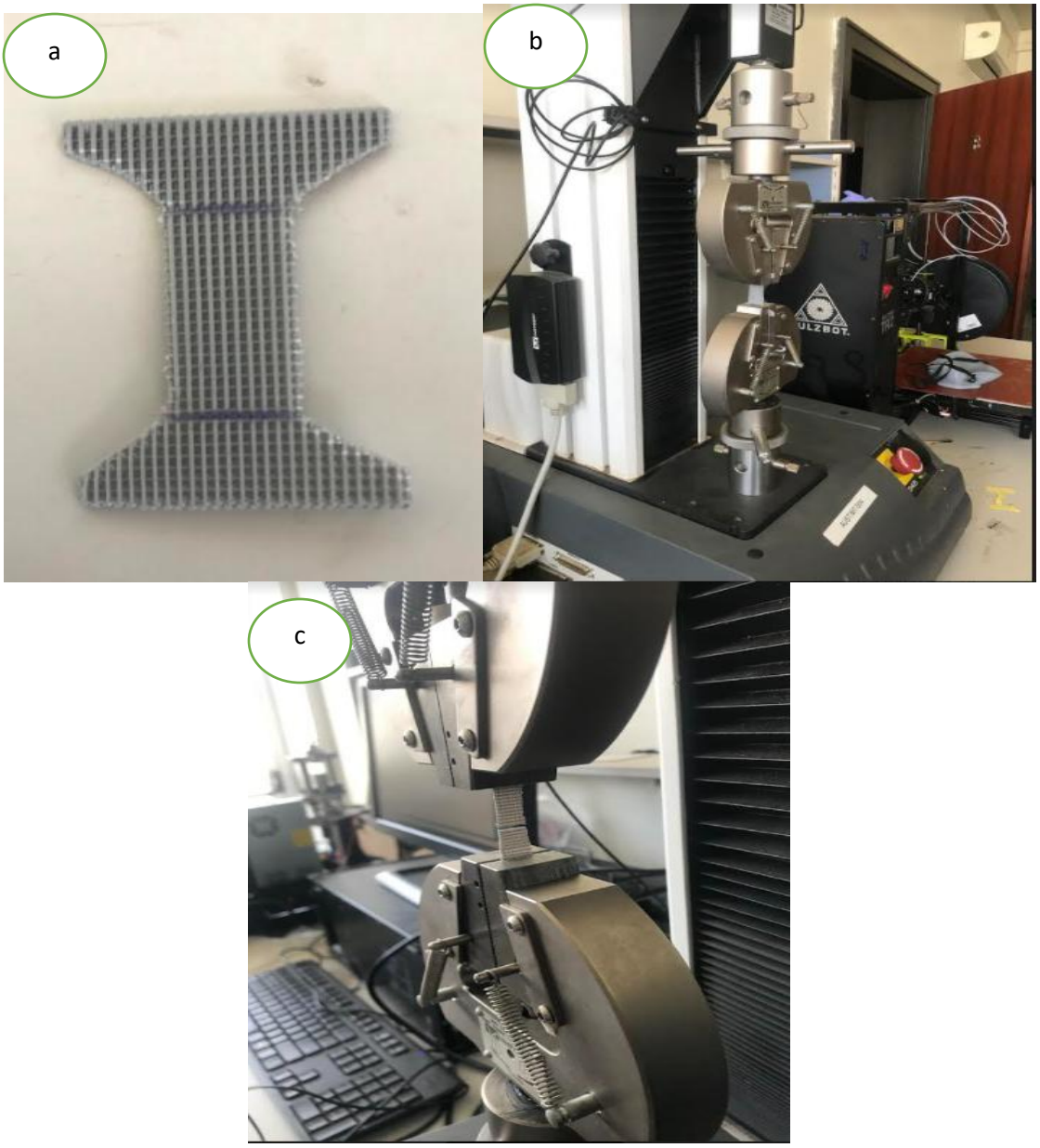


Fig. 9: (a) 3-D Printed dog-bone tensile specimen (b) UTM with the dog-bone shaped scaffold loaded for testing, and (c) Fractured specimen after tensile testing

3.6 Weight Loss

Materials' weight loss during degradation was calculated using a weighing scale at weekly intervals and the various weights were recorded. Before each weight check, we made sure the materials were properly air dried to avoid error due to weight of the liquid (SBF).

3.7 Culturing Cells in Scaffolds

3.7.1 Biochemical Assays

Here, alamarBlue which is a colorimetric indicator was used to ascertain cell proliferation. The cell-laden scaffold was soaked in alamarBlue then left to dry overnight. We then measured the number of cells on the scaffold for different days by obtaining the absorbance of the extracted dye since they are proportional.

3.7.2 Cell Imaging

The cell viability of the osteoblast was visualized using a fluorescence microscope (Zeiss Axio Observer, ZI). This is known as live/dead assay. Here a cell-permeable dye, 4,6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus of the cells and Rhodamine Phalloidin used to stain the cytoskeleton both for about 30 minutes at 37^{o}C. The scaffolds were then cut longitudinally using a sterile blade and each section observed under a fluorescence microscope.

CHAPTER 4

4.1 RESULTS AND DISCUSSION

4.2 Introduction

This chapter presents the results obtained from scaffold fabrication, degradation, mechanical testing, and materials characterization studies that were carried out before and after cell spreading or degradation.

4.3 Scaffold Fabrication

The scaffolds were fabricated using the Lulzbot 3-D filament printer in a woodpile pattern at constant density. The pictures and the SEM images of the printed PLA scaffolds are shown below. They have a well-defined porous structure that are consistent and uniform. The printed PLA scaffolds have pore size of 963.3 µm and strut dimensions of 25 mm X 25 mm X 0.8 mm.

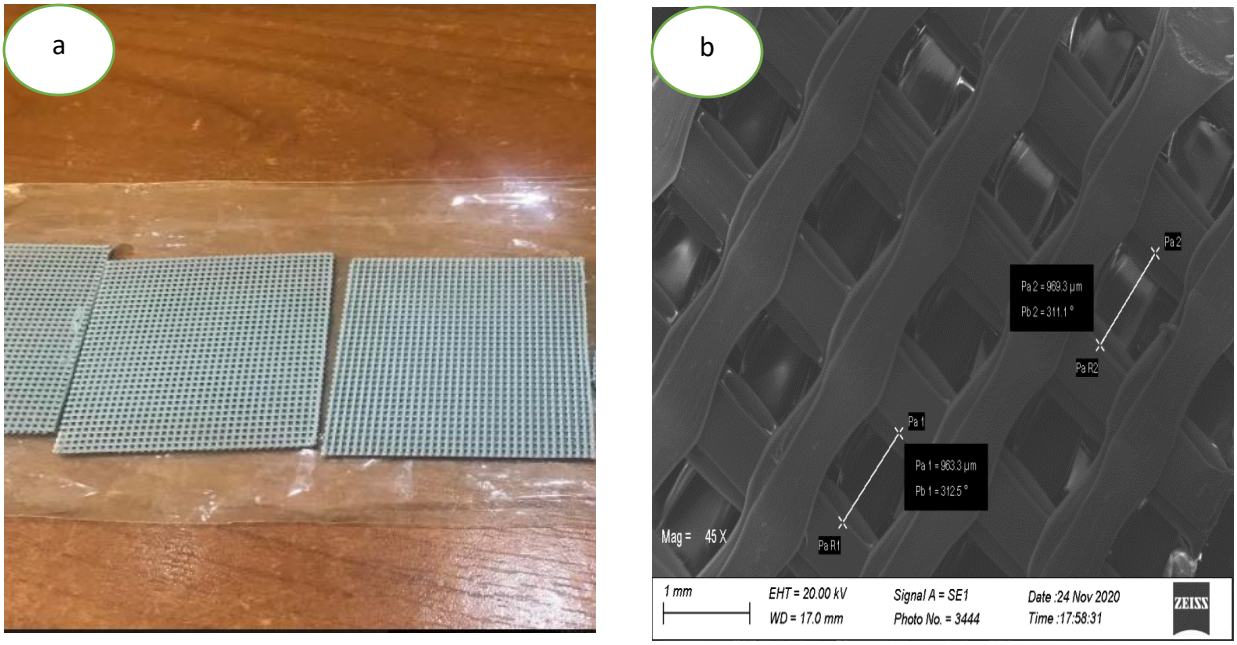
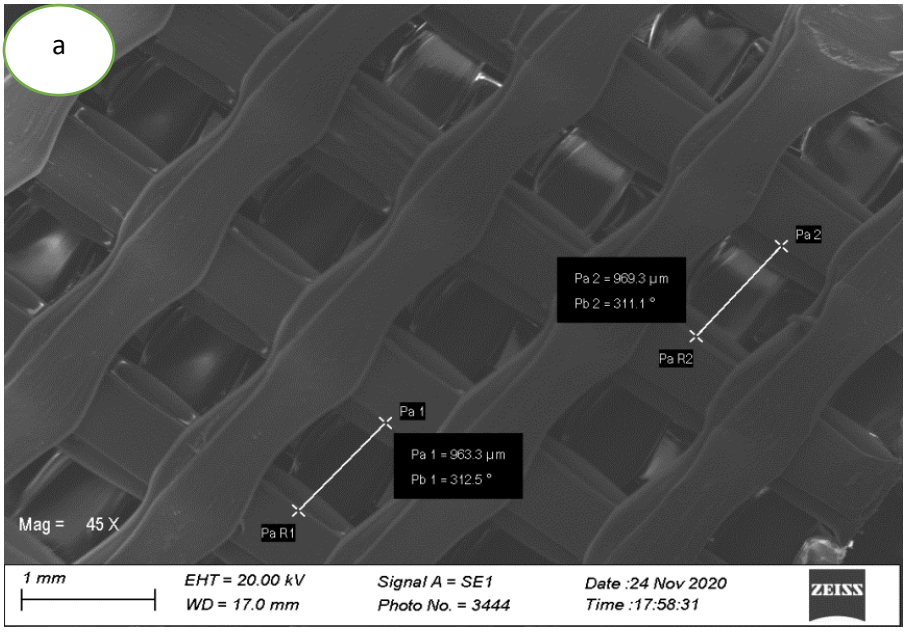
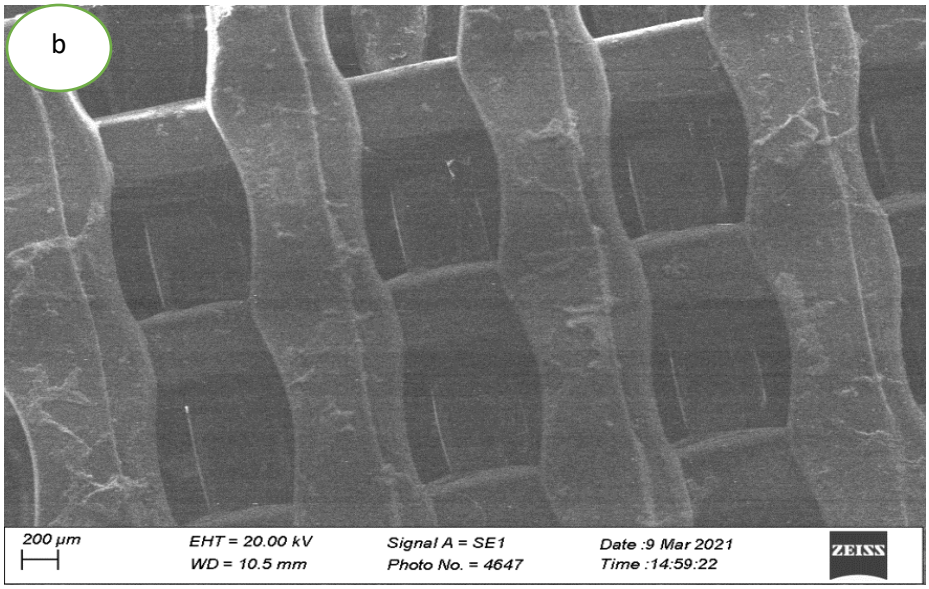


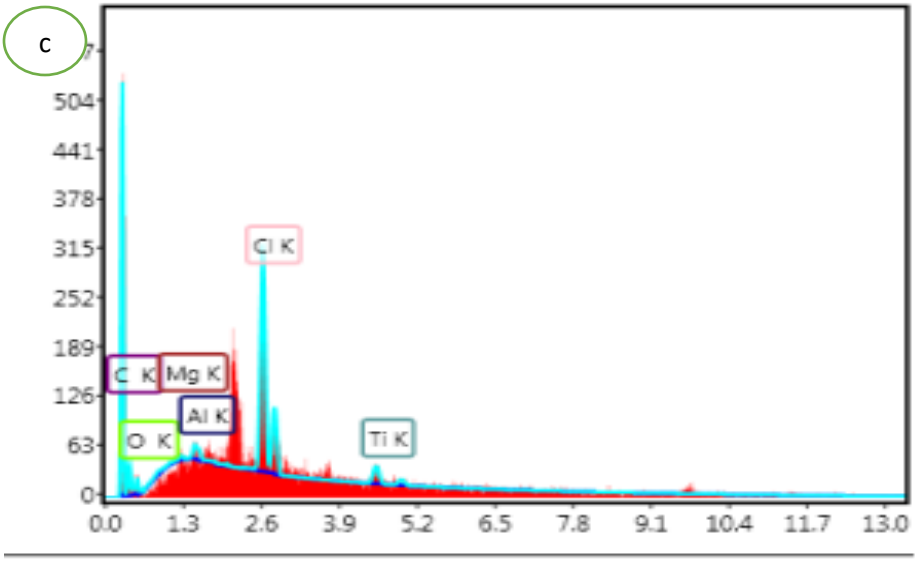
Figure 10: (a) Picture of PLA scaffolds with woodpile pattern (b) SEM image of PLA scaffold showing its pore size of 963.3 µm in both length and width with a scale bar in 1mm

4.4 Degradation Studies

The PLA scaffold was first viewed under SEM and the result in Fig. 11a below was obtained showing the smoothness of the PLA scaffold but subsequently the scaffold showed wears which proves degradation has been taking place as shown in Fig. 11b below. The shape of the pores on the PLA scaffolds became narrower. Therefore, degradation time significantly influences the pore sizes and porosities of the scaffolds. In addition, the weight of the scaffold was checked in a weekly interval same as the pH of the Simulated Body Fluid. EDX results obtained from the scaffold are presented in Figure 11 c. This reveals the presence of C, O, Mg, Cl and Ti in the scaffolds. The pH decreased from about 7.2 to about 6.9 which is due to the acidic nature of PLA (Fig. 11d). Furthermore, the weight of the scaffold was observed to decrease from about 0.2351 gram – 0.2349 gram, as shown in Fig. 11 e below.




b
200 µm
EHT = 20.00 kV
WD = 10.5 mm
Signal A = SE1
Photo No. = 4647
Date :9 Mar 2021
Time :14:59:22
ZEISS


c
504
441
378
315
252
189
126
63
0
Cl K
C K
Mg K
Al K
O K
Ti K
0.0
1.3
2.6
3.9
5.2
6.5
7.8
9.1
10.4
11.7
13.0

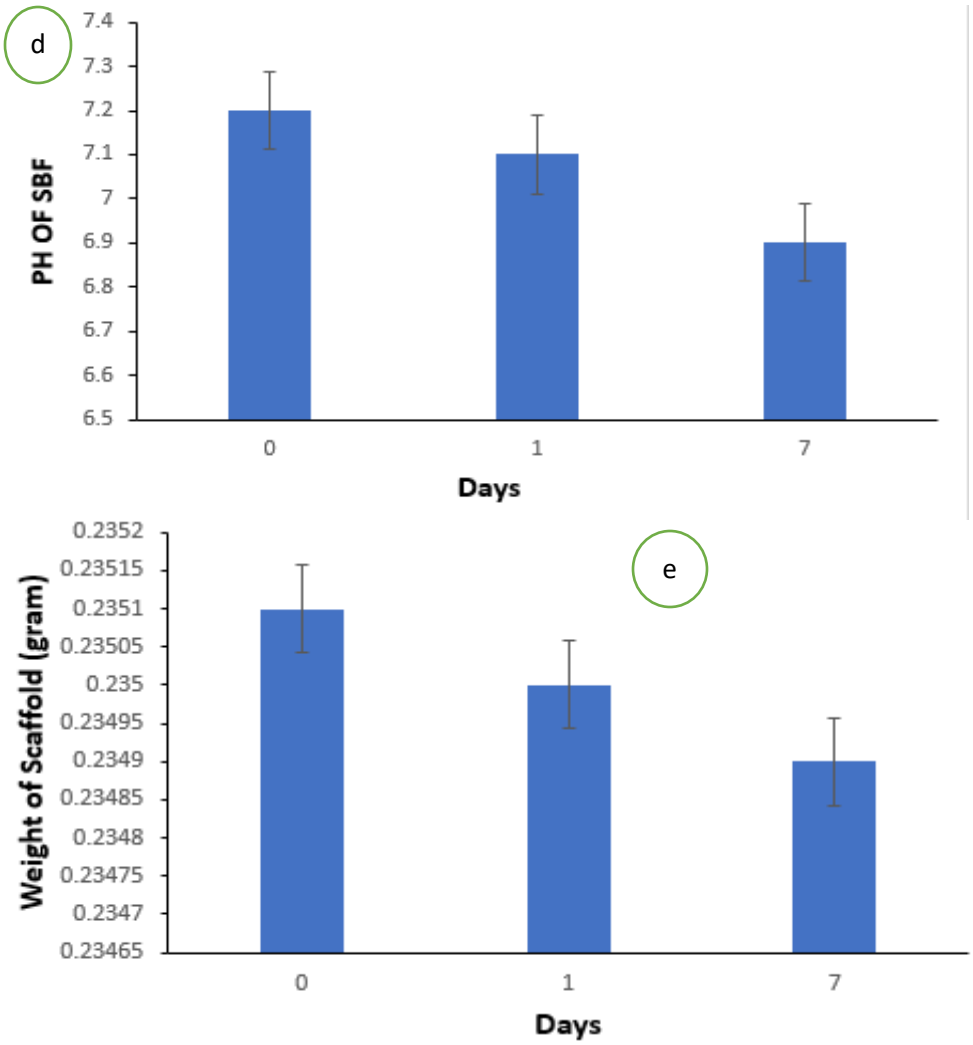


Figure 11: (a) SEM image of the PLA scaffold after 1 week of immersion in a SBF at 37.5^{o}C constant temperature (b) SEM image showing significant degradation at 10th week (c) EDX result of the PLA scaffold (d) Bar chart showing the PH of the PLA scaffold as time passed (e)Bar chart showing reduction in weight of the Scaffold with time.

4.5 Mechanical Characterization

The assessment of the mechanical properties of scaffolds is of importance in most applications of tissue-engineering. Tensile tests of the scaffolds were performed to assess the stress-strain relationship and evaluate their Young's moduli. The tensile strengths were determined to be 8.31, 3.93, 5.98 and 6.40 MPa for weeks 1, 2. 4 and 6 respectively while during cell culture they were 11.77 and 13.91 MPa for weeks 1 and 7 respectively.

Young's moduli during the degradation studies were 15.09, 10.10, 9.81, 12.24 MPa for week 1, 2, 4 and 6 respectively while during culture the young's modulus were 360.44 and 646.69 MPa for weeks 1 and 7 respectively. Results showed that the energy absorption of the scaffolds is greatly reduced with increasing porosity.

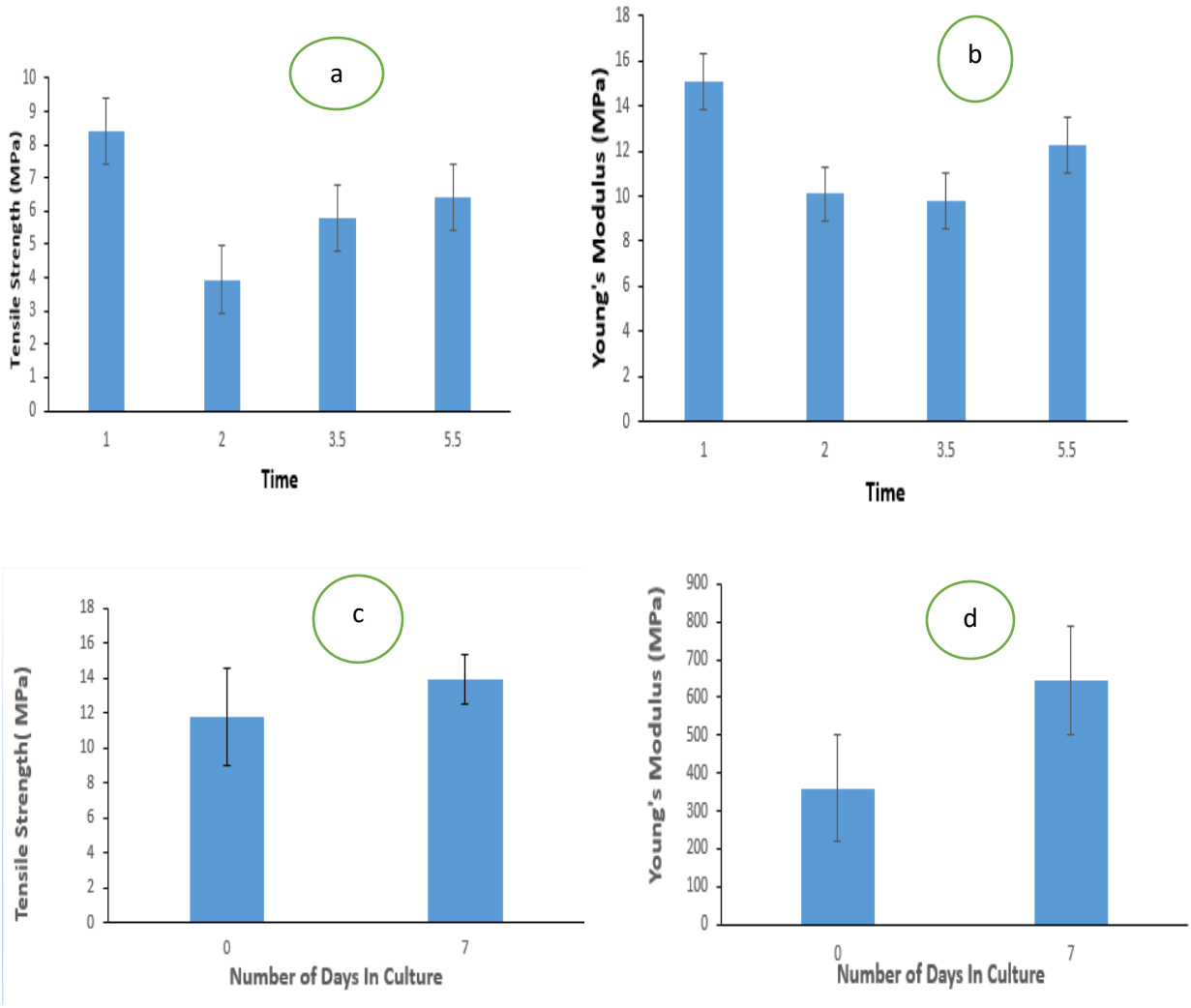


Figure 12: (a) Tensile strength of different PLA scaffolds at various weeks of degradation studies (Error bars = standard deviation of 4 samples (n =4)). (b) Young's modulus of different PLA scaffolds at various weeks of degradation studies (Error bars = standard deviation of 4 samples (n =4)) (c) Tensile strength of different PLA scaffolds during osteoblast culture (Error bars= standard deviation of 2 samples (n=2)). (d) Young's modulus of different PLA scaffolds during osteoblast culture (Error bars= standard deviation of 2 samples (n=2)).

4.6 Cell Imaging

Osteoblast cell adhesion on PLA scaffold was also investigated through SEM as shown in the supplementary figure 13 (a & b). After 7 days of culture, the interaction between cells and the scaffold surface was examined. Cells cultured on PLA scaffold formed a well spread morphology and exhibited excellent cell adhesion

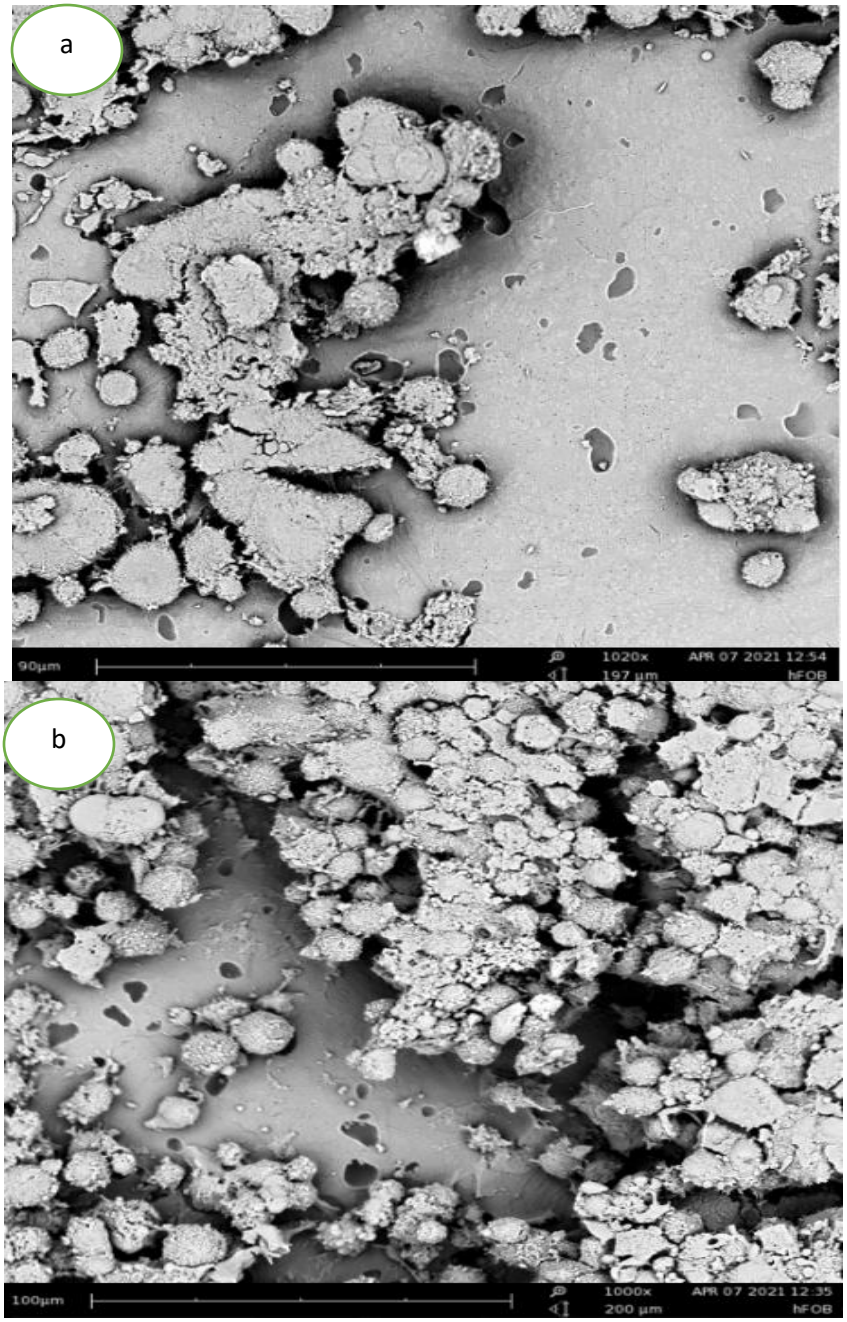


Fig. 13 SEM images showing cell growth on the PLA scaffold for day 0 and day 7 respectively

4.7 Culturing Osteoblast in Scaffolds

It is vital to make the surface of the porous scaffold hydrophilic in order to achieve good cell seeding and culturing (media infiltration). To achieve this, oxygen plasma treatment was carried out making the contact angle to decrease to near 0^{o}. This helped the osteoblast to infiltrate the porous PLA scaffold with ease.

4.7.1 Cell Proliferation, Viability and Function

After cell seeding, cell viability and proliferation in the PLA scaffolds were investigated over a 7 days culture period using biochemical assays and imaging techniques. Cell proliferation in the scaffold was quantified using the alamarBlue® assay. As shown in Fig. 14a, there was linear increase in fluorescence intensity over the period of culture which indicates an increase in the number of cells grown on the scaffold. We further measured the functionality of osteoblast cultured on the scaffolds by calculating the extracellular albumin production (Fig. 14b).

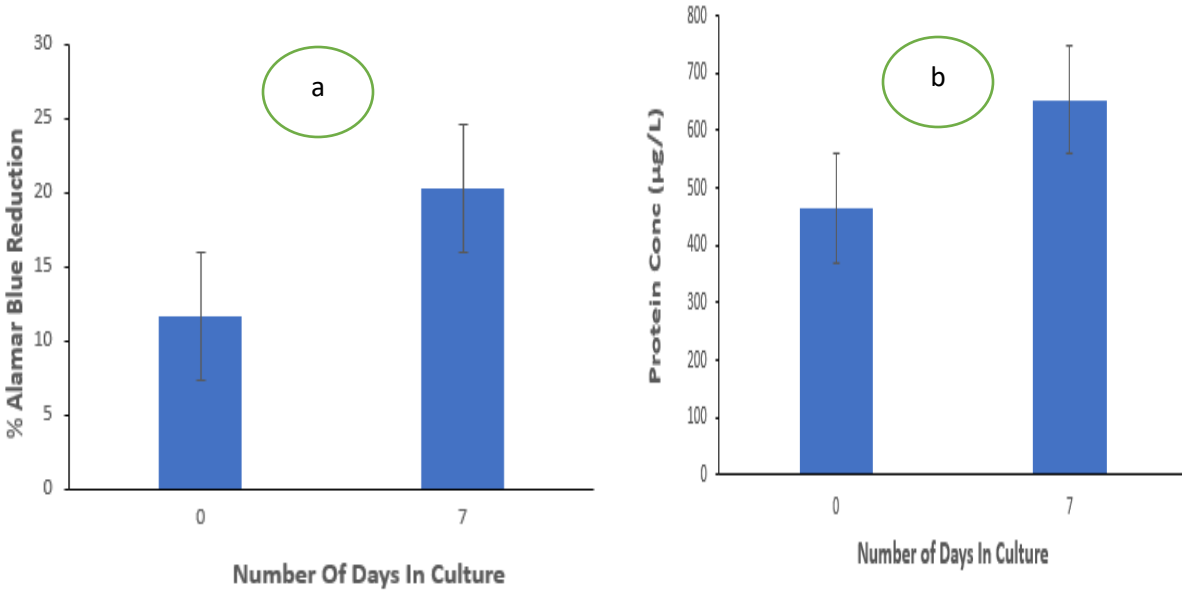


Figure 14: (a) Change in alamarBlue® florescence intensity of scaffolds over culture time. The fluorescence intensity is proportional to the amount of cells. (b) Protein/Albumin production of osteoblast grown in PLA scaffolds over 7 days of culture. Error bars indicate standard deviation of 3 independent scaffolds.

Fig. 15 below shows the fluorescence microscopy images of stained bone cells on days 0 and 7 of the culture period. The blue regions represent the nucleus, while the pink regions correspond to the cytoplasm of the cells. In all cases, no dead cells were observed. So close to 100 % cell viability was maintained throughout the 7-day culture period.

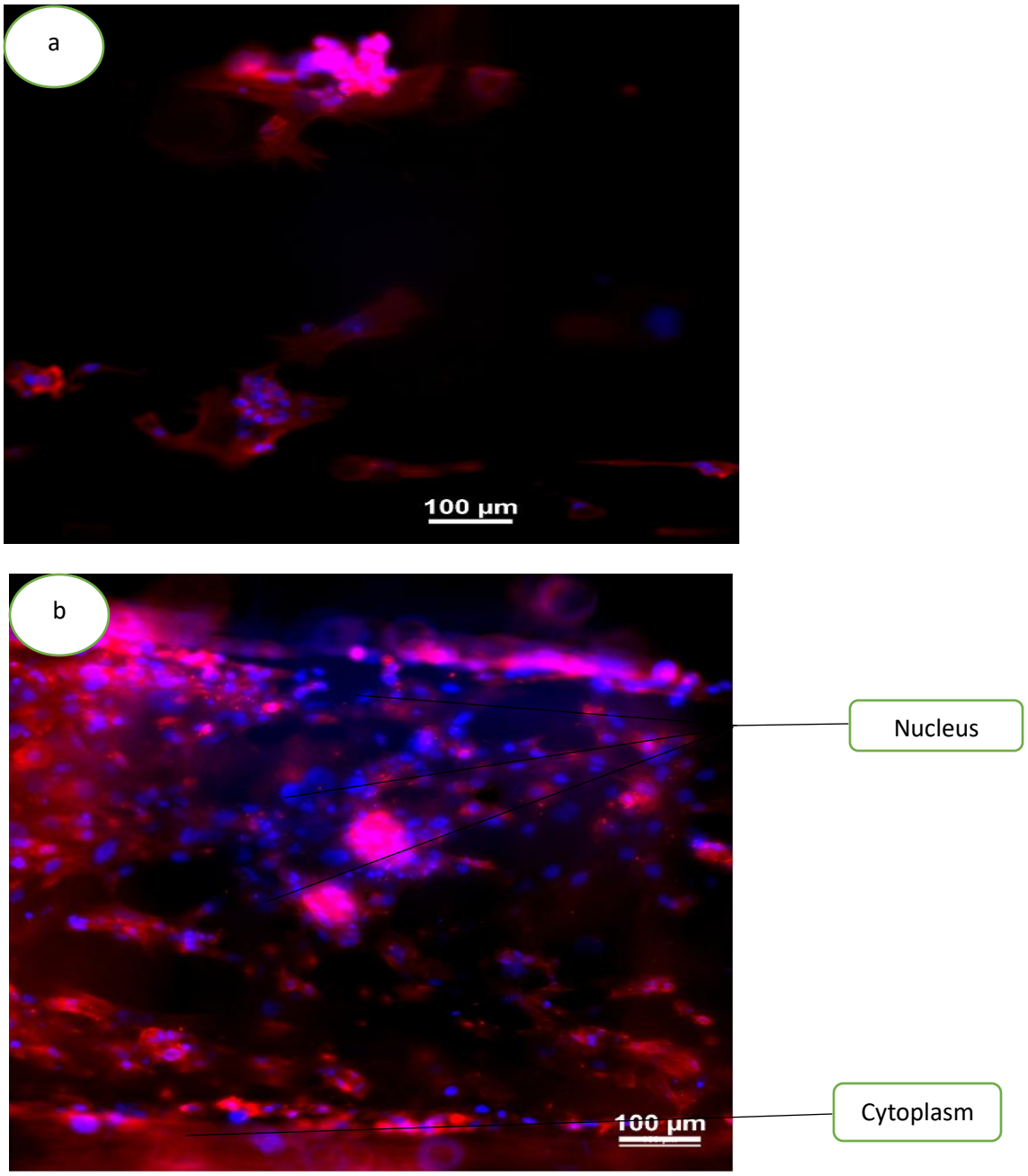


Fig. 15 Fluorescence microscopy images of cell nuclei and cytoplasm obtained at: (a) day 0 and (b) day 7. Images obtained after staining the cells with 4,6-diamidino-2-phenylindole (DAPI) and rhodamine phalloidin.

4.8 Cell Infiltration and Distribution Within the Scaffolds

The osteoblast cells cultured in a growth medium were augmented with fetal bovine serum and maintained in a temperature of about 37 ℃.

To visualize cell distribution through the cross section of the scaffold, it was dissected along its central axis and imaged using fluorescence microscopy. Close to 100 % cell viability was observed (no dead cells could be seen) with cells present throughout the cross section of the scaffold by the end of the culture period.

Scaffolds acquired from day 0 of the culture showed a lower density of cells but with longer culture time, cells appeared to proliferate and are seen to be fairly distributed all over the body of the scaffolds at day 7.

4.9 Implications

The use of PLA in bone regeneration has tremendous scope. It holds the promise of sustainable improvement in the quality of human life, with a reduction in healthcare and life expectancy. It has great potential to offer in the case of nounion and improve clinical outcome for such patients.

The ultimate goal is obviously repair and improvement of bone defects while restoring structural support for movement. The degradation studies in-vitro have also proven the clinical applicability of PLA (in-vitro) for bone regenerations, showing the mechanism which it will undergo down to its excretion.

CHAPTER 5

5.0 SUMMARY AND FUTURE WORK

5.1 Summary

This paper presents the results of an initial study of the structure and mechanical properties of a 3D PLA scaffold that was designed and printed for integration with osteoblasts. A porous structure with pore sizes of 963.3 microns and strut dimensions (25 mm X 25 mm X 0.8 mm) was designed and printed. The initial studies of degradation in simulated body fluid revealed hydrolysis of the PLA scaffold. The degradation also had enhanced effects on the mechanical properties of the PLA scaffold over a period of 8 weeks. Furthermore, the culturing of human osteoblasts onto the porous scaffolds resulted in the spreading and proliferation of human osteoblasts during the initial 7 days of cell culture. The basic mechanical properties (Young's moduli and tensile strengths) of the scaffolds also increased with increasing cell/surface interactions during the 7 days of cell culture. Further work is needed to determine how the cells spread, proliferate and differentiate into bone over longer durations than those employed in this study. These are clearly some of the challenges for future work.

5.2 Future Work

There is need for more time to establish the multiplication of bone cells on the PLA Scaffold. There is need to longer duration of observation during the degradation of the PLA scaffold in Simulated Body Fluid (SBF) explore the effects of longer durations of exposure on PLA scaffold degradation and cell spreading, proliferation and differentiation. Hence, we propose longer term experiments for durations up to 3-6 months. We also suggest the potential use of mechanical stimulation and surface coatings for the enhancement of cell/surface integration and the formation of bone structures with improved mechanical properties.

REFERENCES

Ã, T. K., & Takadama, H. (2006). *How useful is SBF in predicting in vivo bone bioactivity ? $*. *27*, 2907–2915. https://doi.org/10.1016/j.biomaterials.2006.01.017

Ambrosio, A. M. A., Allcock, H. R., Katti, D. S., & Laurencin, C. T. (2002). *Degradable polyphosphazene / poly (a -hydroxyester) blends : degradation studies*. *23*, 1667–1672.

Amini, A. R. (2013). *Bone Tissue Engineering: Recent Advances and Challenges*. *40*(5), 363–408.

Anagnostou, F., Bizios, R., & Petite, H. (2005). *Engineering bone : challenges and obstacles*. *9*(1), 72–84.

Anderson, J. M., Andukuri, A., Lim, D. J., & Jun, H. W. (2009). Modulating the gelation properties of self-assembling peptide amphiphiles. *ACS Nano*, *3*(11), 3447–3454. https://doi.org/10.1021/nn900884n

Athanasiou, K. A., Lanctot, D. R., Agrawal, C. M., & Wang, X. (2000). *Fundamentals of Biomechanics in Tissue Engineering of Bone*. *6*(4).

Babensee, J. E., Anderson, J. M., Mcintire, L. V, & Mikos, A. G. (1998). *Host response to tissue engineered devices*. *33*, 111–139.

Bambole, V., & Yakhmi, J. V. (2016). Chapter 14. Tissue engineering: use of electrospinning technique for recreating physiological functions. In *Nanobiomaterials in Soft Tissue Engineering*. Elsevier Inc. https://doi.org/10.1016/B978-0-323-42865-1.00014-3

Blokhuis, T. J., & Arts, J. J. C. (2011). Bioactive and osteoinductive bone graft substitutes : Definitions , facts and myths. *Injury*, *42*, S26–S29. https://doi.org/10.1016/j.injury.2011.06.010

Bosworth, L. A., Turner, L., & Cartmell, S. H. (2012). State of the art composites comprising electrospun fibres coupled with hydrogels : a review. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 1–14. https://doi.org/10.1016/j.nano.2012.10.008

Boyce, T., Edwards, J., & Scarborough, N. (1999). *ALLOGRAFT BONE The Influence of Processing on Safety and Performance*. *30*(October).

Butler, D. L., & Goldstein, S. A. (2016). *Functional Tissue Engineering : The Role of Biomechanics*. *122*(December 2000).

Deng, M., Nair, L. S., Nukavarapu, S. P., Kumbar, S. G., & Jiang, T. (2008). *Miscibility and in vitro osteocompatibility of biodegradable blends of poly [(ethyl alanato) (p -phenyl phenoxy) phosphazene] and poly (lactic acid-glycolic acid)*. *29*, 337–349. https://doi.org/10.1016/j.biomaterials.2007.09.029

Duran, E., Armando, H., Ballesteros, P., Yesid, D., Quintero, V., Armando, H., & Duran, E. (2012). *OSTEOBLAST ADHESION ON SCAFFOLDS OF PLA-PLG-HYDROXYAPATITE-CHITOSAN-ZINC BY OSTEOBLAST ADHESION ON SCAFFOLDS OF PLA-PLG- HYDROXYAPATITE-CHITOSAN-ZINC BY ELECTROACTIVATION DE PLA-PLG-HIDROXIAPATITA-QUITOSANO POR*.

Elsawy, M. A., Kim, K., Park, J., & Deep, A. (2017). Hydrolytic degradation of polylactic acid (PLA) and its composites. *Renewable and Sustainable Energy Reviews*, *79*(April), 1346–1352. https://doi.org/10.1016/j.rser.2017.05.143

Experimental, A., & Ripamonti, U. G. O. (1991). *The Induction of Bone in Osteogenic Composites of Bone Matrix and Porous Hydroxyapa tite Replicas :*

Felfel, R. M., Poocza, L., Gimeno-fabra, M., Milde, T., Hildebrand, G., & Ahmed, I. (2016). In vitro degradation and mechanical properties of PLA- PCL copolymer unit cell scaffolds generated by two-photon polymerization. *Biomedical Materials*, *015011*, 15011. https://doi.org/10.1088/1748-6041/11/1/015011

Ferreira, J., Gloria, A., Cometa, S., Coelho, J. F. J., & Domingos, M. (2017). *and mechanical properties*. *15*(3), 185–195. https://doi.org/10.5301/jabfm.5000363

Ftipamonti, U. (1996). *Osteoinduction in Dorous hydroxyapatite imilanted in heterotopic sites of different animal models*. *17*(1), 31–35.

Goddard, J. M., & Hotchkiss, J. H. Ã. (2007). *Polymer surface modification for the attachment of bioactive compounds*. *32*, 698–725. https://doi.org/10.1016/j.progpolymsci.2007.04.002

Grabowski, G., & Cornett, C. A. (2013). *Bone Graft and Bone Graft Substitutes in Spine Surgery : Current Concepts and*. *21*(1), 51–60.

Gregor, A., Filová, E., Novák, M., Kronek, J., Chlup, H., Blahnová, V., Barto, M., Ne, A., & Ho, J. (2017). *Designing of PLA scaffolds for bone tissue replacement fabricated by ordinary commercial 3D printer*. 1–21. https://doi.org/10.1186/s13036-017-0074-3

Higashi, S., & Yamamuro, T. (1986). *Polymer-hydrolryapatite composites for biodegradable bone fillers*. *May*.

Ho, E., Lowman, A., & Marcolongo, M. (2006). *Synthesis and Characterization of an Injectable Hydrogel with Tunable Mechanical Properties for Soft Tissue Repair*. 3223–3228.

Hou, Q., Grijpma, D. W., & Feijen, J. (2003). *Preparation of Interconnected Highly Porous Polymeric Structures by a Replication and Freeze-Drying Process*. 732–740.

Hutmacher, D. W. (2000). Scaffolds in tissue engineering bone and cartilage. *Biomaterials*. https://doi.org/10.1016/S0142-9612(00)00121-6

International, T., & Cell, S. (2007). *Characterization of human embryonic stem cell lines by the International Stem Cell Initiative*. *25*(7), 803–816. https://doi.org/10.1038/nbt1318

Jayatissa, N. U., & Bhaduri, S. (2019). *Article 3D Printed Polymer Scaffolds for Bone Tissue Regeneration*. *2*(April), 1–6.

Karageorgiou, V., & Ã, D. K. (2005). *Porosity of 3D biomaterial scaffolds and osteogenesis*. *26*, 5474–5491. https://doi.org/10.1016/j.biomaterials.2005.02.002

Khan, Y. M., Katti, D. S., & Laurencin, C. T. (2004). *Novel polymer-synthesized ceramic composite – based system for bone repair : An in vitro evaluation*. https://doi.org/10.1002/jbm.a.30051

Kim, S., Ph, D., Park, M. I. N. S. U. N., Gwak, S., Choi, C. H. A. Y., Ph, D., Kim, B., Ph, D., & Al, K. I. M. E. T. (2006). Accelerated Bonelike Apatite Growth on Porous Polymer / Ceramic Composite Scaffolds in Vitro. *Biomaterials*, *12*(10).

Krogman, N. R., Singh, A., Nair, L. S., Laurencin, C. T., & Allcock, H. R. (2007). *Miscibility of Bioerodible Polyphosphazene / Poly (lactide-co-glycolide) Blends*. 1306–1312.

Krogman, N. R., Weikel, A. L., Kristhart, K. A., Nukavarapu, S. P., Deng, M., Nair, L. S., Laurencin, C. T., & Allcock, H. R. (2009). Biomaterials The influence of side group modification in

polyphosphazenes on hydrolysis and cell adhesion of blends with PLGA. *Biomaterials*, *30*(17), 3035–3041. https://doi.org/10.1016/j.biomaterials.2009.02.049

Lakshmi, S., Katti, D. S., & Laurencin, C. T. (2003). *B iodegradable polyphosphazenes for drug delivery applications*. *55*, 467–482.

Langer, R., & Vacanti, J. P. (1993). - ARTICLES Tissue Engineering. *Science*, *260*(2012), 1–7.

Lee, J. T. Y., Leng, Y., Chow, K. L., Ren, F., Ge, X., Wang, K., & Lu, X. (2011). Acta Biomaterialia Cell culture medium as an alternative to conventional simulated body fluid. *Acta Biomaterialia*, *7*(6), 2615–2622. https://doi.org/10.1016/j.actbio.2011.02.034

Lee, K. Y., & Mooney, D. J. (2001). *Hydrogels for Tissue Engineering*. *101*(7).

Lutolf, M. P., & Hubbell, J. A. (2005). *Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering*. *23*(1), 47–56. https://doi.org/10.1038/nbt1055

Mohammadi, M., Alibolandi, M., Abnous, K., Salmasi, Z., Jaafari, M. R., & Ramezani, M. (2018). Fabrication of hybrid scaffold based on hydroxyapatite-biodegradable nanofibers incorporated with liposomal formulation of BMP-2 peptide for bone tissue engineering. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *14*(7), 1987–1997. https://doi.org/10.1016/j.nano.2018.06.001

Morgan, E. F., Unnikrisnan, G. U., & Hussein, A. I. (2018). *Bone Mechanical Properties in Healthy and Diseased States*.

Mountziaris, P. M., Spicer, P. P., Kasper, F. K., Ph, D., Mikos, A. G., & Ph, D. (2011). *Harnessing and Modulating Inflammation in Strategies for Bone Regeneration*. *17*(6). https://doi.org/10.1089/ten.teb.2011.0182

Nantulya, V. M., Sleet, D. A., Reich, M. R., Rosenberg, M., Peden, M., & Waxweiler, R. (2010). *Introduction : The global challenge of road traffic injuries : Can we achieve equity in safety ? July 2014*. https://doi.org/10.1076/icsp.10.1.3.14109

Osteogenesis, B. (1997). *Pore Size of Porous Hydroxyapatite as the Cell-Substratum Controls*. *121*(2), 317–324.

Pietrzak, W. S., Kumar, M., & Eppley, B. L. (2003). *The Influence of Temperature on the Degradation Rate of LactoSorb Copolymer*. 176–183.

Pollick, S., Shors, E. C., Holmes, R. E., & Kraut, R. A. (1995). *Bone Formation and Implant Degradation of Coralfine Porous Ceramics Placed in Bone and Ectopic Sites*. 915–922.

Redmond, J. (2015). An Investigation into Osteoblast Adhesion on 3d Printed Scaffolds NanoBioscience Masters Project " An investigation into the adhesion of osteoblasts on 3d printed scaffolds " Jemma Jayne Redmond Student Number : 09260340 Supervisor – Dr Brian Rodriguez Co. *Nanobioscience*, *August 2012*, 1–63. https://doi.org/10.13140/RG.2.1.2722.3209

Rodrigues, C. V. M., Serricella, P., Linhares, A. B. R., Guerdes, R. M., & Borojevic, R. (2003). *Characterization of a bovine collagen – hydroxyapatite composite scaffold for bone tissue engineering*. *24*, 4987–4997. https://doi.org/10.1016/S0142-9612(03)00410-1

Rodrigues, N. (2016). *Manufacture and characterization of porous PLA scaffolds*. *49*, 33–38. https://doi.org/10.1016/j.procir.2015.07.025

Salifu, A. A., Obayemi, J. D., Uzonwanne, V. O., & Soboyejo, W. O. (2020). Mechanical stimulation

improves osteogenesis and the mechanical properties of osteoblast-laden RGD-functionalized polycaprolactone / hydroxyapatite scaffolds. *Biomaterials*, *March*, 1–14. https://doi.org/10.1002/jbm.a.36993

Scaffaro, R., Maio, A., & Nostro, A. (2020). *Poly (lactic acid)/ carvacrol-based materials : preparation , physicochemical properties , and antimicrobial activity*.

Shui, H., Shi, Q., Pugno, N. M., Chen, Q., & Li, Z. (2019). Journal of the Mechanical Behavior of Biomedical Materials Effect of mechanical stimulation on the degradation of poly (lactic acid) scaffolds with different designed structures. *Journal of the Mechanical Behavior of Biomedical Materials*, *96*(April), 324–333. https://doi.org/10.1016/j.jmbbm.2019.04.028

Stevens, M. M. (2008). *Biomaterials for bone Materials that enhance bone regeneration have a wealth of potential*. *11*(5), 18–25.

Sultana, N. (2018). Mechanical and biological properties of scaffold materials. In *Functional 3D Tissue Engineering Scaffolds: Materials, Technologies, and Applications*. Elsevier Ltd. https://doi.org/10.1016/B978-0-08-100979-6.00001-X

Taylor, P., & Pioletti, D. P. (2010). *Computer Methods in Biomechanics and Biomedical Engineering Biomechanics in bone tissue engineering*. *December 2014*, 37–41. https://doi.org/10.1080/10255841003630660

Tripathi, A., Subramanya, P., Murthy, N., Keshri, G., & Singh, M. M. (2011). *Tissue Engineered Osteogenesis in Bone Defects by Homologous Osteoblasts Loaded on Sterile Bioresorbable Coral Scaffold in Rabbits*. *2011*(September), 369–375. https://doi.org/10.4236/ss.2011.27081

Ulery, B. D., Nair, L. S., & Laurencin, C. T. (2011). *Biomedical Applications of Biodegradable Polymers*. 832–864. https://doi.org/10.1002/polb.22259

Wei, G., & Ma, P. X. (2009). *Structure and properties of nano-hydroxyapatite / polymer composite scaffolds for bone tissue engineering*. *25*(2004), 4749–4757. https://doi.org/10.1016/j.biomaterials.2003.12.005

Wei, J., Igarashi, T., Okumori, N., & Igarashi, T. (2009). Influence of surface wettability on competitive protein adsorption and initial attachment of osteoblasts. *Biomedical Materials*, *045002*, 1–8. https://doi.org/10.1088/1748-6041/4/4/045002

Wiese, A., & Pape, H. C. (2010). Bone Defects Caused by High - energy Injuries , Bone Loss , Infected Nonunions. *Orthopedic Clinics of NA*, *41*(1), 1–4. https://doi.org/10.1016/j.ocl.2009.07.003

Yildirim, E. D., Besunder, R., Pappas, D., & Allen, F. (2010). Accelerated differentiation of osteoblast cells on polycaprolactone scaffolds driven by a combined effect of protein coating and plasma modification. *Biofabrication*, *014109*, 1–13. https://doi.org/10.1088/1758-5082/2/1/014109

Zhang, Y., Ni, M., Zhang, M., & Ratner, B. (2003). *Calcium Phosphate – Chitosan Composite Scaffolds for Bone*. *9*(2).