BIOCOMPATIBILITY AND BIOFUNCTIONALITY EVALUATIONS OF METALLIC SCAFFOLDS FOR BONE TISSUE ENGINEERING

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ABSTRACT

Bone grafting has been widely and successfully used in biomedical fields. Current methods of bone grafting that have been used in medical fields are autografting, allografting, and xenografting but there is a limitation of using those bone grafts due to disease transmission, host rejection and other problems. Use of synthetic bone grafting is another alternative method to solve those issues. Various types of materials such as metals, ceramic, and polymer have been used as synthetic bone graft. Polyurethane is commonly used in bone tissue engineering applications because it has excellent biocompatibility. However, polyurethane has lack of bioactive group and it not biostable when it contacts with living tissue that cause device failure in long term implants.

The objective of this thesis was to enhance the mechanical properties of polyurethane. A metallic coating on polyurethane was fabricated to enhance its mechanical properties. Compression test was performed on polyurethane coated samples and effect of stress, strain and the average ultimate strength of the Nb coated, Mg coated and plain polyurethane samples were evaluated. Stress, strain and the average ultimate strength of Nb coated sample was improved significantly whiles stress, strain and the ultimate strength of Mg coated sample did not improve much based on applied coating conditions. Cytotoxicity evaluations of coated samples were conducted using direct and indirect tests. Cells proliferated well with Nb and Mg coated polyurethane samples and the coatings were biocompatible. Moreover, biofunctionality of coated samples were evaluated by performing cell proliferation. Cell proliferation, cell ahhesion and cell morphology of MC-3T3 osteoblasts cells line were observed for 1 day, 4 days and 7 days. The proliferation of cells decreased with coated samples as compared to polyurethane. Optimization of cell culture media is needed to enhance cell proliferation on coated samples.

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CHAPTER 1

INTRODUCTION

1.1 Background

Tissue regeneration and replacement of human tissue and organs are the main purposes of tissue engineering. This field has combined the biological, clinical, and engineering approach in order to meet its goals (Ratner, 2013). Researchers have been developing tissue engineering applications in biomedical fields which focus on human body parts such as skin, the liver, the pancreas, the heart, the bone, as well as others (Ratner, 2013).

Current methods of tissue regeneration and organ replacement that have been practiced in the clinical field include autografting, allografting, and xenografting methods (Porter, Ruckh, & Popat, 2009). These grafting methods are limited because of disease transmission, host rejection, organ shortage, and other problems (Porter et al., 2009). Limiting factors have lead researchers to pursue other methods. Synthetic bone grafting is another type of grafting method that has been widely used for biomedical devices due to the unlimited number of synthetic supplies available in the market. There are many on-going research projects which focus on synthetic bone graft and other related applications, but researches must keep in mind the criteria regarding biocompatibility, degradation rate, mechanical properties and other factors that may affect the patient or medical device used for each bone tissue applications. Therefore, various processes and experiments must be conducted before a device is successfully implanted into a patient.

Metals, ceramics, and polymers have been used as synthetic bone grafts in biomedical devices (Bohner, 2010). Polyurethane, segmented polymer, is from the polymer class family, and it has good biocompatibility (Ratner, 2013). Research has been conducted on polyurethane and

polyurethane based devices. These materials have been largely used in bone tissue engineering applications; however, polyurethane poses some problems.

1.2 Problem Description and Motivation of Work

Polyurethane lacks bioactive groups, which effects its interaction with a living tissue, or cells, that make polyurethane applications limited (M.-N. Huang, Wang, & Luo, 2009). Biodegradation and bioactivity are critical factors to be considered for bone tissue engineering applications (M.-N. Huang et al., 2009). Current polyurethane graft is not biostable which means it does not retain its physical, chemical and mechanical integrity after contacting with living tissue and it is a critical problem in bone tissue engineering applications. Biostability of polyurethane is a critical issue for long term implants; therefore, researchers are investigating ways to enhance biostability of polyurethane for long-term applications (Santerre, Woodhouse, Laroche, & Labow, 2005). Polyurethane had been widely use in biomedical applications but unfortunately it causes a failure in long term implants (Gunatillake & Adhikari, 2011). Researchers used various techniques and strategies to solve these issues; however no optimal solution currently exists for using polyurethane in bone tissue engineering applications. Mechanical properties and degradation rate play important roles in polyurethane applications; hence, mechanical properties of polyurethane need to be improved in order to use it as bone grafts in tissue engineering.

1.3 Objective and Contribution of thesis

The objective of this thesis is to enhance the mechanical properties of polyurethane bone scaffolds, to evaluate the biocompatibility of coated foams and to investigate the cell proliferation and cell adhesion of coated foams. Metallic coatings will be performed on polyurethane scaffolds by using two different types of metallic coatings, magnesium (Mg) and

niobium (Nb). The mechanical properties of coated Nb and Mg polyurethane scaffolds will be evaluated by performing compression test on coated polyurethane samples. Ultimate compressive strength of PU coated foam will be determined. Moreover, cell interactions will be examined on plain polyurethane, Nb coated and Mg coated samples. Biocompatibility and biofunctionality of the coated samples will be evaluated using an *in vitro* environment. Cell proliferation, cell adhesion, and cell morphology of the test samples will also be examined.

CHAPTER 2

LITERATURE REVIEW

2.1 Tissue Engineering

The purpose of tissue engineering is to reconstruct organ function and tissue by applying biological and engineering strategies to clinical problems (Ratner, 2013). The functional failure of tissue and organ cause a serious healthcare problem; however, only a limited number of organ replacements are available to patients (Langer & Vacanti, 1993; Vacanti & Langer, 1999). Although artificial prostheses and mechanical devices help millions of patients, they are not the perfect solution for when mechanical failure occurs during long-term healing processes (Ratner, 2013).

Mechanical devices do not incorporate with host tissue completely because the device does not have the ability to integrate with all functions of host tissue, this can damage the healthy host tissue surrounding the implant (Cristina Castells-Sala et al., 2013; Ratner, 2013). Therefore, tissue engineers are researching possible solutions for tissue repair in order to increase success in the clinical field. It is very important that development of the devices work well in both two-dimensional environments, *in vitro,* and three-dimensional environments, *in vivo* (Cristina Castells-Sala et al., 2013).

The primary goal of tissue engineering is to support the failure of organs/tissues by biological substitute with a combination of engineering, biology, and medical devices (Ratner, 2013). Tissue engineering has appeared as another option for tissue and organ transplantation and it has potential to decrease the immune response of patients (Ratner, 2013; Sachlos & Czernuszka, 2003). This technique could contribute to a long-term solution for damaged organs

and tissues while decreasing need for any additional therapies (Patrick, Zhou, Kwon, Howley, & Tsai, 1998).

In tissue engineering, it is essential to determine how cells interact with the surrounding environment. In fact, the interaction between cell-cell and cell-extra cellular matrix can indicate whether cell proliferate, differentiate, or migrate (Cristina Castells-Sala et al., 2013). Tests are carried out in two-dimensional cultures where cells are grown in non-physiological environments (Cristina Castells-Sala et al., 2013). The three basic components of tissue engineering include scaffolds, cells, and molecules (Cristina Castells-Sala et al., 2013).

These cells have to proliferate, migrate and differentiate into a particular tissue while releasing extracellular matrix components, which are needed to maintain the necessary tissue (Sachlos & Czernuszka, 2003). Scaffolds are made up of polymeric materials and act as three-dimensional templates which provide mechanical and structural support for cell adhesion, cell attachment, and cell proliferation (Chan & Leong, 2008; Ratner, 2013). Scaffolds must react well with cells to produce tissue and proper organ shape and size; therefore, choosing the right scaffolding is very critical in tissue engineering (Sachlos & Czernuszka, 2003).

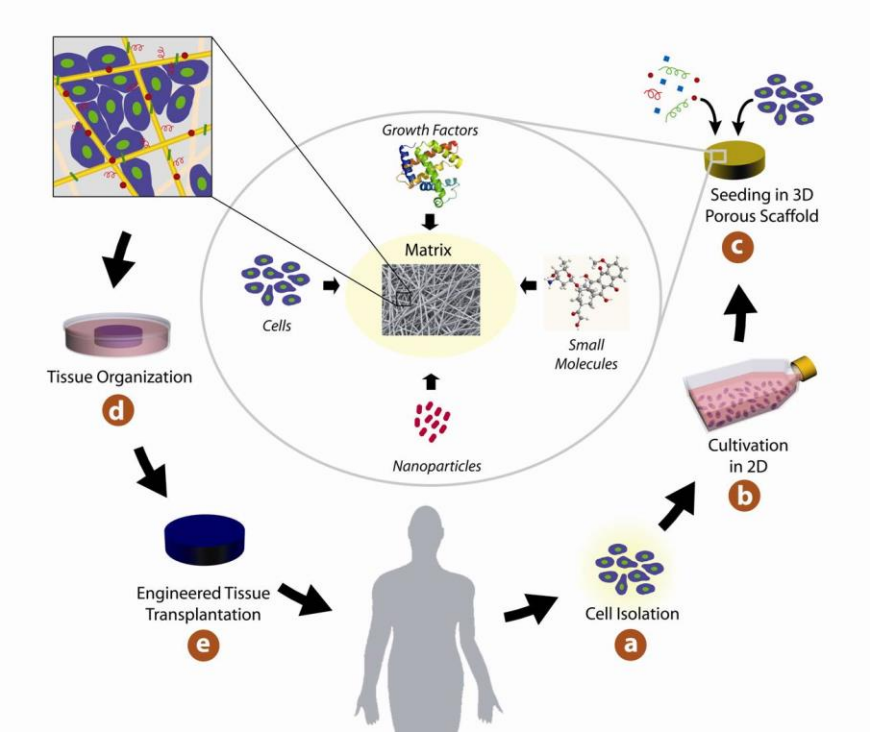

Fig 2.1: Tissue Engineering, figure adapted from ("Nanotechnology Strategies for tissue engineering,")

2.1.1 Applications of Tissue Engineering

The primary key issue in tissue engineering is the regeneration and replacement of human tissues and organs. Several developments have been made in various areas such as skin, liver, pancreas, heart, blood vessels, nervous system, and bone (Ratner, 2013). Another important issue to examine is the shortage of organs. Researchers have been analyzing ways to conduct experiments in tissue engineering in order to solve this matter.

Tissue engineering that focuses on skin is an application that is being examined by many researchers. The purpose of tissue-engineered skin is to repair patient tissue due to critically wounded skin. Skin may be wounded by genetic disorders, acute trauma, surgical interventions, diseases, or injures (Groeber, Holeiter, Hampel, Hinderer, & Schenke-Layland, 2011; Ratner,

2013) . Skin is the largest organ of the human body and it performs as a barrier; in addition, it has the ability to heal by itself. The patients' ability to heal depends on the size and defect of the wounded skin (Groeber et al., 2011). Skin products are available in the market to help skin problems such as burns, ulcers, deep wounds, and other injuries (Ratner, 2013). Collagen, fibrin, hyaluronic acid, and poly (lactic glycolic acid) are mainly used for skin substitutes (Ratner, 2013). Keratinocytes, melanocytes, and fibroblasts produce most of cells in skin tissue (Priya, Jungvid, & Kumar, 2008). Growing and transplanting these cells requires biocompatible matrices for successful skin regeneration (Priya et al., 2008). Furthermore, to attain successful healing, tissue-engineered skin must be connected to the wound and must also prevent immune system rejection (Priya et al., 2008).

Liver transplantation, a final stage treatment for liver, is a tissue engineering application being developed (Ratner, 2013). Various problems concerning drug use, alcohol use, and viruses like Hepatitis can cause intense liver failure (Ratner, 2013). If failure occurs, it is necessary to replace the entire damaged liver, with one that has been received from a donor, or provide temporary support to aid the liver function until an organ donor is available (Kulig & Vacanti, 2004; Ratner, 2013).

Cardiovascular disease causes damages and cardiac tissue failure in many patients which can create arrhythmias and diminished cardiac output (Ratner, 2013). Tissue engineering is examining treatments for myocardial infarction, congenital heart defects, and stenotic valves through restoring cardiac tissues (Ratner, 2013).

Various clinical applications can gain from bone regeneration treatments. These applications include spinal fusion to lessen back pain, temporomandibular joint reconstruction to lessen jaw pain, and reformation of the shape and configuration of carniofacial bone (Ratner,

2013). Although there is a significant improvement in bone regeneration treatments, large bone defect restoration is very critical and must be more closely explored (Ratner, 2013). Even though different material-based scaffolds have been investigated in bone engineering, the development of material is very difficult and requires the finest mechanical properties and degradation kinetics for bone restoration (Muschler, Nakamoto, & Griffith, 2004). Bone regeneration scaffolding should have connected macroporosity to permit three-dimensional bone growth through the scaffold (Gao et al., 2001; Karp, Sarraf, Shoichet, & Davies, 2004; Karp, Shoichet, & Davies, 2003). Different composition of scaffolding creates a challenging outcome between studies and experimentation in the bone engineering field. Composition varies depending on material porosity, surface, chemistry, morphology, degradation rate, pore size(s), and mechanical properties (Ratner, 2013). Methodical studies improve the understanding of osteogenic microenvironment which is necessary to contribute consistent design for the next generation of bone engineering (Ratner, 2013).

2.1.2 General Challenges with Tissue Engineering

Development of tissue engineering has been dramatically increased in the past two decades (Ratner, 2013). The concepts of tissue engineering are dependent on the application of a scaffold/tissue construct (Ratner, 2013). In fact, FDA approved several engineered tissues, which are being used for patient treatment in the clinical setting (Ratner, 2013). In spite of early success, there are still many barriers that must be overcome before tissue engineering achieves its potential objective, curing millions of patients (Griffith & Naughton, 2002).

Tissue engineers are encountering several challenges across a number of areas. Some of the challenges are discussed here. A cell source is one of the main components for a successful tissue engineering strategy (Ratner, 2013). It is important to use reliable cell sources in the

appropriate amount in order to restore the damaged tissues, and to understand how the cells function at the molecular level (Ratner, 2013). It is also essential to understand how cells react to molecular signals and how they produce certain responses (Ratner, 2013). However, locally administered cells die, due to lack of lack of nutrients and oxygen, before they are able to contribute to the healing process (Muschler et al., 2004). The growth of blood vessels, or vascularization, is a barrier in tissue engineering when creating three-dimensional organs (Soker, Machado, & Atala, 2000). In order for cells to survive an adequate amount of oxygen, nutrients, and cell-secreted waste must be removed (Ratner, 2013). There are still various issues of vascularization that must be solved in tissues and organs; therefore, current studies have concentrated on pre-vascularization before implantation (Ratner, 2013). There are many ongoing investigations focusing on how to solve the growth of the blood vessel challenge.

Materials design is another problem in designing biomedical devices. It is still not entirely understood how the scaffold controls cell-matrix interactions although there is a significant improvement in biomaterials development (Place, Evans, & Stevens, 2009). Material design is highly important and it must take into account on how cells proliferate, use growth factor, and other various factors that may have an effect on the device (Ratner, 2013).

2.2 Bone Tissue Engineering

Various types of connective tissue can be found in the body. These include bone, cartilage, ligament, tendon, and muscle (Khan, Magge, & Laurencin, 2013). Bone provides three main functions in the body: (1) muscle attachment, (2) a shield to internal organs, and (3) a storage of necessary ions for the body maintenance process (Sarkar, Wachter, Patka, & Kinzl, 2001). However, bones can fail due to various reasons such as over-load, aging, or diseases which can decrease the strength of the bones (Ratner, Hoffman, Schoen, & Lemons, 2004).

Although the failure of bone can be abrupt, the repair process can be long and difficult; clinical challenges are often encountered (Khan et al., 2013). Although simple bone fractures can be restored back to normal function with the help of external fixation, serious bone injuries cannot be repaired by external fixation (Khan et al., 2013). The structure of the bone is complicated and it needs serious treatments in order to fix a severe fracture (Khan et al., 2013). In addition, surgical intervention may be required for serious types of bone injuries (Khan et al., 2013).

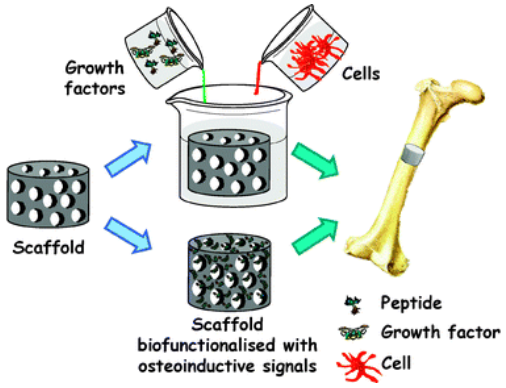

Fig 2.2: Schematic representation of the two main different approaches employed in bone tissue engineering aimed at the formation of new bone, figure adapted from (Vallet-Regí, Colilla, & González, 2011)

2.2.1 Bone Anatomy

Bone is made up of an extracellular matrix and various types of cells which have specific functions (Khan et al., 2013). The matrix is made up of 95% collagen type I and 5% proteoglycans and noncollagenous proteins (Einhorn, 1996). Proteoglycans are high molecular weight proteins (Khan et al., 2013). A proteoglycan is a glycosaminoglycan, and can usually be found in bone and cartilage (Khan et al., 2013). Bone is able to form and deposit crystalline salts, which makes it a special type of tissue (Khan et al., 2013). These crystalline salts produce a

mineralized coating around cells and the extracellular matrix which supplies strength and inelasticity to the tissues (Khan et al., 2013). In addition, the extracellular matrix of bone acts as a reservoir for essential ions such as calcium and phosphate for the body (Khan et al., 2013).

2.2.1.1 Structure of Bone Tissue

Bone, or osseous tissue, is made up of two different types: compact and spongy. The densities of the two bone types are very different. In bone, three major types of cells are present: mature bone cells, osteoblasts, osteoclasts, and osteocytes (Bose, Vahabzadeh, & Bandyopadhyay, 2013). The responsibilities of osteoblasts cell is bone formation, while osteoclasts reabsorb (or break down bone), and osteocytes acts as mature bone cells (Bose et al., 2013).

2.2.1.1.1 Compact Bone (Cortical Bone)

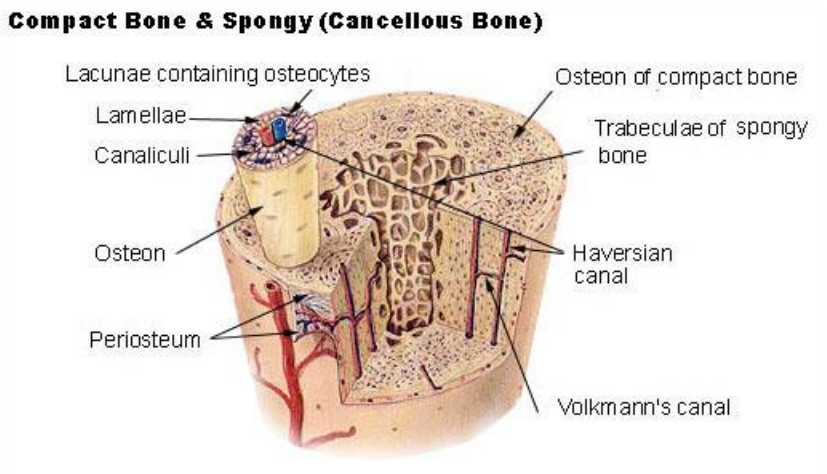

Fig 2.3: Compact Bone & Spongy (Cancellous Bone), figure adapted from (Marieb, 2006)

Compact bone is also known as cortical bone and it is located at the outer layer of the bone (Marieb, 2006). Although the surface area of compact bone is hard and less porous than spongy bone, it is very dense and strong; it not only supports the whole body but also protects the organs (Marieb, 2006). Compact bone is made up of osteons or haversian systems (Clarke,

2008) . Osteons contain the central canal known as the osteonic (haversian) canal (Clarke, 2008). Vertical concentric rings called lamellae surround the osteonic canals (Marieb, 2006). In between lamellace there are small spaces containing osteocytes which are called lacuna, small canals named canaliculi are in between lacuna (Marieb, 2006). These canals connect each cell to make sure there is a continuous supply of necessary oxygen and nutrients to the blood vessels, and nerves (Marieb, 2006).

2.2.1.1.2 Spongy Bone (Cancellous Bone or Trabecular Bone)

Spongy bone is also known as cancellous bone or trabecular bone (Marieb, 2006). Cancellous bone can be found in various places of the body such as at the end of long bones, pelvic bones, ribs, skull, and in the vertebrae of the spinal column (Clarke, 2008). It is very porous, weak, and occupies a large area within the whole bone (Clarke, 2008). Cancellous bone is a supportive stable structure and it contains most of the bone marrow in the body (Clarke, 2008). Bone marrow generates blood cells for repairing damaged or broken bones (Clarke, 2008). Three types of bone repairing cells are present in the cancellous bone: osteoblasts, osteocytes, and osteoclasts (Khan et al., 2013). The repair and renewal of bone to maintain strength and health is a continuous process (Marieb, 2006).

2.2.1.2 Bone Cells

Although various types of cells can be found in the bone, osteoblasts, osteocytes, and osteoclasts are three main cells that play important roles for bone forming, bone absorption, and bone development ("Osteoblast," 2015).

2.2.1.2.1 Osteoblasts

Osteoblasts are bone forming cells and initiate from mesenchymal progenitors, which also generate chondrocytes, muscles cells, and adipocytes (Bellido, Plotkin, & Bruzzaniti,

2014). Osteoblasts release matrix that ultimately encloses them inside the new bone and then are situated far into the bone layer (Khan et al., 2013). Osteoblasts generate plentiful alkaline phosphatase, osteopontin, and bone sialoprotein. They also produce large amounts of osteocalcin when they are mature but less amounts during an early stage of life (Khan et al., 2013). Osteoblasts proliferate and start to produce collagenase at the initial point of their remodeling stage (Khan et al., 2013). They incorporate the final phase of the matrix maturation and the initial phase of the mineralization (Khan et al., 2013). A mature osteoblast has the ability to produce type I collagen, proteoglycans, hormones, and growth factors (Khan et al., 2013). Once the bone formation matrix is completed, some mature osteoblasts stay in the bone as osteocytes, some flatten to protect inactive bone surface as bone lining cells, and the rest of the cells die via apoptosis (Bellido, Plotkin, & Bruzzaniti, 2014).

2.2.1.2.2 Osteocytes

Osteocytes originated from osteoblasts, or mature cells, osteoblasts are enclosed by the products they release ("Osteoblast," 2015). Osteocytes are buried during the bone formation process and are normally delivered right through the mineralized bone matrix (Bellido et al., 2014). Osteocytes, former osteoblasts, are the most abundant cells in bone, forming more than 90% of cells in the matrix (Bellido et al., 2014). Osteocytes have an ability to deposit and reabsorb the bone ("Osteoblast," 2015). Osteocytes are detected when bone resorption is necessary, they transmit the signal to bone lining cells to form bone structures (Bellido et al., 2014). When osteocytes discover fatigue-induced micro-damage and transmit signals to osteoclasts to repair damage bone through remodeling (Bellido et al., 2014). Osteocytes also react to mechanical load changes by causing local bone mass adjustments of geometry through

modeling (Bellido et al., 2014). Furthermore, osteocytes observe changes in hormone levels and react by controlling bone formation and bone resorption rate (Bellido et al., 2014).

2.2.1.2.3 Osteoclasts

Osteoclasts are responsible for bone resorption and are developed from hematopoietic stem cells found in blood circulation (Khan et al., 2013). Osteoclasts are necessary for bone remodeling, which changes bone shape throughout growth, and responsible for keeping unity of the adult skeleton (Bellido et al., 2014). The basic multicellular unit reconstructs bone which contains activity of osteoclasts for bone degradation and bone formation (Bellido et al., 2014). A ruffled border and a clear or sealing zone are the two distant plasma membrane types in osteoclasts cells (Einhorn, 1996; Fitzgerald, Kaufer, & Malkani, 2002) . The clear or sealing zone constructs a wall around the ruffled border for bone resorption (Khan et al., 2013). The osteoclast combines hydrochloric acid and collagenase that are eliminated at the ruffled border with high extracellular concentrations (Khan et al., 2013). Osteoclasts adjust the bone mass whenever it is necessary. Osteoblast precursors are initiated to the bone surface for proliferation, and differentiation of multinucleated cells in order for bone remodeling to occur (Bellido et al., 2014). Once the bone resorption is complete, osteoclasts experience programmed cell death (apoptosis).

2.2.1.3 Fracture Healing

There are several stages of the healing process that take place when a bone has suffered an injury (Carano & Filvaroff, 2003). It is important to understand the structural failure of bone because it involves more complicated sequences than a regular bone break (Khan et al., 2013). Blood provides a certain nutrient supply to the bone; therefore, blood supply may be compromised during a bone fracture whereas other tissues such as ligaments, tendons, and

muscles may or may not have an effect on the bone fracture (Khan et al., 2013). The healing process of bone can be divided into three stages: inflammation, repair, and remodeling (Claes, Recknagel, & Ignatius, 2012).

2.2.1.3.1 Inflammation

When a bone injury occurs, hematoma, platelets, macrophages, monocytes, and polymorphonuclear neutrophils gather at the injury site and start the cleaning-up and restoring process (Khan et al., 2013). A bone break causes cell death which interrupts blood flow to the bone (Khan et al., 2013). The cell level division rate is raised at both the injury site and throughout the whole bone for eight hours after the injury and continuously for the first 24 hours post injury (McKibbin, 1978). Within a few days, this elevated cell division rate decreases throughout the bone (Khan et al., 2013). Osteoprogenitor cells, fibroblasts, and endothelial cells travel to the damaged site from the bone marrow (Khan et al., 2013). These cells join with endogenous growth factors and proteins to start the restoring process (Khan et al., 2013). The renewal process starts as new blood vessels and the bone are formed (Carano & Filvaroff, 2003).

2.2.1.3.2 Soft and Hard Callus Formation

A callus is a fibrous tissue layer that starts to develop around the fracture area as inflammation gradually decreases (Carano & Filvaroff, 2003). Bone continues to reconstruct for several weeks while inside the callus formation (Khan et al., 2013). The shape of the initial callus appears as a soft form and is a cartilaginous/bony tissue. The formation of the cartilage takes place at the callus' edges and bone formation is at the fracture site (Khan et al., 2013). Soft callus supports finite stability to the fracture site. Eventually, cartilage becomes woven bone via endochondral ossification and bone formation starts from mesenchymal stem cells (MSCs) and preosteoblasts in the callus formation (Khan et al., 2013).

2.2.1.3.3 Bone Remodeling

Most organs in the human body do not make any major changes once the adult organ has been completely developed whereas bone is constantly regenerated (Karperien et al., 2014). The regeneration process starts immediately after forming of the first bone; the bone is automatically and continuously replaced by new bone in this process (Karperien et al., 2014). The formation of woven bone in the callus is gradually reconstructed as lamellar bone during the final stage of healing (Carano & Filvaroff, 2003). The mineralized callus is gradually reconstructed through osteoclast resorption of the bone. Osteocytes and osteoblasts are the major contributors in new bone formation (Karperien et al., 2014). The completion of this remodeling stage may take several years but remodeling stage in an adult is limited and is not complete (Khan et al., 2013). However, bone remodeling in children is complete because more cellular, periosteum is present in children which are comparatively thicker than adults (*Orthopaedics: Principles of Basic and Clinical Science*, 1999).

2.3 Motivation for Bone Tissue Engineering

There are various types of bone graft repair methods that have been used in bone tissue engineering. Repair methods and strategies can differ depending on bone injury types. In many situations injured bone may not be able to heal by itself. Traumatic bone loss or tumor resection of a bone defect will not heal with the help of external fixation, so clinical intervention is needed in serious bone defect (Porter et al., 2009). Researchers have found other alternative methods such as autograft, allograft, and synthetic bone graft materials, as well as other applications to solve serious types of bone injuries (Porter et al., 2009).

2.3.1 Current Methods of Bone Grafts

2.3.1.1 Autograft

The autograft, tissue repair method involves, tissue being taken from one area of the patient's body and reconstructed into another area of the same patient (Khan et al., 2013). This method provides an advantage because there is decreased risk of immunogenic response and less disease transfer to the patient since the tissue being used is the patient's own (Khan et al., 2013). Tissue being used for autograft is normally taken from the iliac crest of the pelvis because more muscle can be found in that area (Khan et al., 2013). Trabecular, or porous, bone can also be found in the pelvis crest area (Khan et al., 2013). In addition, trabecular bone cultivated from the iliac crest is more porous and, rich in marrow, blood, stem, progenitor cells, proteins, and growth factors (Khan et al., 2013).

There are certain limitations of using autograft as a bone graft substitute. In autograft treatment, bone is taken away from the pelvis or iliac crest of the patient's own body and is used to repair the bone defect (Porter et al., 2009). Surgical complication rate of autograft procedures is often as high as 30% and may include various issues such as limited accessibility of donor tissue, donor site morbidity, pain, paresthesia, extended hospitalization and rehabilitation, infection, inflammation, and hematoma formation (Khan et al., 2013; Porter et al., 2009) . Autograft tissue harvesting also deals with complications such as hematoma formation, infection, arterial injury, ureteral injury, fracture, pelvic instability, cosmetic defects, tumor transplantation, and sometimes chronic pain (Giannoudis, Dinopoulos, & Tsiridis, 2005).

2.3.1.2 Allograft

For the allograft repair method, tissue is cultivated from the same species, human to human, and used for grafting (Khan et al., 2013). Allograft is carried out with processes such as a

freeze-drying, fresh frozen graft or demineralized bone matrix (Khan et al., 2013). Freeze-dried and fresh frozen grafts allow the microstructure and general structure to remain the same while demineralized bone matrix is a power-like element that does not have its own mechanical properties (Costain & Crawford, 2009). There is little risk of disease transmission from the donor to the receiver in fresh frozen allograft while the freeze-dried process does not transmit disease (Laurencin, Khan, & El-Amin, 2006). The mixture of demineralized bone matrix and solutions will form a material like a plastic which can form any shape (Laurencin et al., 2006). Not all the bone defects can be repaired with autograft and allograft because some severe defects may intervene with surgical procedures, these may include joint replacement, inserting metallic rods, pins, or screws, ceramics, and polymers (Khan et al., 2013).

There are advantages of using allograft as bone grafts. Allograft is another alternative method for bone grafting, but some limitations are involved when using the allograft method for bone graft substitutes. Allografts can cause problems to patients, these may include infection from the donor to the receiver, disease transmissions, and host immune responses (Hou, Yang, & Hou, 2005; Nishida & Shimamura, 2008) .

2.3.1.3 Synthetic Materials

Organ shortage is one of the main problems that the clinical field is facing. The demand of organ transplant is high while there is only a limited supply of organs available to patients. Even if organs are available, there is the chance that a disease could be transmitted from the donor during the organ transplant process. These major challenges cannot be avoided and researchers must find alternative ways to solve the problems presented. An unlimited amount of supplies and elimination of disease transmission are advantages of using synthetic bone graft substitutes (Amini et al., 2012). Materials that are used in bone tissue engineering must be able

to provide biological support (Gunatillake & Adhikari, 2003). In addition, they must be non-toxic, biocompatible, and biodegradable (Gunatillake & Adhikari, 2003). Different types of synthetic materials are used in synthetic bone grafts such as metals, polymers, or ceramics (Bohner, 2010). Metals that are used as synthetic materials for bone grafting are tantalum, titanium, iron, and/or magnesium. Polymers that are used as synthetic materials are polylactides, polyglycolides, polyurethanes, or polycaprolactones. Ceramics that are used include silicate based glasses, calcium sulfate hemihydrates and dehydrate and calcium phosphates (Bohner, 2010).

There are advantages of using synthetic bone grafts. Using synthetic material as bone graft substitute is advantageous because these have materials have potential to eliminate organ shortages, eliminate disease transmission, and reduce infection risk (Porter et al., 2009).

2.4 Polyurethanes (PU)

Polyurethanes are in the polymer class family and have many attractive properties which draw research interest for testing their applications in the medical field (Alves, Ferreira, & Gil, 2012; Ratner, 2013) . Polyurethanes are commercially available and easy to assess which provide advantages for evaluation in research. In addition, they are chemically stable, have good biocompatibility and are easy to process which make them the best choice for use in biomedical applications (Alves et al., 2012; Ratner, 2013) . Polyurethanes have been used as adhesives, coatings, sealants, rigid and flexible foams, and textile fibers (Ratner, 2013). They have been examined in several biomedical applications including pacemakers, breast implants, heart valves, vascular prostheses, bioadhesives, catheters, vascular grafts, wound dressings and other blood contacting applications (Alves et al., 2012; Ratner, 2013) .

2.4.1 Polyurethanes Synthesis

Polyurethanes include three precursor molecules: diisocyanates, diols, and chain extenders (Ratner, 2013). They are also called segmented polymers because they have both hard and soft segments, which can be differentiated based on the polymer molecule that occurs last (Shelke, Nagarale, & Kumbar, 2014). Hard segments will be formed if the last polymer molecules are diisocyanates or, chain extenders; and soft segments will be formed if the last polymers are diols (Ratner, 2013). The soft segment contributes elastic properties to the material due to a low glass transition temperature while hard segment contributes glassy or crystalline properties and gives additional mechanical strength to the materials (Alves et al., 2012; Ratner, 2013) . The hard and soft segment of polyurethane affect morphology, mechanical properties, and the degradation rate (Gunatillake & Adhikari, 2011). Polyurethanes are suitable candidates for high-load bearing applications such as bone scaffolds (Bonzani et al., 2007; Eglin, Mortisen, & Alini, 2009; S. Guelcher, 2008).

2.4.2 Polyurethane and Polyurethane-based Applications in Bone Tissue Engineering

Polyurethane is one of the most in demand biomaterials for biomedical devices because it is a segmented polymer that provides the ability to change its physical properties, blood tissue compatibility, and biodegradation (Santerre et al., 2005). Several researchers examined polyurethane applications in various medical fields because polyurethane has moderately good blood biocompatibility (Santerre et al., 2005). However, polyurethane is not biostable material which means polyurethane does not have ability to keep its physical, mechanical and chemical integrity in long-term implants, *in vivo* (Santerre et al., 2005). Therefore, authors, Santerre, Woodhouse, Laroche, and Labow, analyzed the root problems of PU degradation in vivo and studied the biological processes of PU (Santerre et al., 2005). Modeling technique is one of these

strategies; some researchers inquire how polyurethane applications behave as implants *in vivo* by using the modeling technique (Santerre et al., 2005). Various factors such as biodegradation, environmental biodegradation, and the interaction between polyurethane and biological agents, considered in order to increase the stability properties of polyurethane devices (Santerre et al., 2005). Information about the degradation rate of the implanted devices material is essential because knowing the life span of a biomedical device determines how a device is used. Researchers study the degradation rates of polyurethanes in many different ways. One way that has been used involved monitoring the polyurethane degradation rate in phosphate buffered saline solution at 37˚C while characterization took place over 8 weeks (Guan, Fujimoto, Sacks, & Wagner, 2005). It is important for implants to have appropriate responses while interacting with cells. Therefore, characterizing cell growth of the seeded cells on a scaffold was also examined *in vitro* (Guan et al., 2005). Researchers reported that mechanical and chemical properties of polyurethanes improved; however, no *in vivo* study was conducted to evaluate clinical relevant improvement (Guan et al., 2005).

Researchers have applied different methods to study degradation rates, load bearing, and cell growth of a polyurethane based application (Ruan et al., 2014). Studies investigated the variation of pH values during degradation, water absorption, ratio of weight loss, and molecular weight and degradation rates of polyurethane (Ruan et al., 2014). One such, *in vitro*, experiment was performed for a 12 week period in an environment with a temperature control of 37˚C. Degradation behavior and surface morphologies of the samples at 0, 2, 4, and 12 weeks respectively were studied (Ruan et al., 2014). The tensile strength was tested and the average values of three samples were taken (Ruan et al., 2014). Additionally, a satisfactory pH value was also produced for the cells' growth (Ruan et al., 2014).

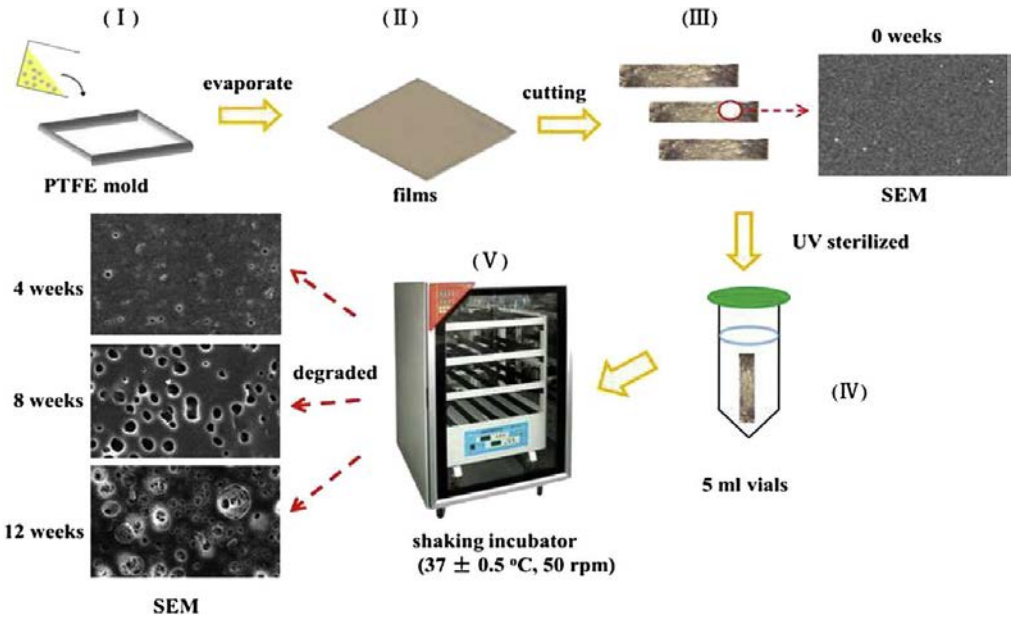

Fig 2.4: Flow chart of formation and degradation experiment of polymer films: (I), (II) and (III), the formation process of polymer films; (IV) and (V), the degradation of polymer filmsand the surface morphologies of P-PUUs degraded at 0, 4th, 8th and 12th weeks, figure adapted from (Ruan et al., 2014)

Some applications must consider materials with different hydrophilicity and/or controlled hydrophilic-to-hydrophobic segment ratios. This means that the hydrophilic segment was based on poly (ethylene oxide-propylene oxide-ethylene oxide) diols (Pluronic1) and the hydrophobic segment was based on poly (ϵ-caprolactone) diol (Gorna & Gogolewski, 2002). High hydrophilic hydrogel-like materials do not support cell adhesion while low hydrophilic materials support cell adhesion and, cell proliferation, which make the low hydrophilic content materials suitable for tissue engineering scaffold applications (Gorna & Gogolewski, 2002). The degradation behaviors of samples in vitro were studied. Samples were placed in phosphate buffered solution with pH values between 7.4 ± 0.2 (Gorna & Gogolewski, 2002). The experiment was examined for 4, 12, 24, and 48 weeks respectively at temperature 37 ± 0.1 ˚C and mechanical properties of the

samples were also tested. The mean of six measurements was found, with a 1 kN load cell operating at a cross-head speed of 200 mm/min (Gorna & Gogolewski, 2002). Both aliphatic polyurethane based ϵ-caprolactone and pluronic based materials showed a decrease in tensile strengths and Young's moduli (Gorna & Gogolewski, 2002). .

2.4.3.1 Limitation of Polyurethane

Developments of implant devices are not simple. The human body is made up of various complex tissues, organs, bones and many other things. Consequently, researchers face many problems while developing the implant devices. Therefore, it is important to study and consider various aspects in order to construct successful implant devices. In bone tissue engineering, synthetic polymer scaffolds such as polyurethane, have to support cells growth, proliferation, and cell attachment ,in addition, the material has to be biodegradable, and bioactive (S. A. Guelcher et al., 2008). Mechanical properties of synthetic polymer scaffolds need to resemble bone (S. A. Guelcher et al., 2008). Polyurethane (PU) networks are made up of two-components: reactive liquid molding of low-viscosity quasi-prepolymers derived from lysine polyisocyanates and poly (3-caprolactone-co-DL-lactide-co-glycolide) triols (S. A. Guelcher et al., 2008). Compression tests of the samples were tested with a speed of 1.33 mm/min at every 2.5s and the compressive strength and Young's moduli of the samples were measured (S. A. Guelcher et al., 2008). Samples were incubated in PBS solutions for 20 days to observe degradation rates, at the same time the cells were seeded into polyurethane networks, liquid molding of low-viscosity quasi-prepolymers derived from lysine polyisocyanates and poly (3-caprolactone-co-DL-lactide-co-glycolide) triols, to observe cell proliferation and attachment (S. A. Guelcher et al., 2008). The outcomes of the experiments gave positive support (S. A. Guelcher et al., 2008).

2.4.3.2 Biostability of Polyurethane

Researchers use different methods to investigate polyurethane applications for long-term implants *in vivo*. Polyurethane is not biostable which mean it is not stable in biologically active environment ;therefore, studies are being done in order to understand the degradation rate of polyurethane scaffolds to improve biostability (Woźniak et al., 2010). An experiment done in an *in vitro* environment is a preliminary step and needs to be carried out before an experiment is conducted in an *in vivo* environment. Mechanical and biological properties of samples are normally tested in bone tissue engineering application. In *in vitro* environments, numbers of cells, cell phenotypes, and cell distribution were evaluated (Woźniak et al., 2010). Human-bone-derived cells (3x 10^5) were seeded on each polyurethane scaffold and performed cell culture experiment *in vitro* environment for 7 days and 14 days (Woźniak et al., 2010). Human-bone-derived cells were used in this study to observe how the cells interact with a polyurethane scaffold *in vitro* for 7 days and 14 days, in addition, cell viability of samples were evaluated using XTT assay by checking absorbance level (Woźniak et al., 2010).

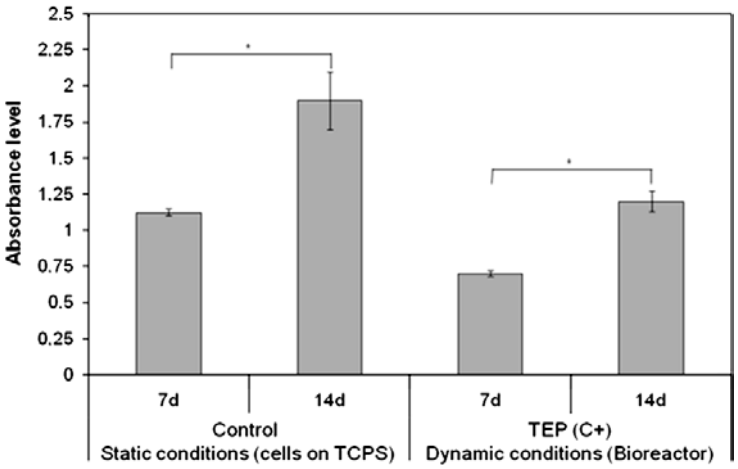

Fig 2.5: The results of the XTT assays performed after 7 and 14 days of culture on the (Tissue Engineering Product) TEP (C+) and control (cells on Tissue Culture Polystyrene plate (TCPS)) samples, figure adapted from (Woźniak et al., 2010)

2.4.3.3 Biodegradability of Polyurethane

A study was done by Joanna Podporska-Carroll, Josephine W.Y. Ip, and Sylwester Gogolewski, in order to determine the biodegradable application of polyurethane scaffold as cancellous bone graft substitutes (Podporska-Carroll, Ip, & Gogolewski, 2014). Several techniques such as proton nuclear magnetic resonance, size-exclusion chromatography, infrared spectroscopy, computed tomography, and mechanical tests were performed in order to make the characterization of scaffolds (Podporska-Carroll et al., 2014). A cell culture experiment was conducted, to investigate how the osteoblast-like MG-63 support cells attached, grew, and proliferated (Podporska-Carroll et al., 2014). The cells were seeded into scaffolds for 1, 2, 3 and 4 weeks and researchers observed the scaffold and cell interactions, as well as DNA content, total protein amount, alkaline phosphate activity, and WST-1 assay of the scaffolds (Podporska-Carroll et al., 2014). (Podporska-Carroll et al., 2014).

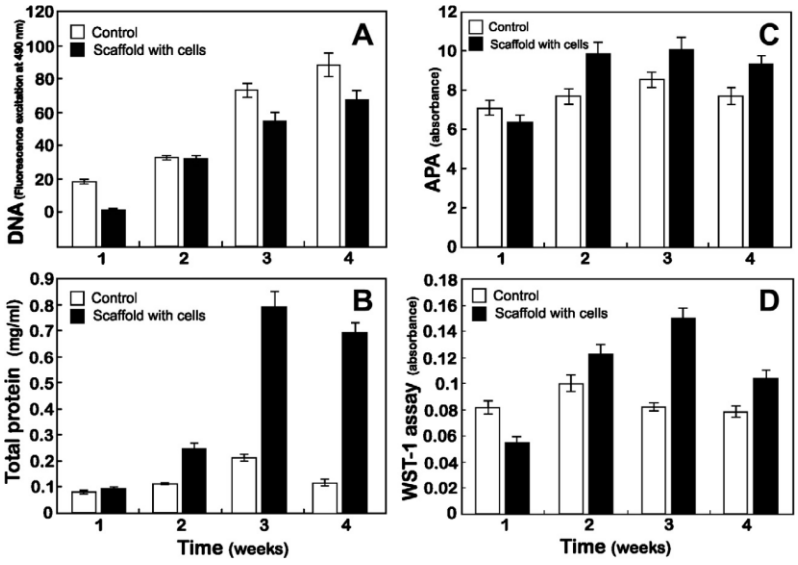

Fig 2.6: (A) DNA content; (B) total protein amount; (C) alkaline phosphatase activity (APA); (D) Wst-1 assay. Error ± SD for n = 6, figure adapted from (Podporska-Carroll et al., 2014)

Researchers found out DNA content increased from week 1 to week 4, total protein increased from week 1 to week 3 but there was a slightly decreased in week 4, alkaline phosphate activity from week 1 to week 4, and WST-1 reached maximum at week 3 and decreased in week 4, indicated that cell proliferation was reduced (in fig 2.6). There is no doubt that the scaffolds promoted cell attachments, growth, and proliferation of the MG-63 cells over the 4 week culture period.

Other researchers also used *in vivo* environment to study scaffolds that can release proper biomolecules/drug(s) to surrounding areas in order to avoid post-operative infections and inflammation after surgery (Gentile et al., 2015). A surface coating of gelatin embedded with polyurethane-based nanoparticles (NPs) was applied on composite scaffolds as well as an anti-inflammatory drug, indomethancin (IDMC) (Gentile et al., 2015). These were meant to, prevent post-operative infections and inflammation and were evaluated by studying the physical properties and cell behavior that took place on and near the scaffolds ;therefore, researchers

examined the experiment *in vitro* environment (Gentile et al., 2015). MG-63 osteoblast-like cells was used to study cell proliferation and cell viability *in vitro* environment and cell culture experiment was examined on NPs for 1, 4 and 7 days (Gentile et al., 2015). Cell viability of materials was observed using 3-dimethylthiazol-2, 5-diphenyltetrazolium bromide (MTT assay) by checking absorbance level and cell viability was increased along with incubation period (in fig2.6) (Gentile et al., 2015). In addition, after the first week of incubation, 65-70% of the drug was released which lead to the finding that the drug was able to prevent post-operative inflammation and infection (Gentile et al., 2015).

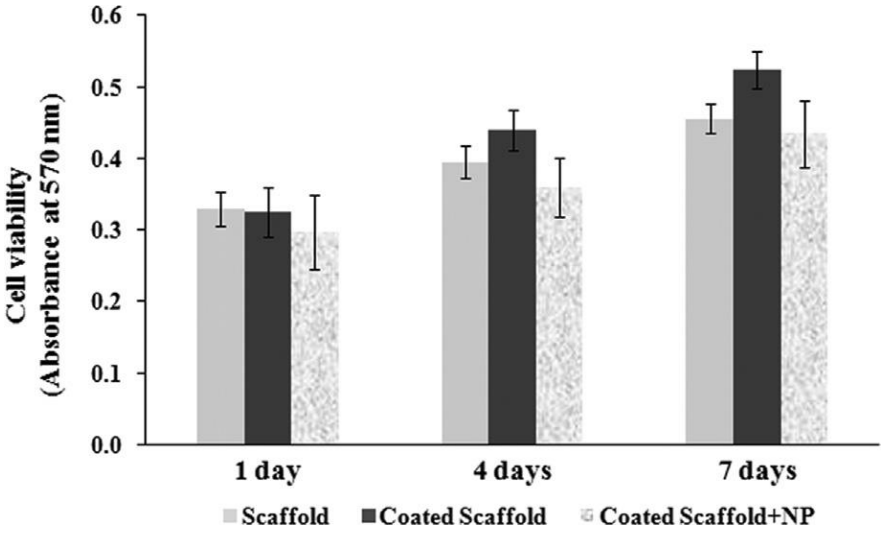

Fig 2.7: MG-63 osteoblast-like cells attachment and proliferation levels assessed using an MTT method on the fabricated porous composite scaffolds before and after collagen gelatin coating without/with incorporation of indomethacin-loaded PU nanoparticles after 1, 4 and 7 days of culture, figure adapted from (Gentile et al., 2015)

Other researchers focused on various issues that occur due to the biomedical device. Infection is a complicated problem in bone tissue engineering applications that cannot be avoided and must not be neglected because an infected wound can lead to danger of the patient's life or loss of a limb (Li, Brown, Wenke, & Guelcher, 2010). For that reason, some researchers

studied how to prevent the infection of the implanted device. The clinical field is presently practicing standard care procedures for treating infected open fractures which involves, first treating the wound with antibiotics then following with bone grafting (Li et al., 2010).

2.4.3.3 Optimization of Polyurethane

Some studies focus on optimization of the structure of polyurethane for bone tissue engineering applications (Bil, Ryszkowska, Woźniak, Kurzydłowski, & Lewandowska-Szumieł, 2010). Optimization is required in all application types and it is crucial, especially in medical devices since the human body system is very complicated. The advantages of using polyurethane in bone tissue engineering are that it has both soft and hard segment ratios. This allows the mechanical and physical properties of polyurethane to change into the appropriate values according to the type of the application that is used in bone tissue engineering (Grad, Kupcsik, Gorna, Gogolewski, & Alini, 2003; Guan et al., 2005; Riboldi, Sampaolesi, Neuenschwander, Cossu, & Mantero, 2005; Zhang, Doll, Beckman, & Hollinger, 2003). Many researchers study various elements including protein adsorption, cell adhesion and blood compatibility of segmented polyurethanes to understand relationships between structure and the properties of segmented polyurethane (S.-L. Huang, Chao, Ruaan, & Lai, 2000; A. Takahara, Tashita, Kajiyama, & Takayanagi, 1985). Some studied and examined the relationship between the surface properties and biocompatibility of PUs based on various types, molar ratios of polyols and diisocyanates. It was found that the type and concentration of polyol on the PU surface had an effect on blood-materials (Lelah, Pierce, Lambrecht, & Cooper, 1985; Atsushi Takahara, Tashita, Kajiyama, Takayanagi, & MacKnight, 1985) . Therefore, some researchers explored the development of polyurethanes with the optimal hard segment content for human osteoblast cultures and observed the reaction and behavior of cells with materials (Bil et al., 2010).

Mechanical properties of samples are normally tested to identify the tensile and Young's modulus values of polyurethanes (Bil et al., 2010). The evaluation of biological testing is also conducted using XTT assays to analyze the viability and number of cultured cells seeded on the test samples (Bil et al., 2010). The reaction of human derived bone cells was influenced by the polyurethane surface due to the hard to soft segment ratio, it also affected by the cell proliferation, and the cell attachment ability (Bil et al., 2010).

Although many cites reported biocompatibility and biofunctionality of polyurethane; however, there is no optimal solution exist for polyurethane scaffolds especially patient spectrum scaffold parameter.

CHAPTER 3

EXPERIMENTAL PROCEDURE AND TECHNIQUES

3.1 Materials

Mechanical properties of uncoated polyurethane samples such as niobium coated polyurethane samples, and magnesium coated polyurethane samples were investigated in this experiment. Before test samples were examined for mechanical testing, test samples were cut out from the polyurethane foam sheet by using a foam cutting machine. The sample was cylindrical shape and had a diameter of 0.39 inch and the height of 1 inch. After the samples were Scut, metallic coatings were applied. Polyurethane samples were coated with niobium (Nb) and magnesium (Mg) to evaluate how the coatings improved mechanical properties.

Fig 3.1: Polyurethane samples were cut by foam cutting machine

Fig 3.2: Polyurethane samples were cut into cylindrical shape

Polyurethane samples were cut by using foaming cutting machine (fig 3.1 and fig 3.2).

3.2 Electrodeposition Process

3.2.1 Fabrication of Metallic Scaffolds

3.2.1.1 Nb Coating

Nb metallic coating was performed using Niobium (V) chloride ($NbCl_5$) salt. Nb coating was examined with two different coating conditions. Tetrahydrofuran was used as ionic liquid in the electrodeposition process. The Nb electrolyte solution contained 1:4 molar ratios of ionic liquid and $NbCl_5$ salt. 4 grams of $NbCl_5$ salt was used in Nb coating condition 1 and added salt slowly into 40 ml water and stirred using a magnetic stir plate until the salt was completely dissolved. After the ionic liquid, 10 ml was added into the solution and stirred with electrolyte solution for a couple of minutes. The electrodeposition process was conducted in Nb electrolyte solution by placing a platinum rod at the cathode and placing a polyurethane sample at the anode. The current was set between 0.03A and 0.09A while the voltage was set between 2.0V and 3.5V for both coating conditions. 6 g of $NbCl_5$ salt was added into 40 ml water and mixed with 10 ml of ionic liquid for Nb coating condition 2 and electrodeposition process was carried out. Electrodeposition was performed two hours for both condition 1 and condition 2 at room temperature. The electrodepositing sample was checked every 5 to 10 minutes and was turned to the side in order to receive an even coat for both conditions. Samples were dried at room temperature in open atmosphere for a few days after electrodeposition. When samples were completely dried, they were proceeded to do mechanical testing.

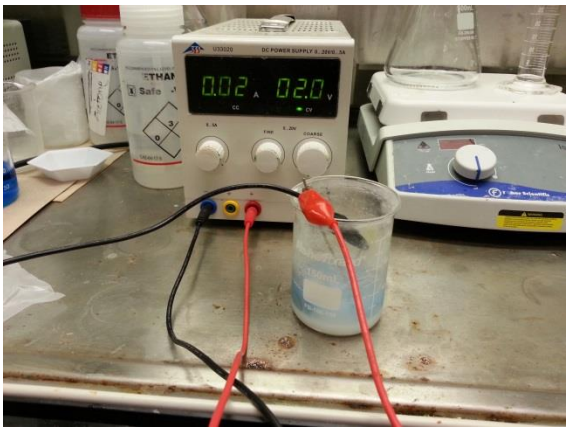

Fig 3.3: Electrodeposition of Nb on polyurethane

Fig 3.4: Nb Coated samples

Fig 3.3 shows the electrodeposition process of polyurethane sample and fig 3.4 shows Nb coated samples after electrodeposition process.

3.2.1.2 Mg Coating

Magnesium (II) chloride ($MgCl_2$) salt was used in metallic coating of Mg. Test samples were electrodeposited with two different conditions. Tetrahydrofuran was used as ionic liquid in the electrodeposition process. The electrolyte solution used contained a 3:4 molar ratio of BF_4 and $MgCl_2$ salt. 18.72 g of $MgCl_2$ salt was slowly added into 40 ml water and the solution was stirred using a magnetic stir plate until the salt dissolved in the water. When $MgCl_2$ salt dissolved in water, 30 ml of ionic was added into the $MgCl_2$ salt solution and stirred until mixed completely. Once the solution was ready, the electrodepositing process was begun. A platinum rod was placed at the cathode and a polyurethane sample was placed at the anode to perform the electrodeposition.

The experiment was set up with a current between 0.05A and 0.1A and the voltage between 3.0V and 4.0V; the sample was electrodeposited for two hours. For Mg coating condition 1, $MgCl_2$ salt was increased by half and the ionic liquid was decreased by half for

another Mg coating condition 2 and the sample was electrodeposited for three hours. The electrolyte solution for Mg conditions contained 28.08g of $MgCl_2$ salt in 60 ml of water and contained 15 ml of ionic liquid. The electrodeposition was checked every 5 to 10 minutes and the samples were rotated to allow for uniform deposits. The experiment was performed at room temperature. The same experiment procedure was carried out for both conditions. After finishing the electrodeposition, samples were placed in a furnace for heat treatment using a temperature of 350˚F. The samples were soaked in water to remove ionic liquid after heat treatment and samples were then dried at room temperature.

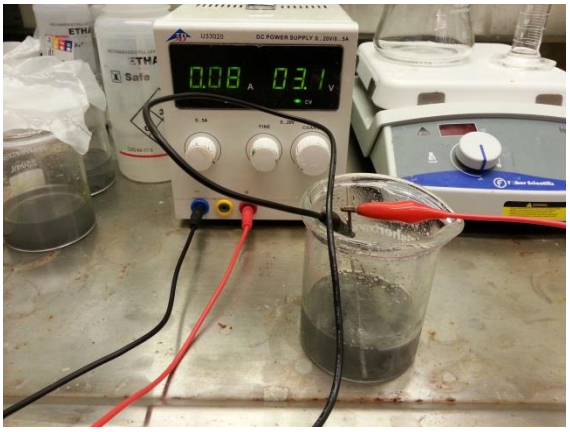

Fig 3.5: Electrodeposition of Mg on polyurethane

Fig 3.6: Mg coated samples

Fig 3.7: Plain polyurethane, Mg coated sample & Nb coated sample

Fig 3.5 and fig 3.6 showed the electrodeposition process of polyurethane and polyurethane samples after being coated with Mg. Fig 3.7 showed photos of plain polyurethane, Mg coated sample and Nb coated sample.

3.3 Evaluation of Mechanical Testing

The mechanical testing was performed when the samples were completely dried. Compressive strength of uncoated polyurethanes, Nb coated polyurethanes, and Mg coated polyurethanes samples were evaluated by using MTS machine. The samples were compressed with a speed of the cross head at 0.1 in/min and measured every second. The weight of the load was 1 lb. Strain and stress was calculated from the data obtained from compression testing. Strain was calculated by dividing crosshead speed over height of the samples and stress was calculated by dividing force over the area.

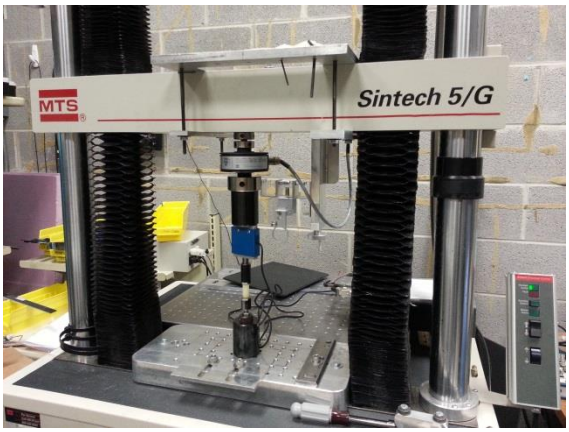

Fig 3.8: Compression test with MTS machine

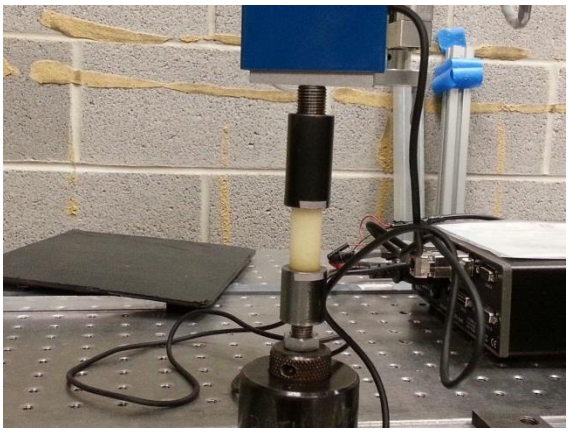

Fig 3.9: Before compression test

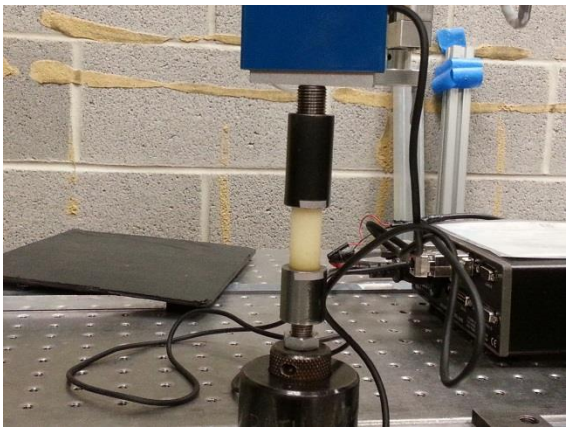

Fig 3.10: Polyurethane sample before the test

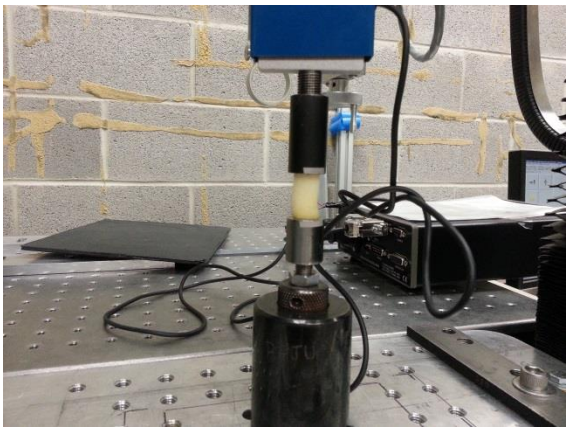

Fig 3.11: Polyurethane sample during compression test

After obtaining metallic coatings on PU, the samples were placed for mechanical testing to check ultimate strength of samples. Compression test was performed using MTS machine which is in fig 3.8, fig 3.9, fig 3.10 and fig 3.11, and shows polyurethane sample before the test and during the compression test.

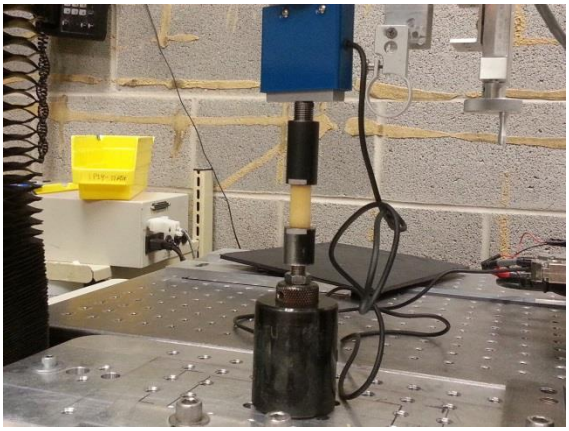

Fig 3.12: Nb coated sample before the test

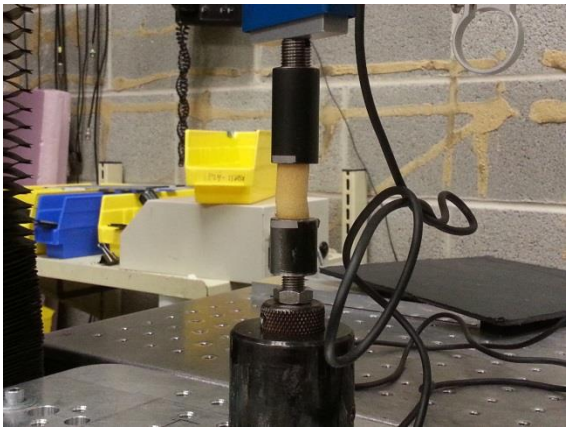

Fig 3.13: Nb coated sample during the compression test

Fig 3.12 and fig 3.13 showed the compression test of Nb coated sample before the test and during the test.

Fig 3.14: Mg coated sample before the test

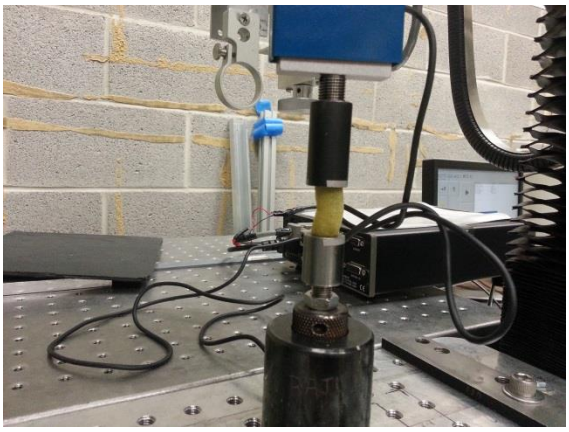

Fig 3.15: Mg coated sample during compression test

Mg coated sample before the test and during compression test is shown in fig 3.14 and fig 3.15.

3.4 Biocompatibility Evaluation

In a tissue culture, the cells were cultivated in an artificial environment which harvests cells, tissues, or organs in a controlled setting (Ratner, 2013). Cell growth during *in vitro* studies requires the same conditions as cells growing *in vivo* (Ratner, 2013). In a cell culture experiment, essential elements are replaced by other products. Culture media acts as the blood, glucose as energy source, salts, and amino acids as essential nutrients, and vitamins as proteins and hormones (Ratner, 2013). The pH control is very important in a cell culture experiment (Ratner, 2013). It is essential to maintain the purification and circulation of the media which must to be changed regularly according to the cells' needs (Ratner, 2013). Since the cells are grown in an artificial environment, there is no immune system to control from fungi and bacteria; therefore, antibiotic must be added into the media (Ratner, 2013). Cell culture experiments need to be carried out in a sterile environment in order to reduce the contamination of the cells and cells must also incubate in a specific temperature (Ratner, 2013). Two different types of cell lines,

L929 fibroblast cell line and the MC3T3-E1 osteoblast cell line, were used to evaluate the biocompatibility and biofunctionality of metallic scaffolds.

3.4.1 Cytotoxicity Evaluation Using Direct Test

PU samples, Nb coated samples, and Mg coated samples were cut into a small size to evaluate cytotoxicity. The test samples were sterilized a couple of days before cytotoxicity evaluation experiment was carried out. Sterilizing of the test samples is very important because unsterilized test samples can contaminate cells and can lead to incorrect cytotoxicity experiment results. L929 fibroblast cells were cultured in a T-75 culture flask for a couple of days beforehand in order for them to grow and reach the desired confluency level. Once the cells reached confluency, they were seeded into a 12-well plate with 200,000 cells per well and incubated at 37˚C in a 5% CO_2 incubator for 1 day. Cell proliferation and cell morphology was checked after 1 day, before the test samples were added into the 12-well plate. The old media was removed before placing the test samples into the 12 wells. Test samples were placed into the cell seeded well plates and complete fresh media was also added. When the samples were placed into the cell seeded well plates, they were incubated for another 24 hours at 37˚C in a 5% CO_2 incubator to evaluate cytotoxicity. Cell morphology and cell proliferation of cells with the test samples were observed after 24 hours period. The entire cell culture experiment was performed in a sterile environment.

3.4.2 Cytotoxicity Evaluation Using Liquid Extract

The test samples were sterilized, before the liquid extraction experiment was carried out. L929 fibroblast cells were cultured in a culture flask for a few days before the liquid extraction experiment started. When the cells reached confluency, a cell count was performed before the cells were seeded into a 12-well plate. 200,000 cells per well were seeded and incubated for a

day. Cell proliferation and cell morphology after incubation for 1 day, 4 days and 7 days were observed. In this experiment test, the test samples were placed in tubes with a culture media and then incubated at 37˚C in a 5% CO_2 incubator for a day. The samples were placed in a 4˚C environment after incubated for 24 hours. Liquid was extracted from the tubes and transferred into seeded well plates; the samples were incubated for another day to evaluate cytotoxicity.

3.5 Biofunctionality Evaluation

Biofunctionality evaluation of metallic scaffolds was examined using the MC 3T3-E1 osteoblast cell line. The osteoblast cells were cultured a few days early to reach the desired confluency. Test samples were sterilized before the experiment was conducted. Once the cells reached confluency, they were seeded into the into a 12-well plate. Cells were counted before they were seeded into the 12-well plate. 20,000 cells were seeded on each test sample and the well plate was incubated for 1 day, 4 days and 7 days. A serial dilution method was used in order to perform the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] calibration experiment. The cells were diluted to a ratio of 1:2, 1:4, 1:8, and 1:16 and diluted cells were seeded in each well. The cell seeded plate was incubated for ≥ 2 hours to allow re-adherence to the surface. An MTT assay was prepared to perform MTT calibration. MTT dissolved in PBS at a concentration of 5mg/ml. MTT assays were added into the 12-well plates after they were incubated for more than 2 hours. Old media was removed from the well plates and fresh media was added, 100µl of MTT solution was also added to each well. MTT solution was added to both cells within test material plates and the calibration plate both were incubated with MTT assay for 2 more hours.

3.6 Cell Characterization

3.6.1 Hemocytometer

Hemocytometer was used to perform cells count. Cells suspensions with dyes were loaded into counting chamber in order to do cell counting. Cell counting was started once the cells were dispersed to grids and only the live cells will be counted. Dilution of cells suspensions need to be performed depending not only on the cell counts but also on the cell seeding as well. Once the cells were adjusted to desired numbers, the cells were seeded into the well plates.

3.6.2 Optical Microscope

The Lecia Dmil LED optical microscope was used to capture the images of the cells and cells with test samples, results were observed. The microscope was attached to a digital camera and a computer that had Leica Vision software. The microscope setting was adjustable depending on the user's choice. Magnifications of the images are also based on requirement of the sample images. The images taken from the camera were analyzed using a computer that had Leica Vision software installed.

Fig 3.16: Leica Dmil Led Microscope

3.6.3 Spectrophotometer (Spectronic 200E)

Spectronic 200, a spectrophotometer, measured the absorbance of the samples. Spectronic 200 came with built-in software; therefore, it was easy to access and analyze the data. The cable was plugged in directly to the source and then settings were adjusted based on user preference. Samples were placed in cuvettes and absorbance was measured by scanning through with UV light. The settings and compartments were adjustable.

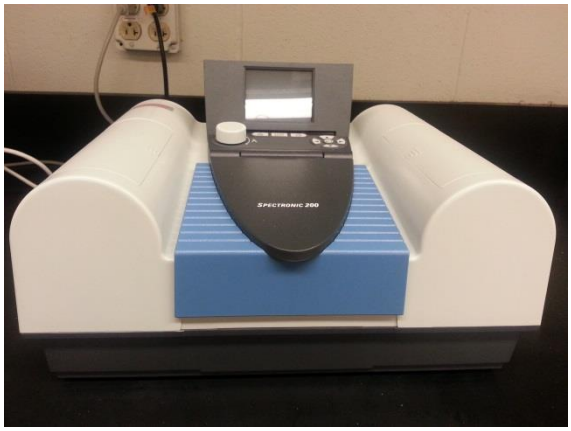

Fig 3.17: Spectronic 200

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Mechanical Testing Results of Metallic Scaffolds

Polyurethane was coated with Nb and Mg to enhance its mechanical properties. Mechanical properties of coated samples were conducted using MTS machine. Fig 4.1 shows the stress and strain result of coated samples.

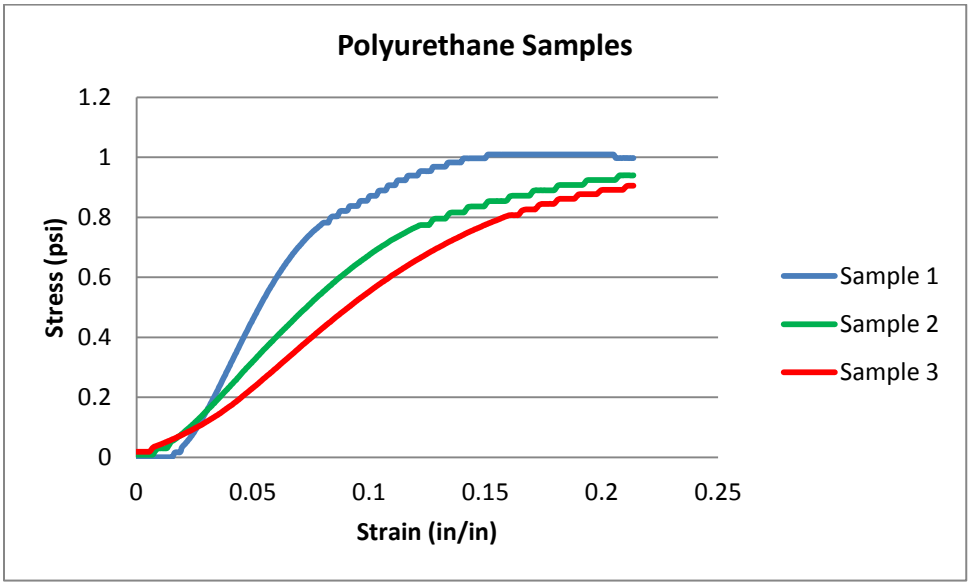

Fig 4.1: Stress and strain diagram of plain polyurethane samples

Stress and strain results of the polyurethane samples are described in the above diagram (fig 4.1). Ultimate stress of the samples was calculated based on stress and strain diagram (fig 4.1) where maximum value of stress was the ultimate stress of the samples.

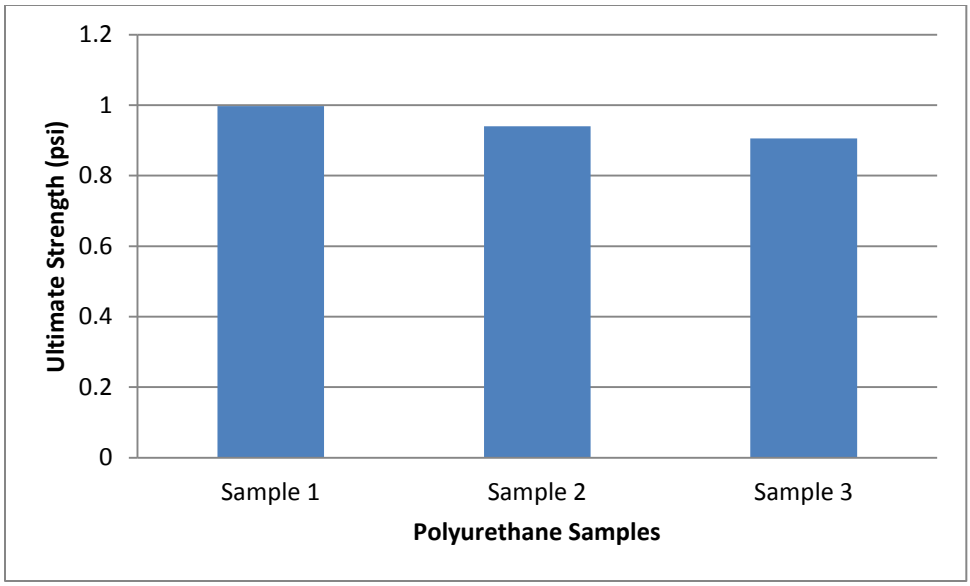

Fig 4.2: Ultimate strength of polyurethane samples

Stress and strain of polyurethane samples were calculated based on data obtained from compression test. Fig 4.1 shows the stress and strain curve for polyurethane. The ultimate strength of polyurethane samples was calculated based on fig 4.1 stress and strain diagram. The ultimate compressive strength of the samples ranged between 0.9-1.0 psi and the average value of samples and standard deviation was calculated. The average ultimate strength was 0.95 psi ± 0.048.

4.1.1 Nb Metallic Coating

Compressive strength of Nb coated samples was also examined and stress and strain results of Nb coated samples are described in the diagrams below.

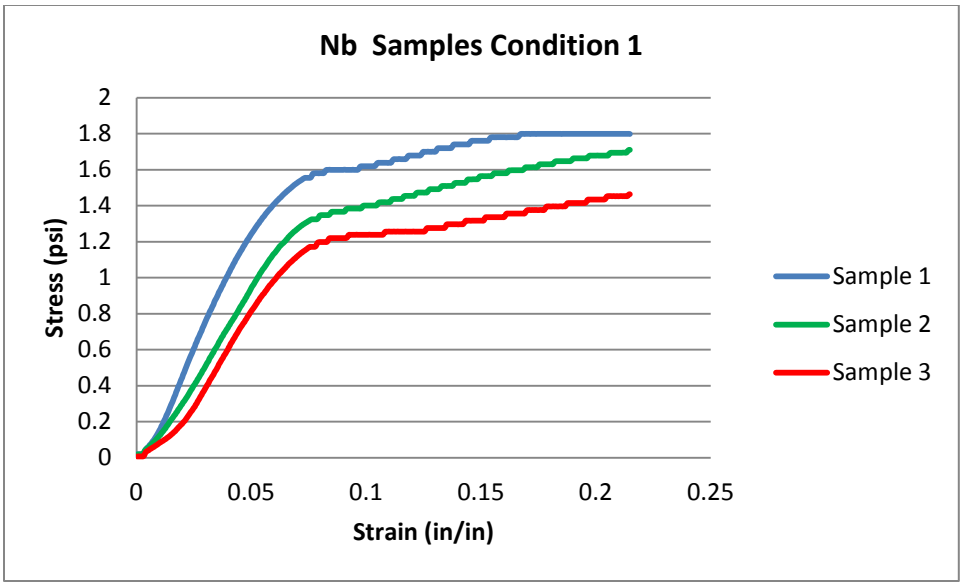

Fig 4.3: Stress and strain diagrams of Nb coated polyurethane samples in condition 1

Stress and strain curve of coated samples were described in above diagram (fig 4.3).

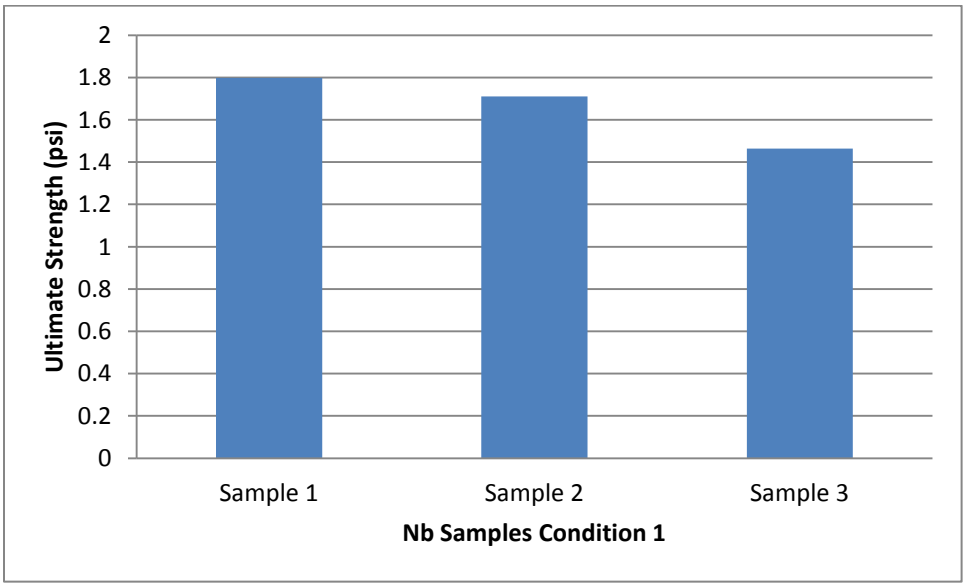

Fig 4.4: Ultimate strength of Nb samples condition 1

Fig 4.3 described strain and stress diagram of Nb sample condition 1 while fig 4.4 showed ultimate compressive strength of samples. The ultimate strength of Nb coated samples

was between 1.5 - 1.8 psi. The average value of compressive stress for Nb samples condition 1 was 1.66 psi ± 0.17.

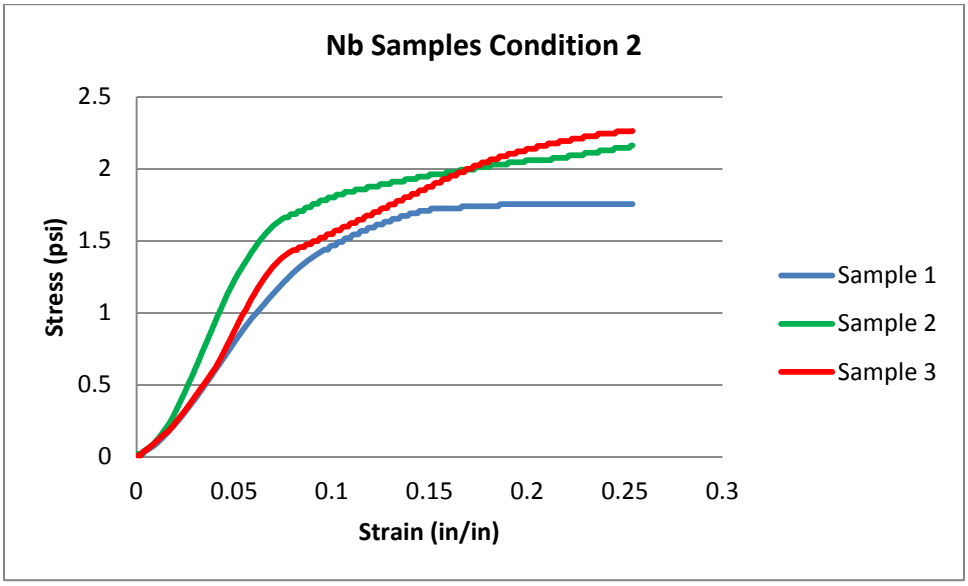

Fig 4.5: Stress and strain diagrams of Nb coated polyurethane samples in condition 2

The approximate value of ultimate strength was 1.7, 2.1 and 2.2 psi while corresponding strain was approximately 0.26 in/in for Nb coated sample condition 2.

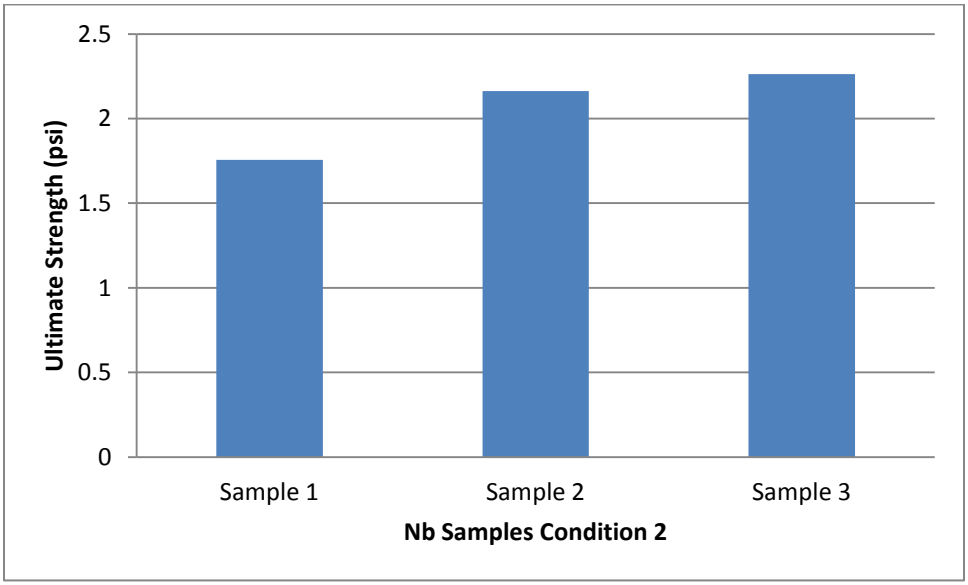

Fig 4.6: Ultimate strength of Nb samples condition 2

Strain and stress and ultimate compressive strength of Nb samples conditions were mentioned in fig 4.5 and fig 4.6 respectively. Ultimate compressive strength of samples was between 1.7- 2.2 psi. The average ultimate strength was calculated and the value was 2.061 psi ± 0.27.

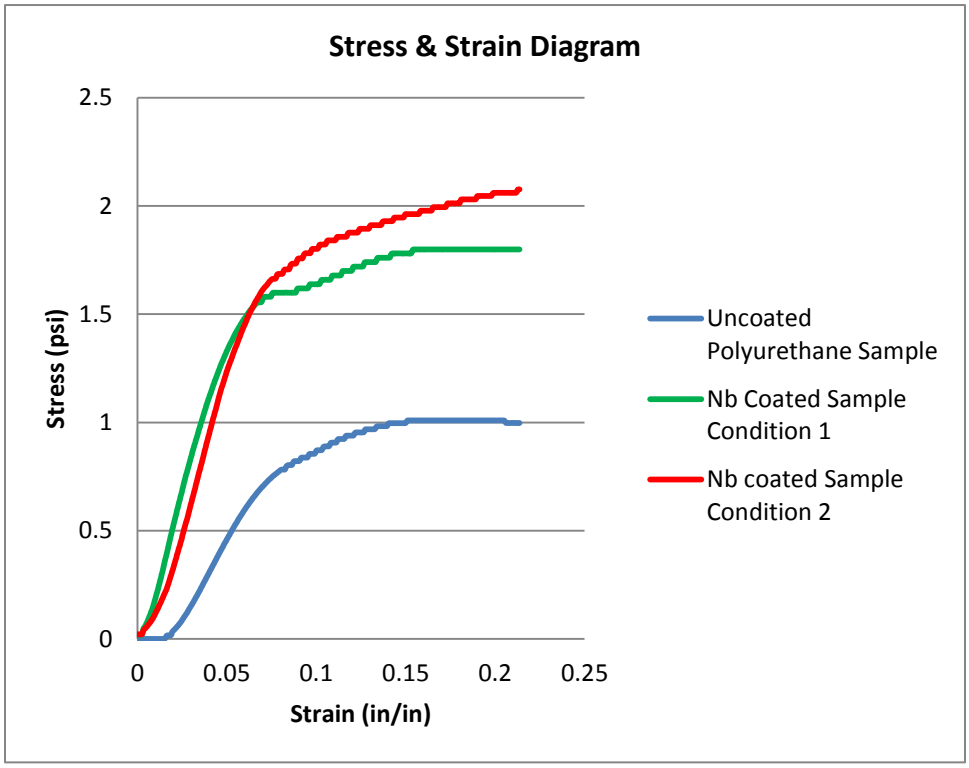

Fig 4.7: Stress and strain diagrams of uncoated polyurethane sample, Nb coated polyurethane samples in Condition 1, and Nb coated polyurethane samples in Condition 2.

Representative stress and strain result of uncoated polyurethane, Nb coated sample condition 1 and Nb coated sample condition were described in fig 4.7. Stress and strain of Nb coated sample for both conditions were higher than uncoated polyurethane and improved significantly compared to plain polyurethane samples. Approximate value of stress of uncoated polyurethane, Nb coated sample condition 1 and Nb coated sample condition 2 was 0.99, 1.8 and 2.1 psi.

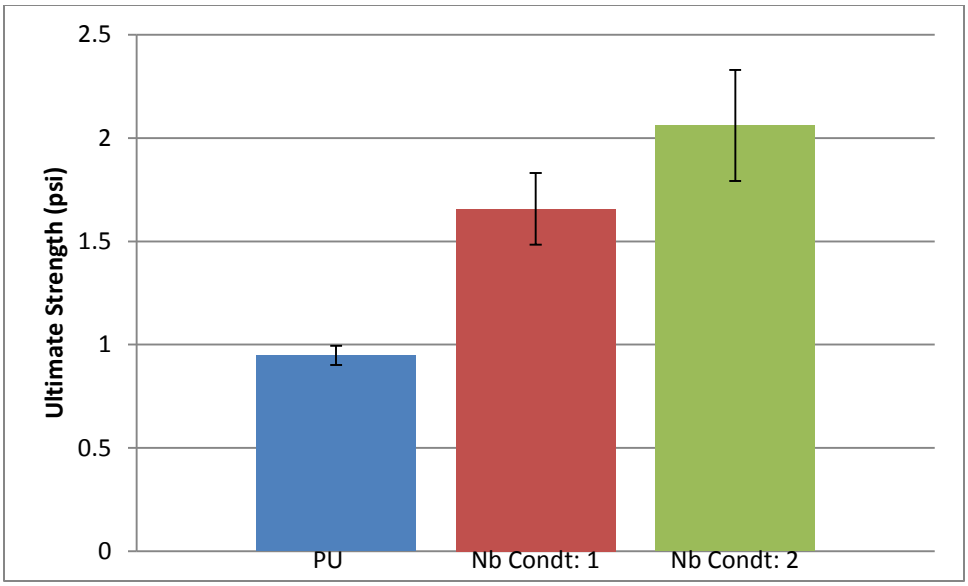

Fig 4.8: Average ultimate strength of polyurethane (SD ± 0.046), Nb condition 1(SD ± 0.17) and Nb condition 2 (SD ± 0.27)

Strain and stress results of the Nb coated polyurethane samples were illustrated in the diagrams (fig 4.6, fig 4.7 & fig 4.8). The results were different depending on the coating conditions. Nb coating condition 1 contained 4 g of salt and Nb coating condition 2 contained 6 g of salt in electrolyte solutions and 10 ml of ionic liquid was used for both conditions. Both conditions were electrodeposited for two hours. Average ultimate strengths were also calculated. Based on above diagram fig 4.8, average ultimate strength of polyurethane was 0.95 psi ± 0.046 while average ultimate strength of Nb condition 1 was 1.66 psi ± 0.17. The ultimate strength of Nb condition 1 was improved when compared to polyurethane. Moreover, average ultimate strength of Nb condition 2 also marginally improved and the value was 2.061 psi ± 0.27 when compared to Nb condition 1. Mechanical properties of both Nb coated polyurethane samples in both conditions improved significantly. In addition, average ultimate strength of Nb condition 2 samples was higher than Nb condition 1. Meantime, average weight of Nb coated samples was recorded before and after electrodeposition process. The average weight was 0.079 g and 0.11 g

before and after electrodeposition for condition 1 when the average weight was 0.08 g and 0.16 g before and after electrodeposition for condition 2. The average weight of coated samples could also effect on ultimate compressive strength for Nb coatings. Hence, mechanical properties of Nb coated polyurethane samples improved when they were compared to plain polyurethane samples.

4.1.2 Mg Metallic Coating

Strain and stress results of Mg coated samples are described in the diagrams below.

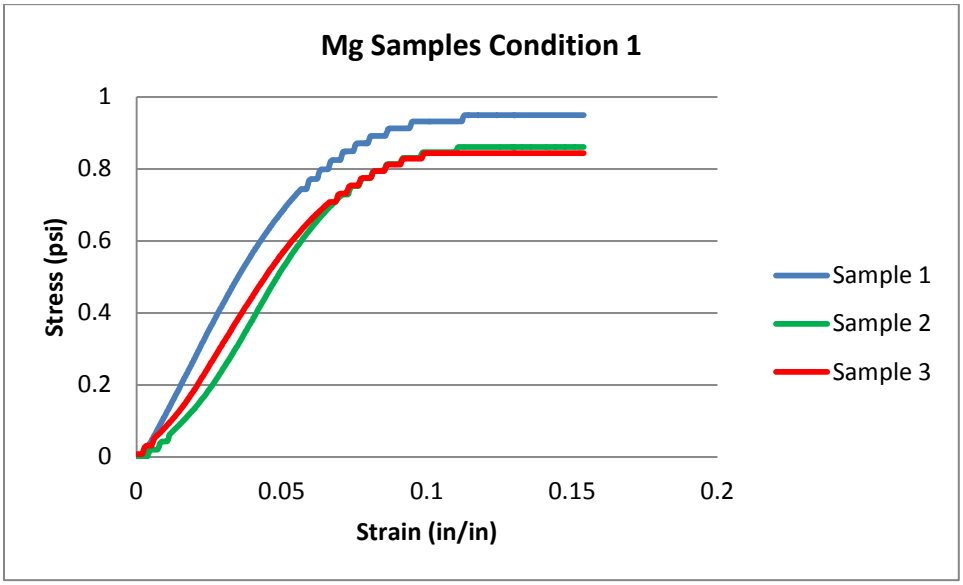

Fig 4.9: Stress and strain diagrams of Mg coated polyurethane samples in condition 1

Stress and strain curve of Mg coated samples condition 1 were linear and the approximate stress values of Mg coated condition 1 were 0.95, 0.86 and 0.84 psi when approximate strain value was 0.16 in/in for all the samples.

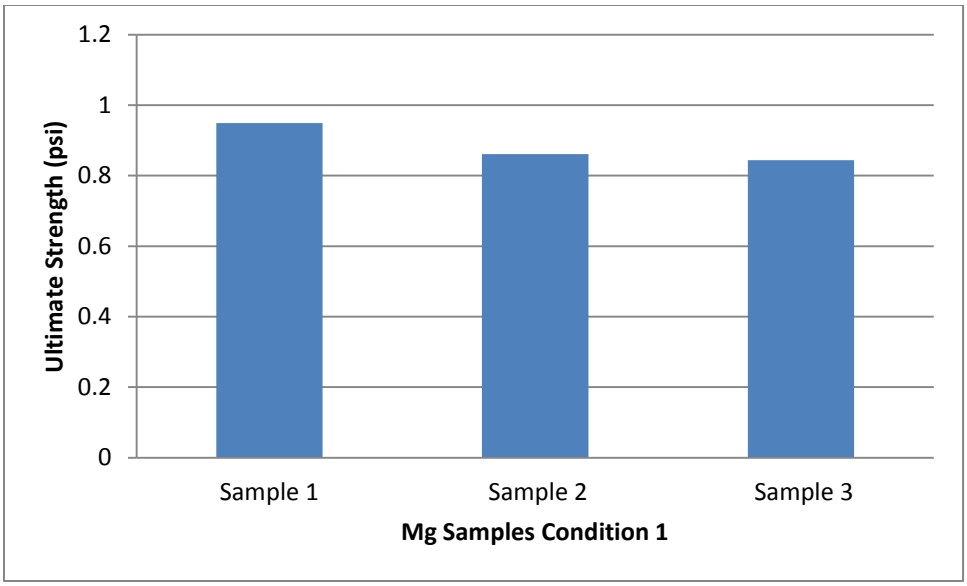

Fig 4.10: Ultimate strength of Mg samples condition 1

Stress and strain values of Mg coated samples condition 1 were also calculated based on the compression test results. Stress and strain values of Mg coated samples were mentioned in fig 4.9. In addition, ultimate strength of Mg coated samples were calculated from strain and stress diagram. The ultimate compressive strength of condition 1 samples was between 0.8 - 0.9 psi. The average ultimate strength was also calculated and the value was 0.88 psi ± 0.057.

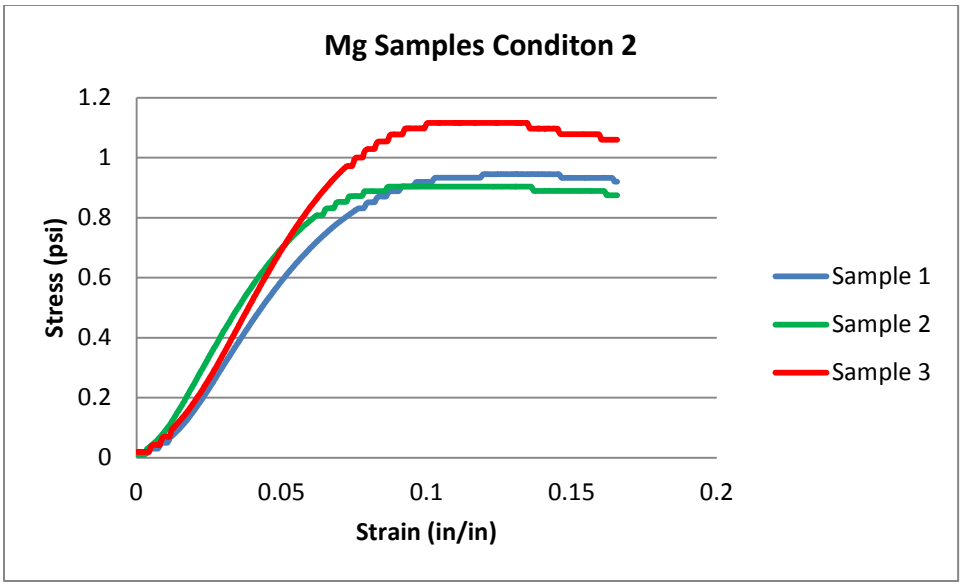

Fig 4.11: Stress and strain diagrams of Mg coated polyurethane samples in condition 2

Stress and strain curve of Mg coated condition2 were linear (fig 4.11) while the ultimate strength of samples were the maximum strength of the test samples.

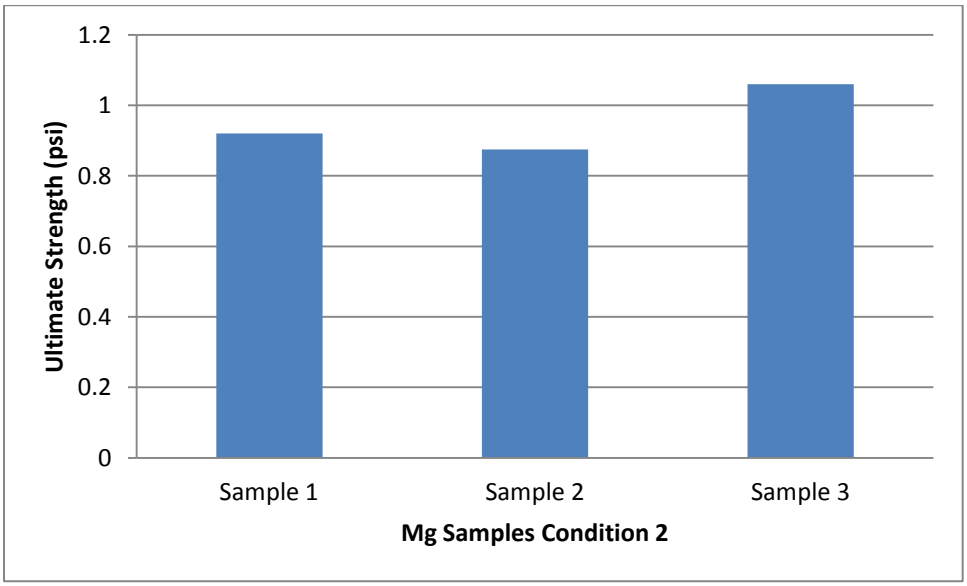

Fig 4.12: Ultimate strength of Mg samples condition 2

Stress and strain values of Mg sample condition 2 were calculated from data obtained from compression test. The stress and strain diagram is described in fig 4.11. Maximum

engineering stress which is also known as ultimate compressive strength of the samples were also calculated and described in fig 4.12 and values were between 0.8-1.1 psi. The average value of compressive stress was 0.95 psi ± 0.097.

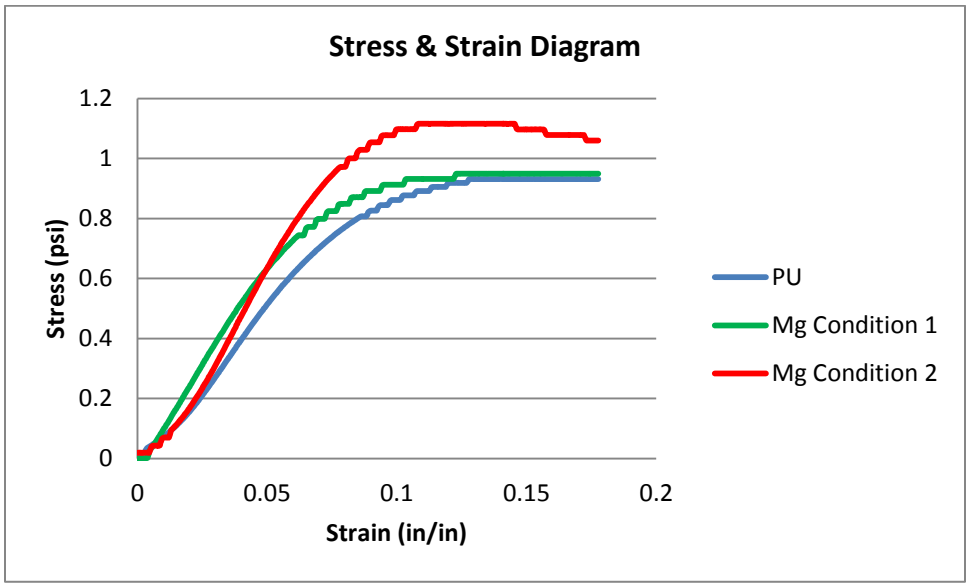

Fig 4.13: Stress and Strain Diagram of Uncoated Polyurethane, Mg coated sample condition 1 and Mg coated sample condition 2

Fig 4.13 shows the values of stress and strain diagram of uncoated polyurethane, Mg coated sample condition 1 and Mg coated sample condition 2.

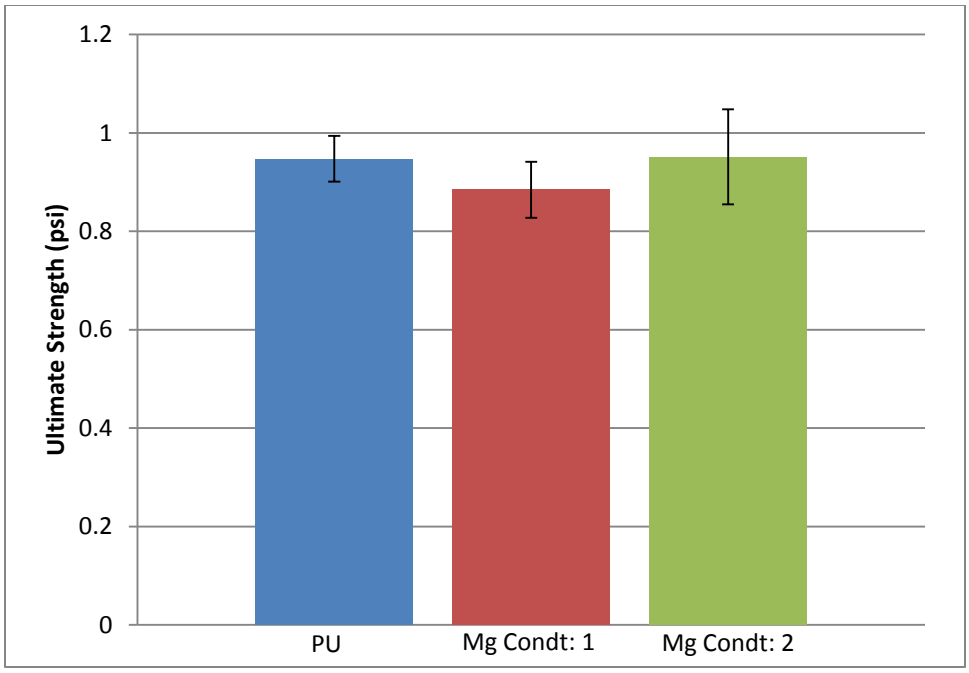

Fig 4.14: Average ultimate strength of polyurethane (SD ± 0.046), Mg condition 1 (SD ± 0.057) and Mg condition 2 (SD ± 0.097)

The results of the sample were different depending on the different coating conditions. Mg samples condition 1 were electrodeposited for two hours with 3:4 molar ratios of Magnesium (II) chloride ($MgCl_2$) and Tetrahydrofuran while electrodeposition time for Mg condition 2 was increased to three hours with 1.5:6 molar ratios. Based on the diagram (fig 4.14), average ultimate strength of polyurethane samples was 0.95 psi ± 0.046 while Mg samples condition 1 was 0.88 psi ± 0.057 and Mg sample condition 2 was 0.95 psi ± 0.097. There was no significant improvement of ultimate strength for Mg sample condition 1 when compared to plain polyurethane sample. The ultimate strength was improved for Mg sample condition 2 when compared Mg sample condition 1 which means there was a significant mechanical improvement on Mg coated polyurethane samples in condition 2. However, the average ultimate strength of Mg coated condition 2 was not mechanically different as compared to plain polyurethane sample. Results suggest that further optimization of electrodeposition parameter is necessary to obtain

scaffold with improved properties. However, Nb coating result indicate that enhancement of mechanical properties is feasible via this technique.

4.2 Biocompatibility Evaluation of Direct Test

4.2.1 Cell Proliferation and Cell Morphology

Once test samples were incubated with cells at 37˚C in a 5% CO_2 incubator, they were observed for cell proliferation after 24 hours. Cell proliferation and cell morphology of the test samples were observed under the microscope by checking the cells around the test samples, images of the test samples were taken to examine cell viability. PU samples were used as positive control and the results were compared with the PU sample's result. Healthy cells around PU, Mg coated, and Nb coated samples were spindle shaped and adhered to the surface of the test samples. There were a few cells lost which were floating around the sample area.

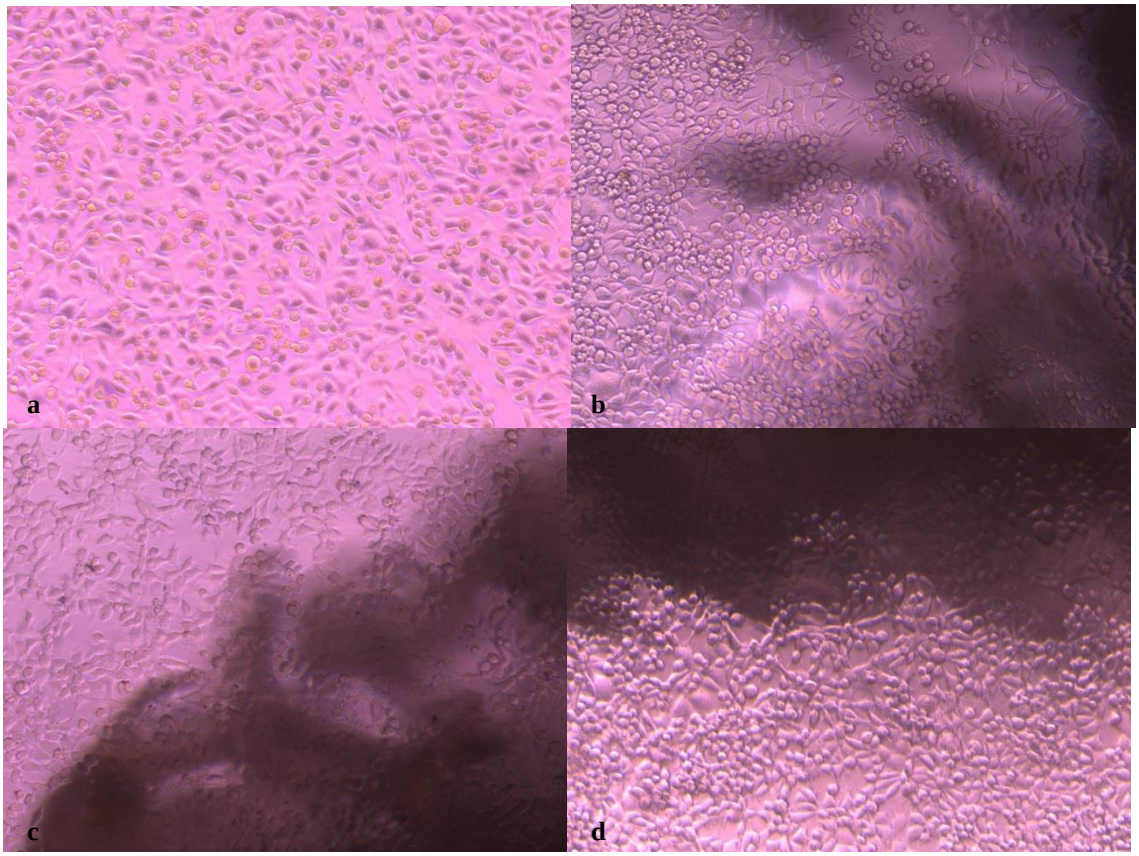

Fig 4.15: a) Image of fibroblast L929 cells b) Cell proliferation on PU sample
c) Cell proliferation on Mg coated sample d) Cell proliferation on Nb sample

Fig 4.15 showed the image of L929 fibroblast cells and cell proliferation on plain polyurethane sample, Mg coated sample and Nb coated sample. Cells were adhered to the edge of the surface and the shapes of the cells were spindle shaped. Based on the result the healthy cells were adhered to the surface of the test samples; therefore, samples are not toxic and they are biocompatible.

4.3 Biocompatibility Evaluation of Liquid Extract

4.4.1 Cell Proliferation and Cell Morphology

Cytotoxicity evaluation of liquid extract of test samples was observed after the cells were incubated for one day. Cell morphology and proliferation of test samples was evaluated under the microscope by observing shape changes and observing, viable, and nonviable cells before the cell count procedure was carried out. Experiment was carried out for 1 day, 4 days and 7 days period. Before the cell count was performed, images of test samples were taken using optical microscope. Cell count was performed for PU, Mg coated samples, and Nb coated samples after 1 day, 4 days and 7 days. The images and the results of samples are described in the diagrams below.

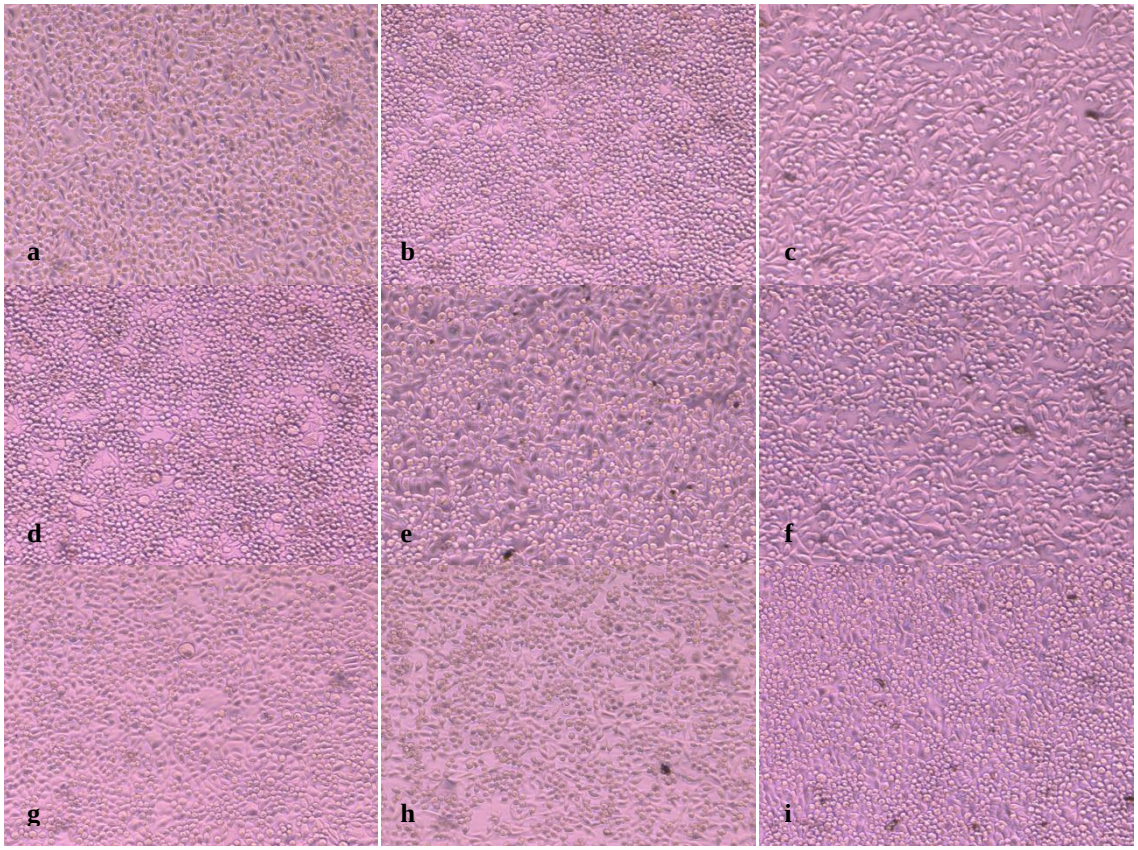

Fig 4.16: L929 fibroblast cells images of

a) PU sample on 1 day, b) PU sample on 4 days, c) PU sample on 7 days

d) Mg sample on 1 day, e) Mg sample on 4 days, f) Mg sample on 7 days

g) Nb sample on 1 day, h) Nb sample on 4 days, i) Nb sample on 7 days

Images of cells were taken on 1 day, 4 days, and 7 days before cell count was performed (fig 4.16). The shapes of the cells were spindled and cell proliferated well with all the test samples.

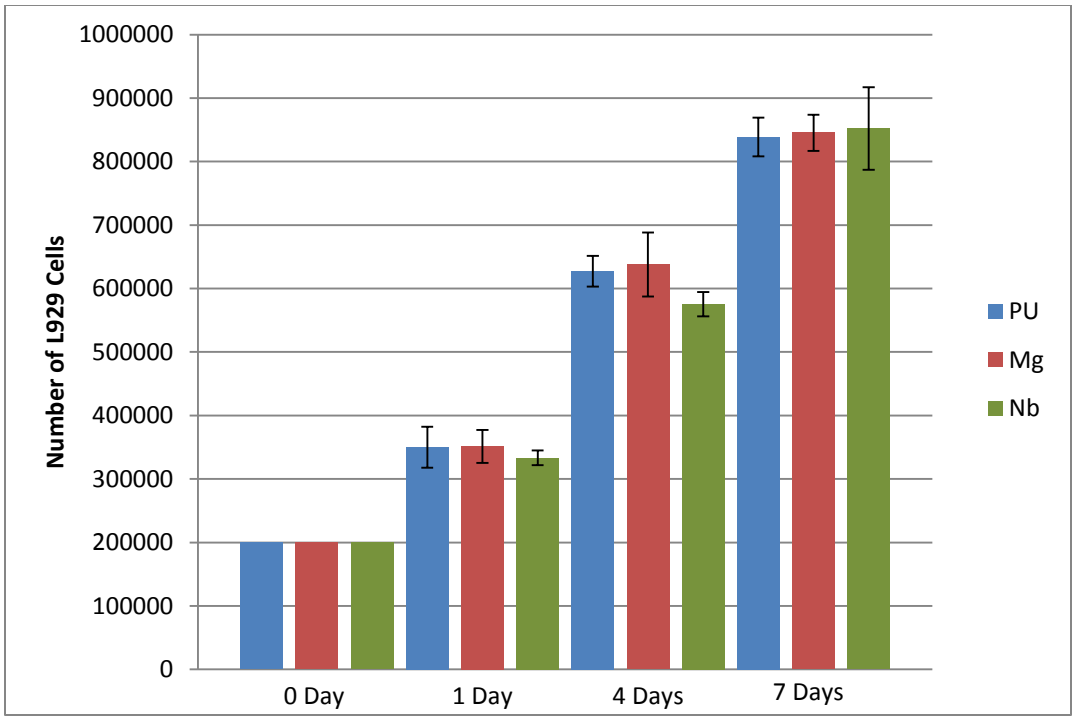

Fig 4.17: Cell growth on test samples 0 day, 1 day, 4 days and 7 days

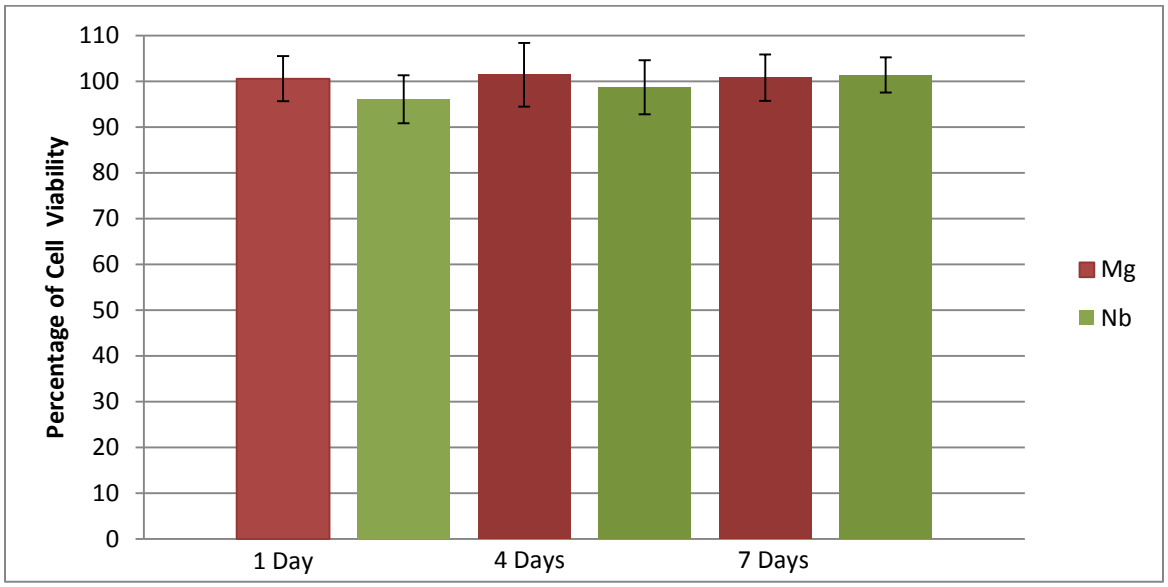

Fig 4.18: Cell viability of Mg coated and Nb coated samples on 1 day, 4 days and 7 days

Average numbers of cell were calculated and described in fig 4.16. 200,000 of cells were seeded for PU, Mg and Nb sample on day 0. Average numbers of cell growth on PU sample (350,000 ± 32,186.95), Mg sample (351,333.3 ± 25,794.06), and Nb sample (333,333.3 ± 11,718.93) were almost same in day 1. The amount of cells growth for the test samples was increased in 4 days on all the samples while cell growth on PU sample (627,333.3 ± 241110.86), Mg sample (638,000 ± 50,477.72) and Nb sample (575,333.3 ± 19008.77) were almost the same as Mg sample and the cell growth on Nb sample was the lowest when compared to other test samples. The average number of cells growth on PU sample (838,666.7 ± 30,615.9), Mg sample (845,333.3 ± 28,378.4) and Nb (852,000 ± 65023.07) sample were almost the same in 7 days. Percentage cell viability of Mg coated samples and Nb coated samples were described in fig 4.18. The average cell viability of coated samples was calculated based on cell growth as compared to cell growth on PU. Percentage cell viability of Mg coated samples on 1 day was 100.56 ± 4.91, 101.40 ± 6.93 in 4 days and 100.76 ± 5.07 in 7 days while percentage cell viability of Nb coated was 96.06 ± 5.26 in 1 day, 91.85 ± 5.91 in 4 days and 101.37 ± 3.87 in 7 days. The cells were grown well on 1 day, 4 days and 7 days cell culture experiment. Based on the results, test samples were not cytotoxic and cells proliferated successfully with all the samples.

4.4 Biofunctionality Evaluation

4.4.1 Cell Adhesion and Cell Proliferation

Test samples were incubated with osteoblast cells for a day in order to evaluate biofunctionality. MTT assays were added into well plates containing test samples and cells as well as added into the calibration plate with four different cell dilutions (1:2, 1:4, 1:8, and 1:16). All plates were incubated for more than 2 hours at 37˚C in a CO_2 incubator. MTT formazan

crystals of test samples were observed after incubation for more than two hours. The crystals are needle-shaped and are with dark purple in color.

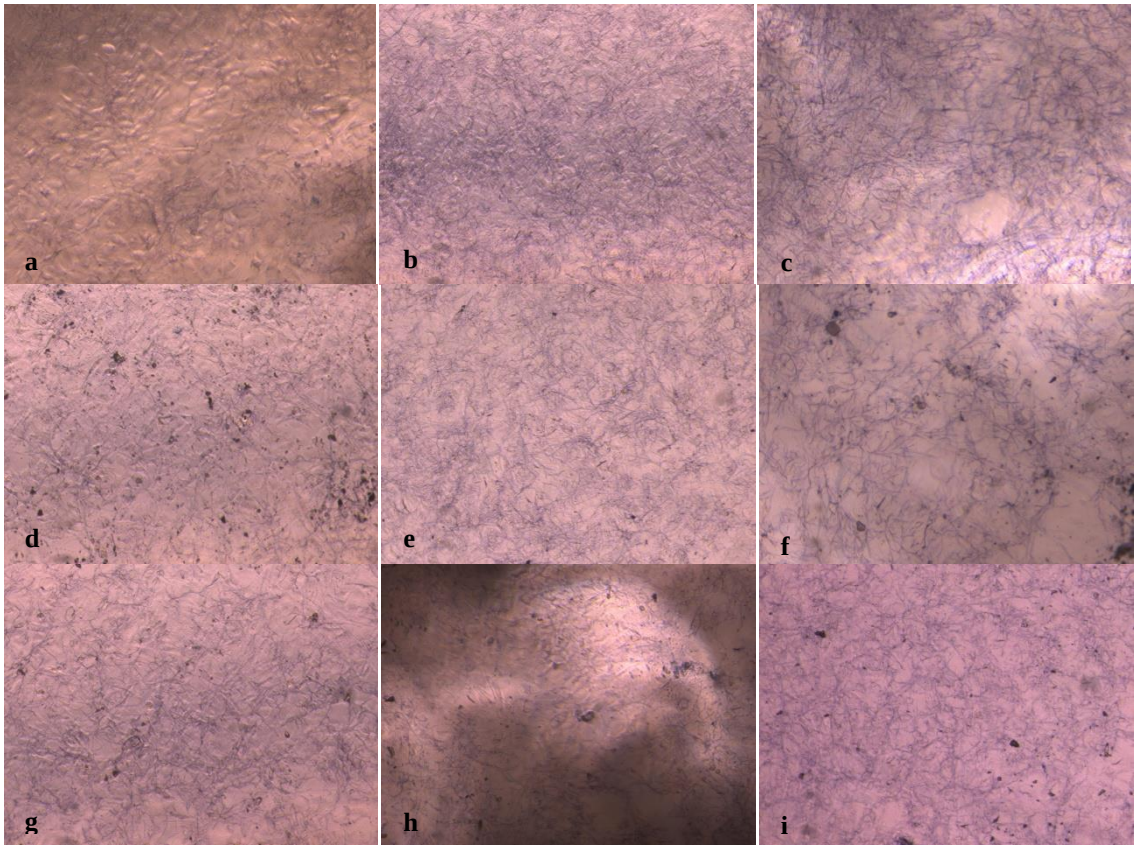

Fig 4.19:

a) MTT formazan crystal of PU sample 1 day, b) PU sample 4 days, c) PU sample 7 days

d) MTT formazan crystal of Mg sample 1 day, e) Mg sample 4 days, f) Mg sample 7 days

g) MTT formazan crystal of Nb sample 1 day, h) Nb sample 4 days, i) Nb sample 7 days

Osteoblast cells were changed into needle-shaped crystals when the cells were contact with MTT assay. MTT formazan crystal of PU, Mg , and Nb samples were shown in fig 4.19 for 1 day, 4 days and 7 days culture period.

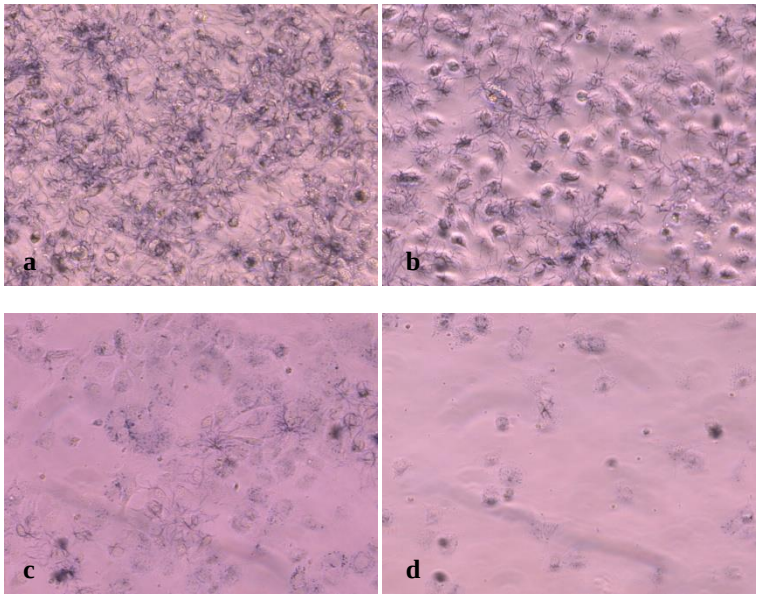

Fig 4.20: MTT formazan crystal of calibration plate

a) MTT formazan crystal on well #1 , b) MTT formazan crystal on well #2

c) MTT formazan crystal on well #3 , d) MTT formazan crystal on well #4

When observation of MTT formazan crystal samples was completed, the test samples were proceeded for absorbance measurements. Dimethyl sulfoxide (DMSO) was added into the well plate in order to dissolve MTT formazan crystals completely. DMSO solutions from the well plates were transferred into microcentrifuge tubes and centrifuged for 3 minutes, then solutions were transferred into cuvettes to measure their absorbance. Absorbance of test samples was measured using spectrophotometry with a wavelength of 570nm and the number of cells was calculated based on the absorbance value. The calibration curve of 3T3 cells is shown in the diagram below.

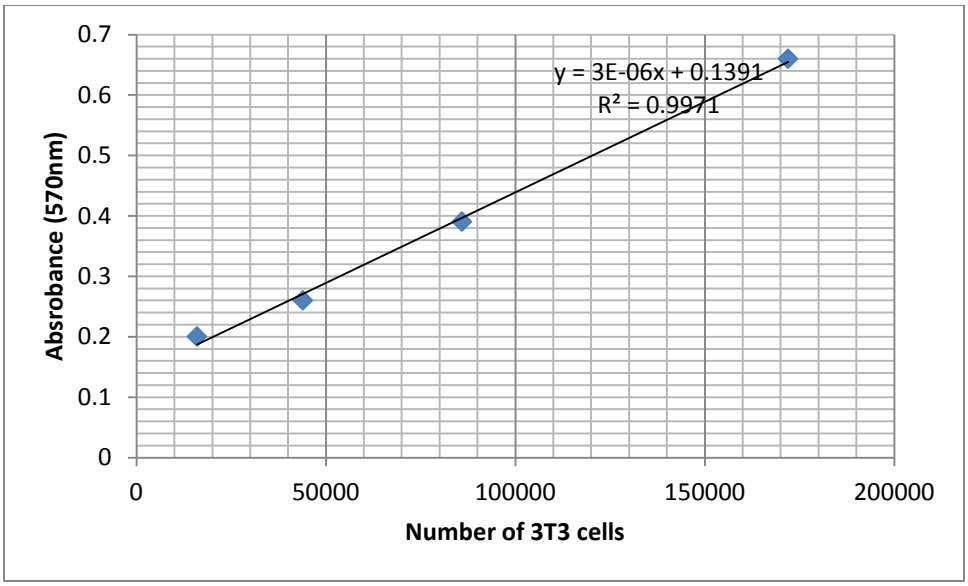

Fig 4.21: MTT assay calibration curve for MC3T3-E1 cells

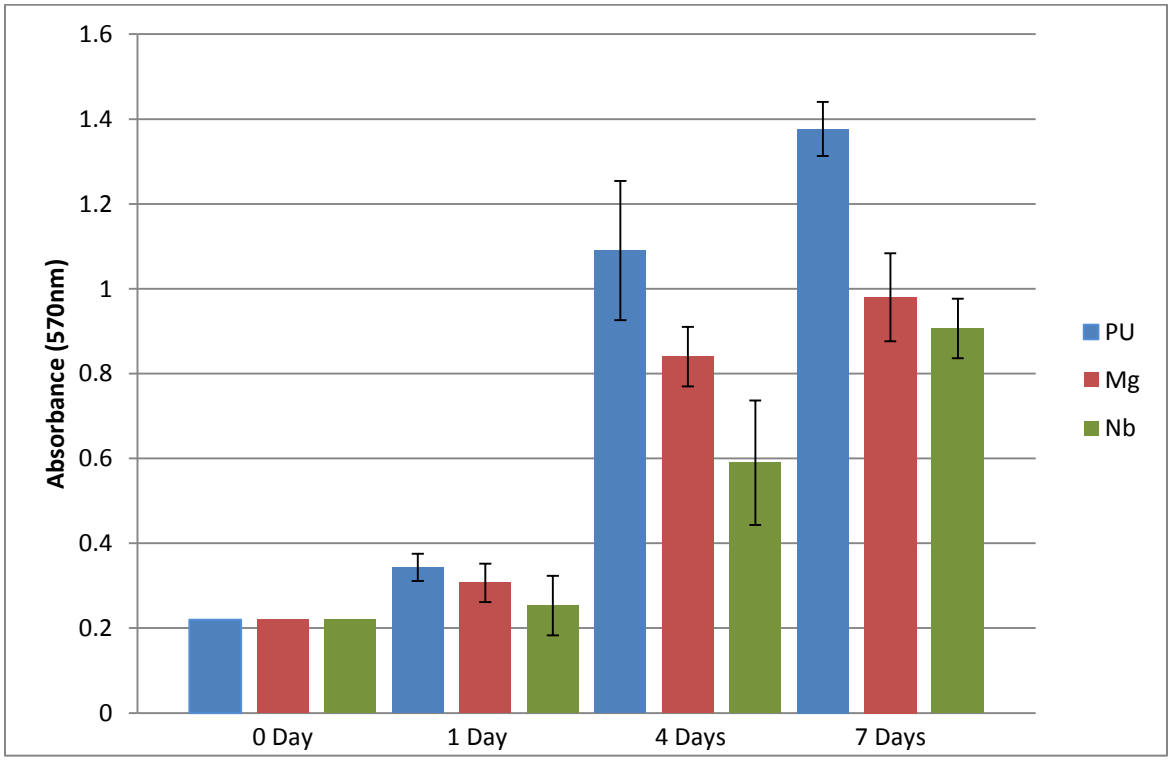

Fig 4.22: Absorbance level of PU, Mg coated samples and Nb coated samples were measured at 570nm and test samples were evaluated for 0 day, 1 day, 4 days and 7 days

Obsteoblast cells were cultured for 1 day, 4 days and 7 days to evaluate biofunctionality. Absorbances of the samples were measured after osteoblast cells were incubated with MTT assay. Absorbance level of test samples were measured on day 0 was 0.22. Average absorbance level of PU (0.34 ± 0.032) is in day when Mg sample (0.31 ± 0.045) is slightly lower than PU and average absorbance of Nb sample (0.25 ± 0.07) is lowest in day 1. Average absorbance level of PU (1.09 ± 0.16) in 4 days and (1.38 ± 0.064) 7 days evaluation and it was the highest, when Mg sample was the second highest (0.84 ± 0.07) in 4 days and (0.98 ± 0.104) 7 days culture period. The average absorbance of Nb sample was the lowest in (0.25 ± 0.07) in 1 day, (0.59 ± 0.15) 4 days and (0.90 ± 0.07) 7 days.

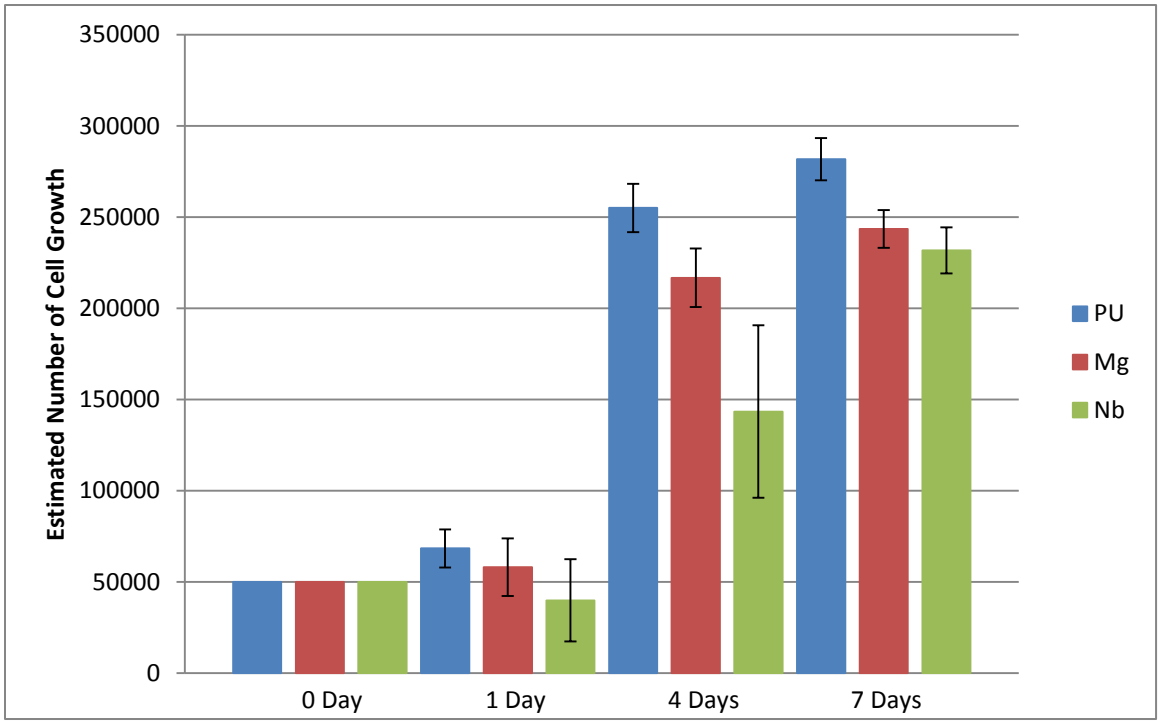

Fig 4.23: Estimated number of cell count on PU, Mg coated samples and Nb coated samples were measured at 1 day, 4 days and 7 days

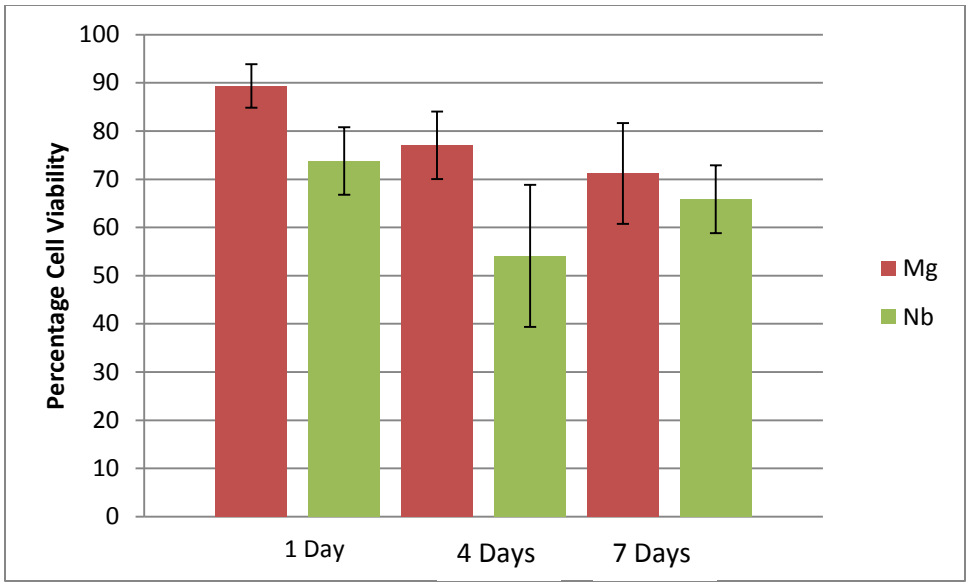

Fig 4.24: Cell Viability percentage of PU, Mg coated samples and Nb coated samples were measured at 1 day, 4 days and 7 days

Estimated number of cells was calculated based on absorbance values (fig. 4.22). Absorbance value of 50,000 number of 3T3 cells was 0.22 based on MTT calibration curve. The average number of estimated cell count for PU sample is 68,333.33 ± 10,408.33 in 1 day, 255,000 ± 13228.76 in 4 days, and 281,666.67 ± 11547 in 7 days. The average number of estimated cell count for Mg sample is 58,000 ± 15,715.23 in 1 day, 216,666.67 ± 16072.75 in 4 days, and 243,500 ± 10,331.99 in 7 days. The average number of estimated cell count for Nb sample is 39,833.33 ± 22,540.70 in 1 day, 143,333.33 ± 47,258.16 in 4 days, 231,666.67 ± 12,583.06 in 7 days. The cell growth on Mg and Nb coated samples were not as good as PU samples. As state earlier, bone growth factors could be introduced to minimize effect on coating to ensure optimal cell growth.

Cell viability percentage of PU, Mg coated sample and Nb coated samples were also calculated based on MTT calibration curve in fig 4.21. Although there is a cell proliferation on

the PU sample, there is a decrease in cell proliferation on both Mg coated and Nb coated samples. This suggest that optimization of cell culture medium may be needed to counteract that, for example bone growth factors could be introduced to ensure optimal cell growth.

Chapter 5

CONCLUSION AND FUTURE WORK

5.1 Conclusion

The main objective performed in this thesis was to improve the mechanical properties of polyurethane scaffolds and to evaluate the biofunctionality and biocompatibility of the coated polyurethane scaffolds. Electrodeposition of metallic coatings was conducted for polyurethane scaffolds to increase the mechanical properties of the scaffolds. Compressive strength of uncoated PU, Nb coated, and Mg coated samples were tested using the MTS test machine. The average ultimate strength of samples was calculated. The mechanical properties of Nb coated samples were improved significantly while Mg coated samples did not show much improvement when compared to plain PU samples. Further optimization of electrodeposition parameters is needed to obtain required enhancement of mechanical properties with Mg coated foam.

The coated samples were examined for biocompatibility and biofunctionality after mechanical property tests were done. All biology testing in this study was carried out in an *in vitro* environment. Test samples were evaluated through different biology testing methods including a direct contact test, liquid extract, and biofunctionality test to evaluate cell morphology, cell proliferation and cell adhesion. Based on the results obtained from the direct and liquid extract tests, it can be concluded that cells proliferate well with Mg and Nb coated PU material samples. But there was no significant improvement of cell growth for biofunctionality test. Optimization of cell culture system such as introduction to growth facts may be needed to obtain desired cell behavior.

5.2 Future Work

Experimental results proved that mechanical properties of Mg coated sample did not improve much compared to Nb coated samples; characterization and optimization of Mg metallic coating need to be performed in the future. Cell attachment and cell proliferation of test samples was examined in an *in vitro* environment. Moreover, biofunctionality tests need to be conducted again in the future in order to prove that the samples support cell attachment, and stimulate bone ingrowth. Since the experiment was conducted *in vitro* environment, studies *in vivo* environment must be performed in the future in order to prove that metallic scaffold coatings are suitable for implant devices.

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