INVITRO STUDIES ON ELECTROSPUN POLYURETHANE SCAFFOLDS

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ABSTRACT

Electrospun polyurethane scaffolds have been used in the past for neural cells and cartilage. They have been used effectively in growing tissues and mimicking the extracellular matrix. Polymer scaffolds are also more easily available when compared to natural materials such as collagen that need to be harvested from living organisms and this may raise ethical issues. For this reason, polyurethane can also be used with osteoblast cells instead of natural materials, due to their mechanical strength, durability, biodegradability and relative ease of availability. In this experiment, two types of polyurethane scaffolds were fabricated using electrospinning. One of the scaffolds has parallel fibers with 0-degree alignment while the other has non-parallel fibers with 45-degree alignment. The polyurethane scaffold with non-parallel fibers contains 93.3% Polyurethane and 6.7% Hydroxyapatite whereas the polyurethane scaffold with parallel fibers has 100% Polyurethane. The cells used were human fetal osteoblast cells (HFOB), they are capable of very quick cell divisions and differentiation under appropriate conditions. Throughout the study, the cell culture protocols were followed in the lab and the cell counting and cell viability assays were carried out frequently. The results of this experimental study will prove if the electrospun polyurethane scaffolds are biocompatible and allowed cell attachment. Furthermore, it can be concluded if the addition of 6.7% hydroxyapatite to the polyurethane scaffold will have any noticeable changes on cell growth and attachment when compared to the scaffold with 100% polyurethane. This is done to observe whether a 100% polymer scaffold or a polymer scaffold with some natural mineral content (hydroxyapatite) would be better for HFOB growth and proliferation.

ABSTRAK

Perancah polyurethane elektrospun telah digunakan pada masa lalu untuk sel saraf dan tulang rawan. Mereka telah digunakan dengan berkesan dalam tisu tumbuh dan meniru matriks ekstraselular. Perancah polymer juga lebih mudah didapati jika dibandingkan dengan bahan semulajadi seperti collagen yang perlu dituai daripada organisma hidup dan ini boleh menimbulkan isu etika. Atas sebab ini, poliuretana juga boleh digunakan dengan sel-sel osteoblast dan bukan bahan semulajadi, kerana kekuatan mekanikal mereka, ketahanan, kelembapan biodegradasi dan kemudahan relatif ketersediaan. Dalam eksperimen ini, dua jenis perancah poliuretana dihasilkan menggunakan elektrospinning. Salah satu perancah mempunyai serat selari dengan penjajaran 0 darjah manakala yang lain mempunyai gentian bukan selari dengan penjajaran 45 darjah. Perancah polyurethane dengan serat bukan selari mengandungi 93.3% Polyurethane dan 6.7% Hydroxyapatite sedangkan perancah polyurethane dengan gentian selari mempunyai 100% Polyurethane. Sel-sel yang digunakan adalah sel osteoblast janin manusia (HFOB), mereka mampu divisi sel yang sangat cepat dan pembezaan di bawah keadaan yang sesuai. Sepanjang kajian, protokol kultur sel telah diikuti di makmal dan pengiraan sel dan ujian daya tahan sel telah dijalankan dengan kerap. Hasil kajian eksperimen ini akan membuktikan jika perancah poliuretana elektrospun adalah biokompatibel dan lampiran sel yang dibenarkan. Selanjutnya, dapat disimpulkan jika penambahan 6.7% hydroxyapatite pada perancah polyurethane akan mempunyai sebarang perubahan ketara pada pertumbuhan sel dan lampiran jika dibandingkan dengan perancah dengan polyurethane 100%. Ini dilakukan untuk mengetahui sama ada perancah polymer 100% atau perancah polymer dengan kandungan mineral semulajadi (hydroxyapatite) akan lebih baik untuk pertumbuhan dan percambahan HFOB.

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1.0 CHAPTER 1: INTRODUCTION

The aim of tissue engineering is to fabricate three-dimensional scaffolds that can be used for the reconstruction and regeneration of damaged or deformed tissues and organs. The most interesting materials today are biodegradable polymers due to their mechanical properties, ability to control the rate of degradation and similarity to natural tissues. Polyurethanes (PUs) are attractive candidates for fabricating scaffolds as they are known to be biocompatible, have excellent mechanical properties and mechanical flexibility.

Polyurethane scaffolds can be fabricated using electrospinning. The process of electrospinning has been done in the past and has resulted in the production of fibers of different alignment. However, it is difficult to interpret which arrangement of fibers would be better suited and compatible with tissue growth. Different electrospinning parameters such as applied voltage, distance between syringe and collector affect the scaffold that is fabricated using this technique. In this research, the electrospun composite polyurethane scaffolds will be integrated into cell cultures containing human fetal osteoblast cells (HFOB) to carry out in vitro studies. The growth and compatibility of cells and tissues under controlled conditions were closely monitored in the cell cultures (Grada, Kupcsika et al. 2003).

In the past, many tissues have grown successfully in the presence of polyurethane scaffolds including bone tissues. Polymers are known to have strong mechanical properties, can be easily combined with other materials and are usually biodegradable. The process of electrospinning was chosen as it is easier to set up and it produces fibrous scaffolds using different materials with a large surface area and many effective scaffolds were fabricated in previous studies (Grada, Kupcsika et al. 2003).

In this experiment, scaffolds containing varying amounts of polyurethane and hydroxyapatite were electrospun to be used in cell cultures with HFOB cells. The cell growth profile was carried out before seeding the cells into scaffolds. The results were recorded in graphs after cell counting and cell viability studies.

1.1 Objectives of the study

- To integrate the electrospun polyurethane scaffolds in cell culture in vitro containing Human fetal osteoblast cells and observe if cell growth and cell attachment occurs.
- To observe if the electrospun polyurethane scaffolds are biocompatible with the HFOB cells.
- To conclude, if the addition of hydroxyapatite to polyurethane scaffolds affects HFOB cell growth and attachment.

2.0 CHAPTER 2: LITERATURE REVIEW

The development in the field of tissue engineering have resulted in the fabrication of scaffold or matrix-based culture systems that are meant to replicate the biological, physical and biochemical atmosphere of a natural ECM. Even though their medical applications in regenerative medicine seem to acquire the most attention, other areas of biomedical research could also prosper by the use of influential technology that has already been worked on, in tissue engineering (Hutmacher, Loessner et al. 2010).

Cells in a living organism are present in a 3D environment called the extracellular matrix (ECM). This structure supports connection between cells, the basement membrane as well as the neighboring matrix; it affects the carrying of oxygen, hormones and nutrients, the elimination of wastes, and migration of different types of cells (Hutmacher, Loessner et al. 2010).

The endeavors in the field of tissue engineering have been highlighted by the fabrication and integration of hydrogels or scaffold-model tissue engineered constructs (TECs) that produce controlled and supportive microenvironments similar to the in vivo surroundings (Hutmacher, Loessner et al. 2010).

Latest developments are geared towards the utilization of scaffolds with new and complex techniques that would lead to the production of guidance channels, which accurately imitate the natural process of repair inside the human body (Yang, Murugan et al. 2005).

2.1 Materials in Tissue Engineering

Natural ECMs are acquired the from dermis, small intestine submucosa (SIS), fascia lata, and pericardium, and these are altered then marketed as patches. ECM devices available in the market, are obtained from a number of organisms such as human, porcine, bovine, equine and tissues such as dermis, SIS, fascia, pericardium and they must undergo many preparation and sterilization methods. It must be considered that extracellular matrix grafts have received FDA approval for use in medicine as augmentation devices and not as replacement structures to replicate human body systems or give complete mechanical support in tendon repairs. Table 2.1 shows many commercially available ECM grafts available along with their origin and applications (Aurora, McCarron et al. 2007).

Ceramics like hydroxyapatite (HA) and β-Tricalcium phosphate (β-TCP) have been used frequently in bone tissue engineering as they are osteoconductive. However, these materials on their own are brittle and possess a low mechanical stability, which limits their effectiveness in the regeneration of large bone defects (Veiga and Branco 2015).

Table 2.1 A list of some commercially available ECM devices and scaffolds in regenerative medicine (Aurora, McCarron et al. 2007)

Material	**Origin**	**Sterilization**	**Applications**
Restore™	Un-cross linked Porcine Small intestinal Sub mucosa	Electron Beam irradiation	Abdominal wall Gastrointestinal issues
Cuff patch™	Cross-linked Porcine small intestinal Sub mucosa	Electron Beam irradiation	Rotator cuff repair Musculotendinous issues
GraftJacket ™	Human Dermis	None. It is regarded as tissue transplant	Gastroinstestinal issues

		by the FDA (Not an ECM device)	Musculotendinous issues
TissueMend™	Bovine Dermis	Ethylene Oxide	Healing Tendon injuries Rotator cuff, Achilles tendons and patella
Permacol™	Porcine dermal collagen	Gamma Radiation	Abdominal wall repair
Poly L lactic acid scaffold (PLLA)	Thermoplastic polyester from renewable resources	Gamma Radiation	Neural tissue engineering
non-woven Polyglycolic acid (PGA)	Thermoplastic polymer. From glycolic acid	Ethylene oxide	Adipose tissue engineering Absorbable sutures
Type I collagen sponge	Porcine tendon	Ethylene oxide	Adipose and skin tissue and Bone regeneration
Calcium Phosphate ceramics	Minerals containing Calcium ions and phosphate ions	Gamma radiation	Bone tissue repair and regeneration

Natural ECM devices are largely available for treating tendon and muscular injuries as mentioned in table 2.1. Restore™, Cuff patch™, Graftjacket™, TissueMend™ and Permacol™ are excellent for gastrointestinal problems, musculotendinous issues cardiovascular and soft tissue healing. They are harvested from a variety of tissues as listed above in table 2.1. These materials must undergo decellularization, removal of DNA content as it is known to cause inflammatory response and sterilization before use (Gilbert, Freund et al. 2009).

Type I collagen, the major constituent of the ECM in the human body, has shown an impressive biocompatibility in the body and has been medically used as a dermal replacement to effectively treat skin ulcers, adipose tissue engineering as well as in bone regenerative medicine (Itoi, Takatori et al. 2010).

Hydrogel-based scaffolds are a very important class of tissue engineering constructs as well. They are often able to mimic the natural ECM effectively. Hydrogels are hydrophilic and cross-linked in nature, and hence they can soak up large amounts of water without dissolving or destroying the polymer. This gives them physical properties that resemble internal tissues. Hydrogels have a high affinity for oxygen, nutrients and water-soluble substances. Hydrogels can be of natural or synthetic origin (Zhu 2010).

Natural hydrogels are primarily made up of natural components, like the proteins collagen, gelatin and fibrin as well as polysaccharides like alginate chitosan, hyaluronic acid and dextran. Synthetic hydrogels have synthetic polymer origins like polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyacrylamide (PAAm), polyethylene glycol (PEG), and polypeptides (Laschke, Strohe et al. 2010).

2.2 Polymers and tissue engineering

Poly L Lactic acid (PLLA) is a polymeric material that can be used in neural tissue engineering as a scaffold. The nano fibrous and micro fibrous scaffolds can be fabricated using electrospinning in different diameters and studied in a neural stem cell culture. It was observed that the neural stem cells had grown and the neurite had grown parallel to the fiber direction of the aligned scaffolds. The study also showed that the Neural stem cells (NSC) differentiation rate was faster

for nanofibers as compared to micro fibers, but it was not dependent on the fiber alignment (Yang, Murugan et al. 2005).

Polyglycolic acid (PGA) is a synthetic biodegradable material that has been largely used as a substance for the production of absorbable sutures. PGA has been used in studies relating to adipose tissue engineering. Non-woven PGA scaffold was used in an adipose stem cell culture (ASC). Although, there was successful cell infiltration and proliferation, type I collagen had better results and a higher rate of cell differentiation in the ASC culture. PGA can still prove to be a suitable scaffold due to it biocompatibility and biodegradability in other applications. Polymers possess excellent mechanical strength and high toughness (Itoi, Takatori et al. 2010).

2.3 Polyurethanes in different applications

Mechanically, polymers are known for properties such as extensive deformation and high toughness whereas ceramics like hydroxyapatites (HA), are known for their increased compressive strength but brittle nature (Chen, Liang et al. 2013).

Polymers commonly used in the fabrication of porous scaffolds in different geometric structures are polyhydroxy acids like polyglycolides, polylactides, and their copolymers as discussed earlier. Scaffolds derived from such polymers were noted to maintain the adherence, expansion and proliferation of chondrocyte cells as well as other types of cells such as osteoblasts (Grada, Kupcsika et al. 2003).

Other alternative materials for scaffolds are the polyurethanes. For a long time, polyurethanes are being utilized frequently in different implantable units such as pacing leads insulation, intra-aortic balloons, catheters as well as breast implants. One of the required properties for polyurethanes is their molecular stability in vivo and nowadays there has been some interest in fabricated

biodegradable polyurethanes which degrade to un-poisonous by-products in vivo. As of late, biodegradable polyurethanes were used successfully as bone graft replacements in animals (Grada, Kupcsika et al. 2003).

In a study, where chondrocyte cells were seeded into the polyurethane scaffolds, the results conclude that this scaffold proved excellent for tissue growth when compared with other polyhydroxacids. A dynamic sell culture ended in the adherence of 60% to 70% of cells into the scaffold only a couple of days after integration. The controlled hydrophobicity of the scaffold surface also had a role in good cell infiltration. However the polymer shows a low degradation rate in vitro, which is alright because it coincides with the low turnover rate of cartilaginous tissue. However, a big problem in utilizing porous scaffolds is that they are unable to retain most of the extracellular matrix proteins synthesized by cells and this is the reason that the chondrocytes don't have a proper surrounding or stimuli to produce an organized ECM. A good idea to overcome this issue might be the reduction of pore size to improve nutrient supply to cells, but it should be noted that if pore size is too small it may impair cell distribution in the scaffold and limit nutrient supply (Grada, Kupcsika et al. 2003).

In another experiment, three polyurethane (PU) foams (PU, PUL 30%, PUL 40%) with varying calcium phosphate levels in the matrix were gained by a single step bulk polymerization and were to be used in vitro with Human osteosarcoma (Saos-2) cell culture. Alamar blue solution was utilized in order to observe the metabolic activity of the cells, when doing the cytotoxicity and cytocompatibility test. Comparing the different analyzed scaffolds, PUL 40% is unique for its properties. It has pore size 200–400 μm and porosity (>75%) and can be applied in bone tissue regeneration and showed higher stiffness. PU and PUL 40% scaffolds appear to be reliable materials to be used for bone tissue regeneration showing good results pertaining to cell adhesion,

proliferation and differentiation. Since this experiment was performed in a static culture condition, a dynamic culture condition can also provide improved cell proliferation and differentiation levels, since osteoblast metabolism is noted to rely on external stimulus, such as shear stress caused by fluid flow (Veiga and Branco 2015).

2.4 Fused deposition modeling

A controlled porosity polymer-ceramic scaffold was developed using the fused deposition technique. The polymer, polypropylene (PP) was chosen for its high mechanical strength and previous success in the healthcare as grafts. The ceramic to be used is tri-calcium phosphate (TCP) as it is very similar to the bone in mineral composition but is too brittle to be used as a scaffold on its own. Development by FDM requires three important steps:

- To select a non-toxic polymer which can be easily extruded with ceramic powders. The polymer chosen was Polypropylene HGV-090 as the backbone. It has a low density of 0.90 g/cm3 with a heat deflection of 107^{o}C at 0.455 MPa. These conditions make it easy to extrude PP.
- Development of feedstock for the Fuel Deposition Modelling process.
- Fused deposition modeling of extruded filaments

TCP, a bio-ceramic, was chosen to be the ceramic phase. Bio-ceramics disintegrate when implanted into the host as new tissues begin to grow around the biomaterial.

During this procedure, PP-TCP composite filaments were altered by using a single screw extruder and after that, fused deposition of the PP-TCP filaments lead to the fabrication of porous structures. These porous scaffolds with various textures were composed and manufactured using FDM machine. These scaffolds were characterized for their physical, mechanical, and biological

properties for their application as bone grafts. Samples that underwent physical and mechanical characterization had a pore size of 160 am while the pore volume was about 36%, 40%, and 52%. Compression testing demonstrated that scaffold strength decreased as the pore volume increased (Kalita, Bose et al. 2003).

2.5 3D printing scaffolds

3D printing creates 3D structures by inkjet printing a liquid binder to combine material powders. Since biomaterials are often found in either solid or liquid phase, a large variety of biomaterials have been used right away in 3D printing. With 3D printing it is possible to have computerized management over spatial distribution of composition and microstructure by printing diverse matter using various printing parameters. In order to print whole scaffolds, a solvent binder in liquid state could be printed over polymer pieces and a powder bed of porogens (porogens are particles or substances that form pores in scaffolds). The solvent binder will lead to the dissolution of the polymer and then evaporation. The polymer will re-precipitate to form a solid model. Final porosity is acquired after particulate leaching and removal of the solvent (Lee, Dunn et al. 2005).

It should be noted that this method has its limitations. Firstly, the pore size is a critical parameter in making scaffolds. The desirable pore size for bone tissue is about 200–400 mm however a typical 3D printed incremental layer thickness of 150 mm is required to increase inter-layer connectivity. To distribute the porogen particles in correct sizes (200 to 400 mm) an incremental layer thickness of every layer must be expanded to the size range of the porogens (Lee, Dunn et al. 2005).

2.6 Electrospinning

Polymer fibers that have a range of diameters from a few nanometers to microns can be easily utilized in the tissue engineering process. During the electrospinning process, a polymer solution is added to a syringe with a thin needle facing a collection plate. A high voltage is then applied to this solution resulting in the formation of a jet as the applied electric field overcomes the surface tension of the solution. As the jet travels towards the collecting plate it becomes thinner due to solvent evaporation and fibers begin to form. Fibers may also be produced due to the deposition of fibers on the grounded target and these are the non-woven, highly porous nets. If a rotating collector (electrically charged disc of metal) is placed at the grounded target, this allows the deposition of fibers with some alignment. Figure 2.1 shows the process of electrospinning in a flowchart and figure 2.2 shows the components of the electrospinning apparatus that is used in the lab (Buttafoco, Kolkman et al. 2006).

Electrospinning is an ideal technique for the fabrication of small diameter fibers. It was found that the concentration of solutions affected the alignment and diameters of fibers. Solvents such as Glutaraldehyde act as the cross-linkers of the fibers and are added to the polymer solution prior to electrospinning (Buttafoco, Kolkman et al. 2006).

The process of electrospinning is based on the extension of a solution that is viscoelastic into nanofibers or microfibers by utilizing a large electrostatic force. A large DC voltage is applied across the solution, resulting in repulsive forces between similar charges within the liquid. Under the surrounding electric field, a cone shape is formed at the tip of the liquid this is called the Taylor cone. When the applied voltage is strong enough to exceed the surface tension on the Taylor cone, a thin jet of liquid is produced from the Taylor cone and it proceeds towards the collector. An

electrode of either the opposite charge or neutral charge is placed close by to pull and collect the fibers. As the liquid jet proceeds to move through the ambient and towards the collector, the solvent from this fiber jet evaporates and is removed leaving a solid fiber behind on the collector (Hasan, Memic et al. 2014).

Generally, in biomedical applications electrospun polymer fibers, both biocompatible or biodegradable are required to be three-dimensional scaffold models that can effectively mimic the natural in vivo ECM and the proliferation of supporting cells. Electrospinning produces a loose network of 3D porous mats that have high porosity and surface area that appears identical to the cell ECM structure and therefore it makes an ideal component for use in tissue engineering. These porous scaffolds with nanoscale architectures having a large surface area can effectively absorb proteins and manifest many binding sites for receptors on cell membranes. The non-woven fibrous sheets generated by electrospinning imitate the ECM components much far more than those generated by other conventional techniques. Table 2.2 shows the effects of changing electrospinning parameters on the morphological structure of electrospun fibers. (Carlberg, Axell et al. 2009).

2.7 Advantages of Electrospinning

2.7.1 The fabricated material has a high surface area to volume ratio

The nano-fibers generated from the electrospinning technique have a high surface area to volume ratio; this makes the material very attractive for applications that require a high surface area (Teo, W. E. 2013).

2.7.2 Many different types of polymers and materials can be electrospun

The few requirements for electrospinning make it easier to mix polymers or materials to be produced as fibers. Although the process is often used to fabricate polymeric nano-fibers, ceramic and metal nano-fibers have also been constructed indirectly through electrospinning of their precursor material (Teo, W. E. 2013).

2.7.3 Electrospinning equipment is easy to set-up and effective training can be provided

The set-up cost for electrospinning in a laboratory setting is relatively cheap as compared to many other systems. The technique is also quite easy to learn and effective training can be provided (Teo, W. E. 2013).

Table 2.2 Parameters that affect the morphology of electrospun nano-fibers

Parameters	**Effect on the morphological structure of electrospun nano-fiber mats**
Viscosity of solution	A threshold viscosity value should be reached to produce An effective polymeric jet during electrospinning. As the viscosity increases, the diameter of fibers increases. Additionally, the fibers formed are more uniform (Nasefa, Abbasia et al. 2012)
Molecular weight	As the molecular weight and the solution concentration rises, the fiber diameter increases as well. The average nano-fiber diameter range was observed to be between 175.83 and 735.69 nm (Akduman, Kumabasar et al. 2014)

Surface Tension	Various solvents can produce specific surface tensions. When the concentration is fixed, decreasing the solution's surface tension can cause the fabrication of smooth fibers (Hasan, Memic et al. 2014)
Electric field or applied voltage	If the applied voltage is higher than the threshold voltage, charged jets may be released from Taylor Cone. However, there is no significant relationship between the voltage and the nano-fiber diameter (Li and Wang 2013)
Flow rate	The polymer solution flow rate within the syringe should be less in order to obtain smooth fibers of smaller diameters. A lower flow rate allows more time for the polymer solution to polymerize (Li and Wang 2013)
Distance between the needle and the collector	If this distance is decreased, the fiber will not be able ample time to harden before reaching the collector and if the distance is too long then the resulting fibers would be thicker (greater diameter, bead fiber). An optimum distance is recommended, one that is not too long or too short in order to obtain thinner fibers (Li and Wang 2013)

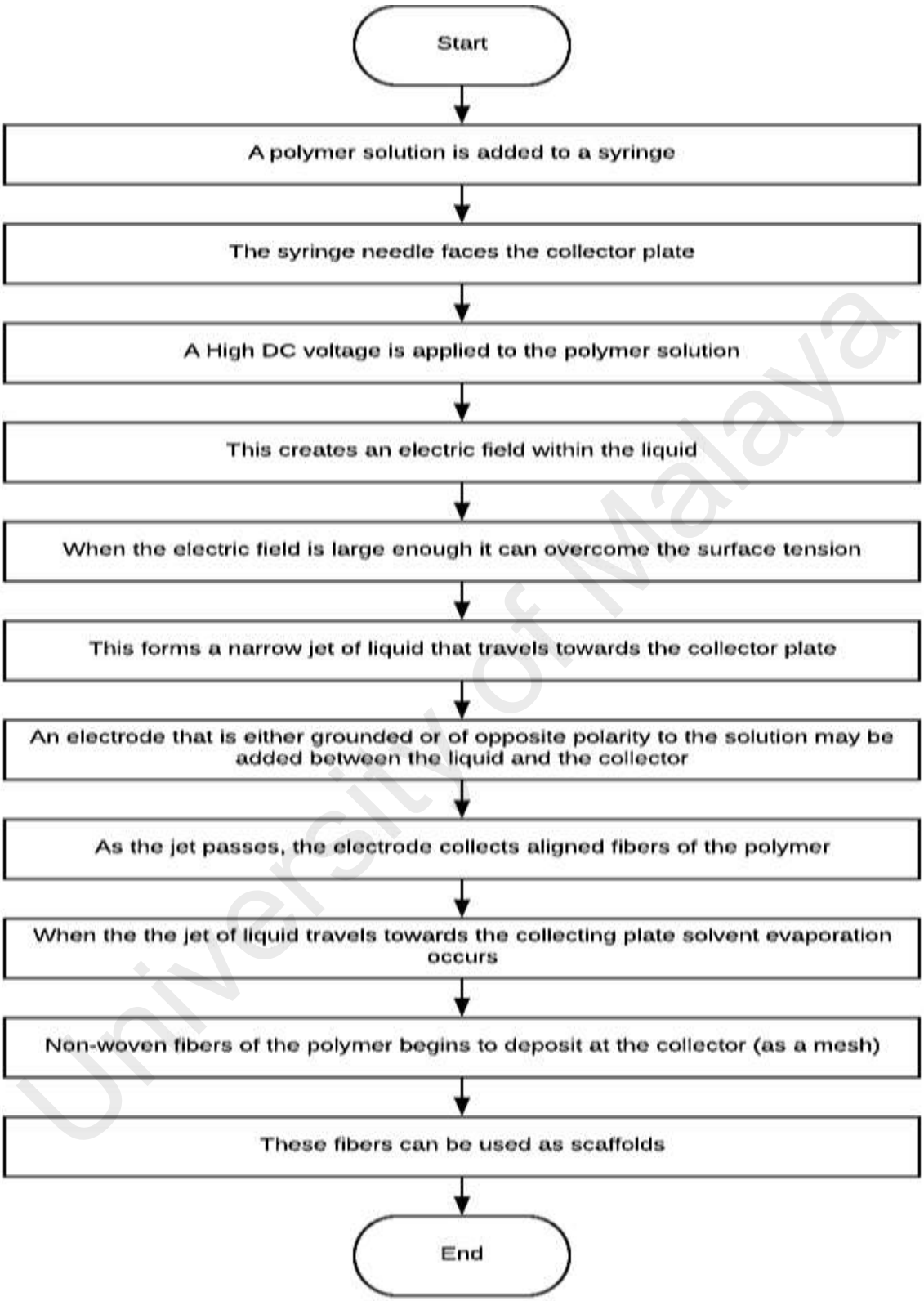

Figure 2.1 Flowchart explaining the process of electrospinning

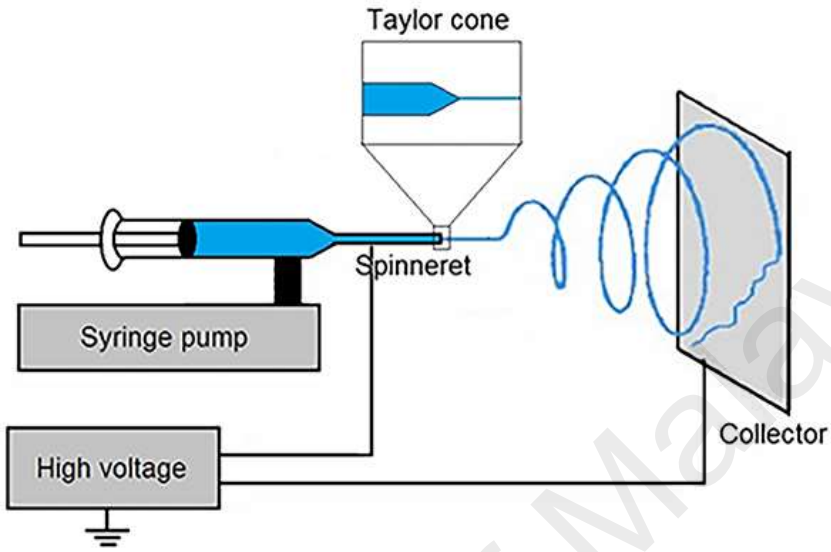

Figure 2.2 This diagram illustrates the process of electrospinning and the required components for the nano-fibers to form (Zhao, Shi et al. 2011).

2.8 Cell culture protocol for human fetal osteoblast (hFOB) cell line

The human fetal osteoblast cells are precursors to the human osteoblast cells or bone tissue. These cells are able to proliferate very quickly so they are ideal for studying human osteoblast differentiation and bone tissue substitutes. Histo-chemical studies that have been conducted in the past have resulted in a list of features that are known to be related to the osteoblast phenotype. This includes:

- Increased alkaline phosphatase activity. Alkaline phosphatase is an enzyme released by osteoblast cells.
- Production of type I collagen.

- Synthesis of a mineralized matrix. Associated with increase alkaline phosphatase activity.
- Manifestation of protein containing bone 7-carboxyglutamic acid, osteonectin, fibronectin, proteoglycans, sialoprotein, collagenase along with many more proteins. (Rodan, Yasuo Imai et al. 1987).

2.9 Culture medium solution for HFOB cells

HFOB cells may be grown in Dulbecco's modified eagle medium (DMEM) in the presence of L-glutamine, amino acids, glucose, vitamins, phenol red and salts in an atmosphere that's humid with 95% air and 5% CO^2 temperature 37ºC. It is necessary for the temperature and humidity to mimic the conditions inside the body. Cells are washed with Dulbecco's phosphate buffered saline before undergoing dissociation. This is done enzymatically by trypsinization using trypsin enzyme (Okumura, Goto et al. 2001).

The cells are treated with antibiotic solutions to reduce the chances of microbial contamination. Penicillin-streptomycin may be used for this purpose. The cells may be fed a medium containing 10mm glycerophosphate and 50 microgram/ml ascorbic acid in order to induce mineral formation. In TCP plastic 6-well plates, the cells are seeded onto electrospun aligned polyurethane fibers. Polyurethane fibers must be washed with 70% ethanol solution before transferring cells into them. The medium should be changed frequently. The cells are preserved for 2-3 weeks (Carlberg, Axell et al. 2009).

2.9.1 Light Microscopy

For histological examination the samples were immersed in a frozen medium Tissue-Tek® OCT solution, in a vacuum condition for four hours and then cryosectioned at a temperature of twenty degrees Celsius. These cryosectioned parts were transferred onto glass slides stored at a

temperature of 50 degrees Celsius overnight (In order to prevent detachment, these may be stained with trypan blue and examined by light microscopy). The staining also allows us to count the number of viable cells. Trypan blue can be used to count cells under the microscope. Viable cells appear to glow, while the dead cells are stained blue (Ciapetti, Ambrosio et al. 2003).

2.9.2 Scanning Electron Microscopy

At this stage, the samples are fixed with a percentage of paraformaldehyde. An example of a solution that could be used is the Karnovsky's fixative containing 2% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer, pH 7.2–7.4. The sample should be fixed for at least 1 hour. Before examination under a scanning electron microscope, the sample should be coated with palladium-gold and then scanned at a recommended voltage of 15 kV. This procedure allows us to effectively image the cells. The organization of materials and cells that are planted onto the polymer scaffold and the synthesis of mineral ECM can be simulated using light and scanning electron microscopy. Scanning electron microscopy graph analysis is done to find and observe the fiber diameter distribution across the polyurethane scaffold (Hassna Rehman Ramay 2003).

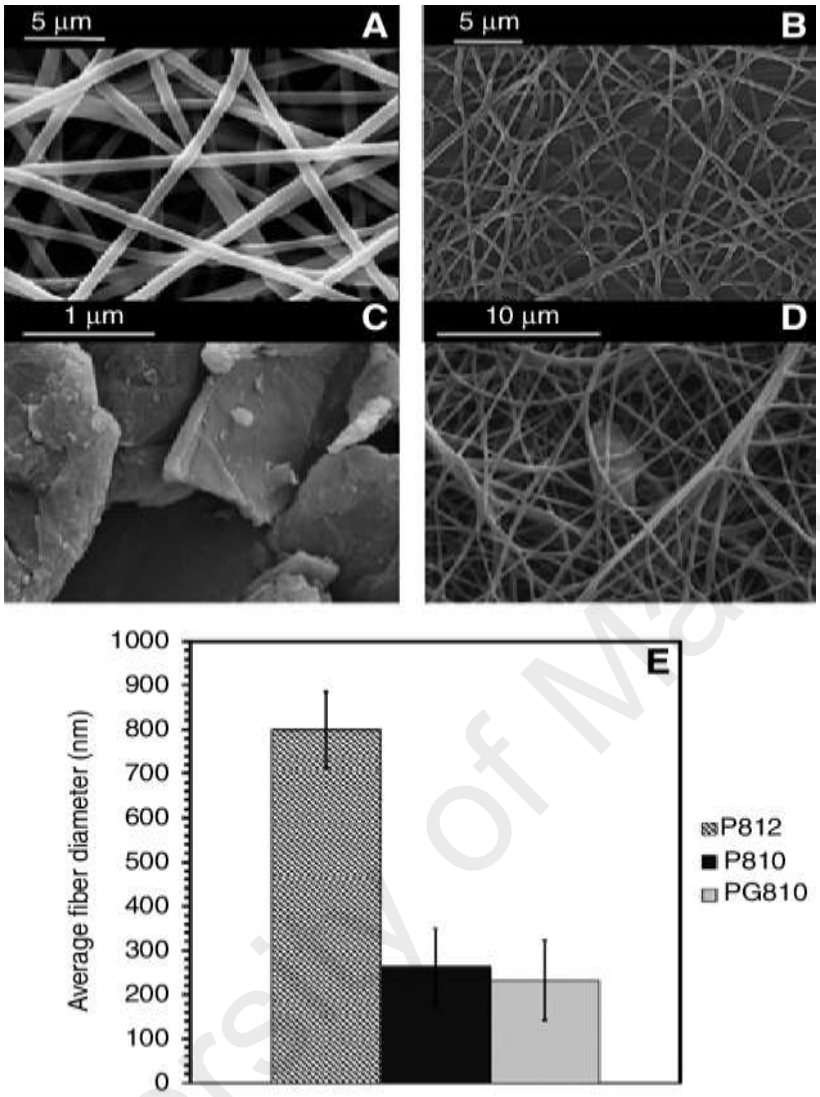

Figure 2.3 Scanning Electron Microscopy graphs (A-D) and mean fiber diameter (E) of electrospun polyurethane scaffolds (Safikhani, Zamanian et al. 2017)

2.9.3 Alamar Blue assay

The Alamar blue assay is used to measure the resulting products of redox reactions that happen within the mitochondria of live cells, making it easy to conclude the metabolic state of the cells. The samples are incubated at 37 degrees Celsius for 4 hours with this stain and the results are

measured in relative fluorescence units (RFU) using Cytofluor 2350 (Millipore Corporation). This quantification can be done on selected days such as day 1, 3, 7, 14 and 21 (Ciapetti, Ambrosio et al. 2003).

2.9.4 Alkaline phosphatase assay (ALP assay)

ALP activity can be measured by the incubation of 100 ml of the cell samples in the presence of 100 ml of p-nitrophenylphosphate solution in a glycine buffer for 15 min at 37 degrees Celsius. The reaction gives p-nitrophenol when ALP I present and this is measured by monitoring light absorbance by the solution. The results are expressed in mmol/l. Alkaline phosphatase is an enzyme produced by osteoblast cells and is a good indicator to determine if the tissues are developing and growing (Ciapetti, Ambrosio et al. 2003).

2.9.5 DNA quantification by PicoGreen® DsDNA assay

For DNA analysis and gene expression in the cells, the PicoGreen® assay may be used for the samples. The reagent is fluorescent and can be used to examine the mRNA and other enzymes. To determine DNA fragment size, samples may be separated by electrophoresis on an agarose gel with ethidium bromide at 60 V for 1 hour, stained, and visualized with ultraviolet transillumination (Gilbert, Freund et al. 2009).

2.9.6 Cytotoxicity assay

The goal of cytotoxicity assay is to analyze the cell viability. The tests performed are to check the membrane integrity, membrane metabolic activity, loss of monolayer adherence and death of cells while the cell seeding is done on scaffolds. This assay is ideal for use in in vitro studies of cell

culture to identify the cytotoxic or cytostatic potential of the scaffold used and how they affect the cells by making use of commercial test kits that are easy to work with.

3.0 CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Fabrication of scaffolds

In this experimental study, two types of scaffolds are used. These scaffolds were prepared by a graduate researcher from biomedical engineering department, University of Malaya. The scaffolds were fabricated using electrospinning. One of the scaffolds was prepared using 14 grams of polyurethane resin and 1 gram of HA added to 100 ml of solvent containing N, N-dimethylformamide (DMF) and tetrahydrofuran (THF). The ratio of DMF and THF is 1:1. This solution is electrospun to produce a PU scaffold with non-parallel fibers (45 degrees alignment). The applied voltage for scaffold fabrication was 15kV, the solution injection rate was 0.1 ml/hr and the fiber diameter was 2µm. The distance between the syringe tip and collector plate was 15cm. The final scaffold was composed of 93.3% of polyurethane and 6.7% of Hydroxyapatite.

The second scaffold is prepared by using 15 grams of polyurethane resin added to 100ml of DMF and THF. The ratio of DMF and THF is 1:1. This solution is electrospun to fabricate a PU scaffold with parallel fibers (0 degrees alignment). The parallel fibers are obtained by placing an electrode between the syringe containing this solution and the collector plate. The applied voltage for scaffold fabrication was 15kV, the solution injection rate was 0.05 ml/hr and fiber diameter was 2.8µm. The distance between the collector and syringe tip was 15cm. The final scaffold is composed of 100% polyurethane.

3.2 Media for cell growth

The medium solution used is Dulbecco′s Modified Eagle′s Medium (DMEM) with phenol red and 10% concentration of penicillin-streptomycin antibiotic. Fetal bovine serum is used as a source of

protein and nutrients for the cells. These reagents were purchased from Gibco, Invitrogen Corp USA.

3.3 Characterization of scaffolds

The electrospun scaffolds were studied using Field Emission Scanning Electron Microscopy (FESEM). The scaffolds were freeze-dried, coated with silver and scanned at a voltage of 5kV. They were then fixed with 2% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer for one hour. The scanning electron microscope examination was then carried out at 5kV. This procedure allowed us to effectively image the scaffolds and receive images with the fiber distribution and fiber diameter values. The organization of fibers in the scaffold were made visible.

3.4 HFOB cell revival

The biosafety cabinet is where all the cell culture procedures were carried out. Before use, all equipment including pipettes, pipette tips, petri dishes and t-flasks were UV sterilized inside the cabinet. Enzymes, cells, medium and reagents should not be UV sterilized. Figure 3.1 shows the biosafety cabinet with the required consumables and equipment.

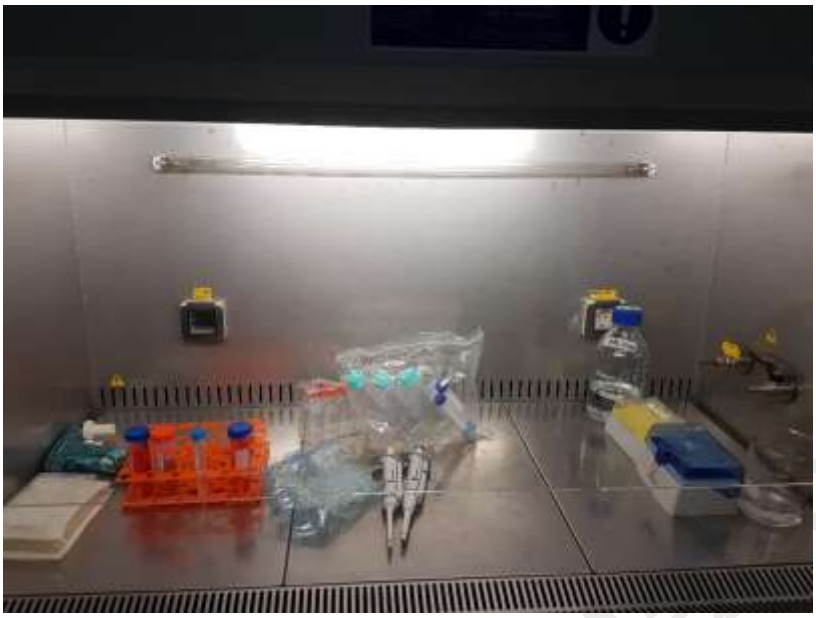

Figure 3.1 The biosafety cabinet

To prepare the medium, 1liter of DMEM F-12 with phenol red containing 10 % pen-strep antibiotics was transferred into three 50ml tubes. Each tube is filled with 45ml medium. Fetal bovine serum (FBS) is added to each tube in quantity 5ml.

A cryo-vial containing hFOB 1.19 ATCC with passage 6 (P6) is taken from the -80^{o}C fridge to be revived. The cells are thawed at a temperature of 37 degrees Celsius using a water bath. The medium solution prepared earlier warmed to 37 degrees in an oven. Both the cryo-vial and medium tubes are sprayed with 70% ethanol and moved to the biosafety cabinet. In a centrifuge tube, 10ml of medium solution and 1ml of hFOB cells from the vial is added. Centrifuge the tube at 1000rpm for 10 minutes. The medium and cells separate. The media on top is decanted and a pipette is used to transfer the cells at the bottom to a T75 flask.

After that, 6ml of medium is transferred into the T75 flask. Figure 3.2 shows the T75 flask containing cells and the medium solution in lab. The flask is shaken gently and observed under the

microscope for attachment and contamination. It is then placed into the incubator to allow proliferation and growth of cells. To observe attachment, confluency and contamination, place the flask under the microscope and activate the device that provides interface with the desktop computer. The software used is called NIS elements (imaging software). This allows us to capture images in grayscale according to the magnification, position, brightness and contrast that is set on the microscope. For the study, each day we took images at 4x and 10x magnifications and observed the cells.

Figure 3.2 A T75 flask containing medium and cells, after two days

3.5 Trypsinization

When the cells reached 70% to 90% confluency, trypsinization can be done. Phosphate buffered saline or PBS is prepared by using PBS tablets (Sigma-Aldrich USA). 1 tablet was added to 500ml of distilled water and autoclaved. Trypsin enzyme was purchased from Gibco, Invitrogen Corp USA.

The medium in the T75 is decanted into a beaker of ethanol. After that, 2.5ml of PBS is added inside the flask and shaken gently to wash the cells for 10 seconds. The PBS is discarded into the ethanol, before adding 2.5ml of trypsin. The trypsin enzyme is shaken in the flask for 5 minutes to ensure all the cells on the flask wall are detached and floating.

3.6 Cell counting

After trypsinization, using a pipette, 20µl of the solution containing detached cells is drawn and added to a 1.5ml tube. Using a pipette, 20µl of trypan blue stain (Sigma-Aldrich USA) was introduced into the tube and shaken to mix well with the cells. The hematocytometer with a glass slide is prepared beside the microscope. Using the pipette, the trypan blue and cell mixture is added on the hematocytometer, enough to cover the grid. The cells inside the four boxes are counted under the microscope. Trypan blue stains the cells such that the viable cells appear to glow and have a light color while the stain enters the membranes of dead cells making them appear dark blue. The number of viable and dead cells in each of the four boxes is counted and recorded for measurement later.

3.7 Calculation of cell growth per ml

The number of cells per ml of solution can be calculated using the formula below

Concentration = Number of cells / Volume of hematocytometer

Volume of hematocytometer = 1mm x 1mm x 0.1mm = $0.1mm^3$ or 0.0001ml (10^{-4}ml)

Number of cells per ml = Total number of viable cells in all boxes x 2 / 4 x 10^{-4}

Or (Total number of viable cells in all boxes x 2 / 4) x 10^4 = x cells/ml

Where 2 is the dilution factor that is multiplied with the total number of viable cells and the equation is divided by 4 because the cells are counted in the 4 boxes of the hematocytometer.

For Example

Box 1 = 16 viable cells and 2 dead cells

Box 2 = 18 viable cells and 2 dead cells

Box 3 = 21 viable cells and 1 dead cell

Box 4 = 13 viable cells and 1 dead cell

Number of cells = (68 viable cells x 2 / 4) x 10^4 = 340,000 cells/ml

3.8 Cell growth profile

For the cell growth profile, 6-well plates are used. This study is performed three times in order to calculate an average and the plot cell growth profiles. To do this, 2ml of fresh medium solution is added to each of the 6 wells from the medium and FBS solutions that were stored in the three 50 ml tubes at the time of cell revival. In the proliferation study, it was decided to distribute 500,000 cells per well in the 6-wells so 0.28ml (according to a cell count after passage). A 6-well plate used for the cell growth profile can be seen in Figure 3.3.

Over the course of 1 week, each day excluding the weekend, the cells in each well are observed under the microscope for confluency, then trypsinization and cell counting is carried out for all wells. This is done to gather data which could be used to plot the cell growth profile later. For the weekend, an approximate number of cells data were added to complete the plot, as it was not possible to measure the data during that time.

3.9 Cell viability

To measure the percentage of viability after cell counting and proliferation, the following formula is used:

Cell viability as a percentage = (viable cells / [viable cells + dead cells]) x 100

Where viable cells and dead cells are counted using trypan blue during cell count

Figure 3.3 A 6-well plate for cell growth profile

3.10 Cell seeding into scaffolds

After cutting the scaffolds into a size to fit the wells, these scaffolds were immersed in ethanol overnight and then the next day, they are rinsed with PBS three times and also rinsed with DMSO (Solvent dimethyl sulfoxide).

In a 6-well plate, all 6 wells are filled with 2ml medium, 0.28ml cell solution and the PU scaffolds are added as well. The PU scaffolds with HA is added to 3 wells, while the PU scaffolds without

HA are added to the other three wells as a control. The purpose of the control wells is to observe whether the scaffolds are affected by the addition of HA or not.

The trypsinization and cell count is carried out for each well. This is done to measure how many cells have attached to the fibers. This is carried out over a span of 1 week, similar to the growth profile. After collecting all the data from this study, a graph to observe the cell growth trend with the addition of scaffold is plotted.

Figure 3.4 Picture of the polyurethane and hydroxyapatite scaffold with non-parallel fibers

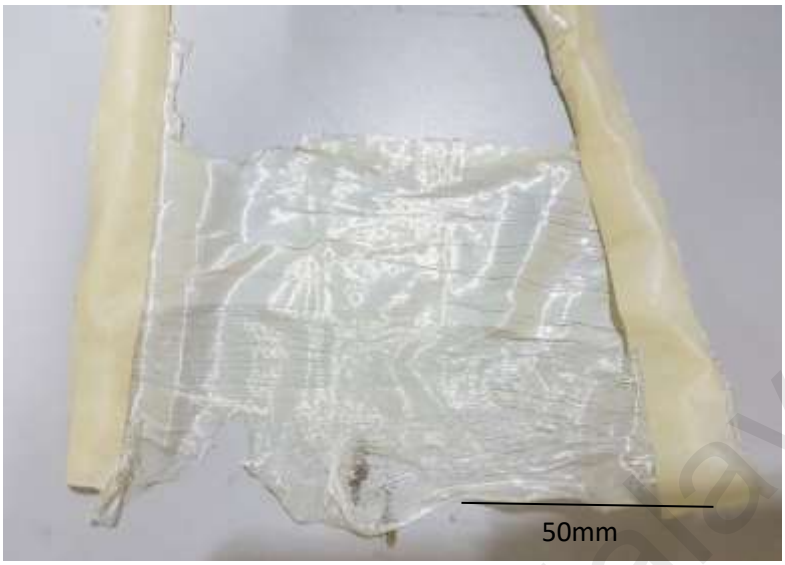

Figure 3.5 Picture of the polyurethane scaffold with parallel aligned fibers, no HA added

4.0 CHAPTER 4: RESULTS

The following are the images that were scanned using scanning electron microscopy. These are the scaffolds used during the experimental study. The graphs that were plotted using the data from the cell growth profile and cell seeding are also displayed and compared below.

4.1 Images of the polyurethane scaffolds taken by FESEM

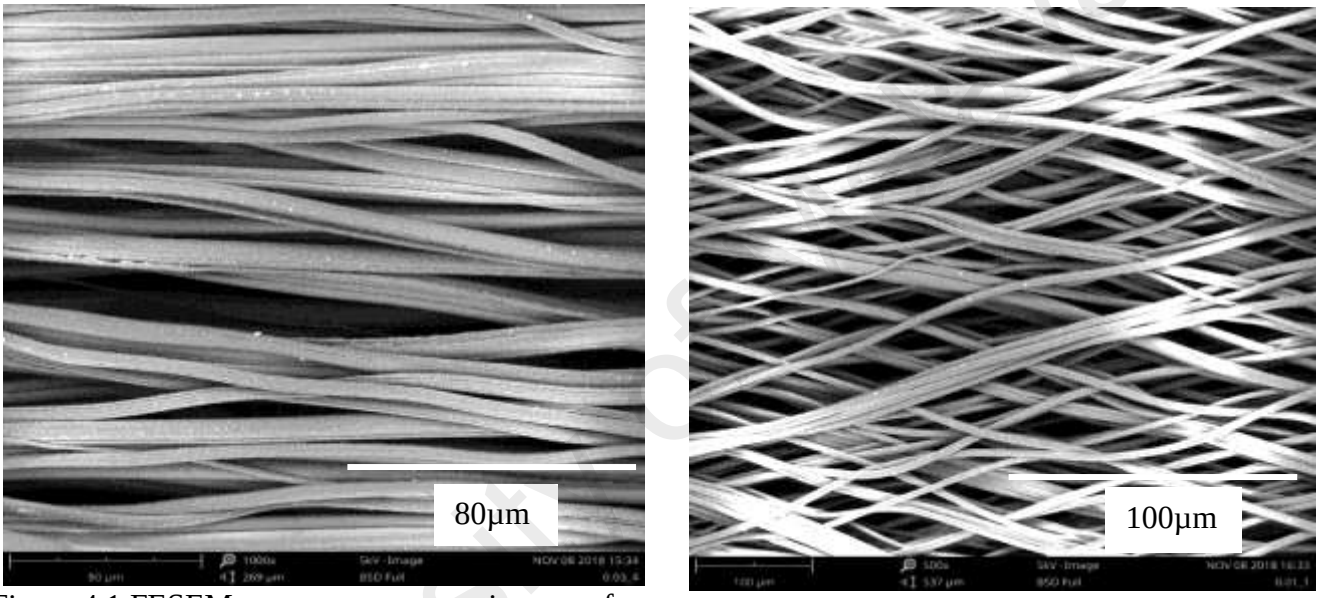

Figure 4.1 FESEM images of a) Polyurethane scaffolds (a) Scaffold with parallel fibers and 0% HA 1000x magnification (b) Scaffold with non-parallel fibers and 1%HA 500x magnification b)

Figure 4.1(a) is the image of the polyurethane scaffold with parallel fibers. The fiber diameter was measured to be 269µm. The scaffold was imaged before cell seeding. Figure 4.1(b) is the image of the polyurethane scaffold with 1% HA and random fibers at an angle of 45 degrees. The fiber diameter was measured to be about 537µm, making the fibers larger in diameter compared to the previous scaffold. The scaffold was imaged before cell seeding.

4.2 The Cell growth profile without scaffolds

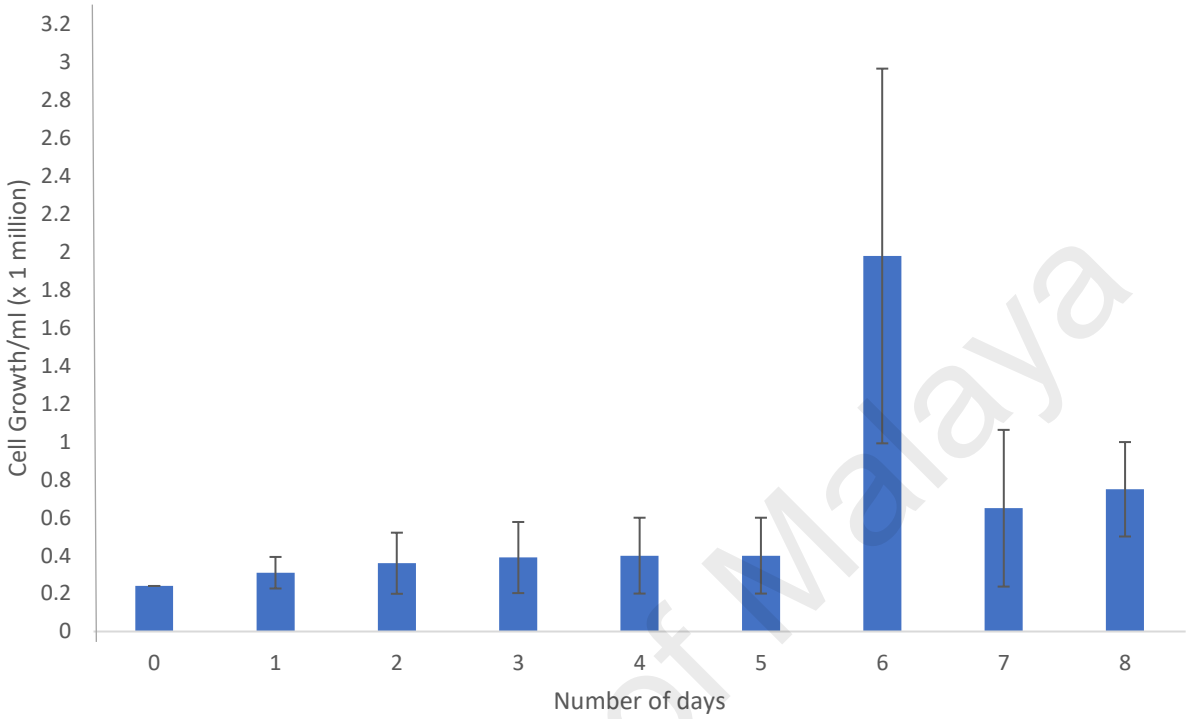

Figure 4.2 This chart shows the average cell growth or number of cells per ml that was measured for eight days. The cells were grown without scaffolds. The error bars represent the standard deviation (n=3)

The bar graph of cell growth (in millions) against number of days was plotted. Three cell growth profiles were carried out in the lab in separate 6-well plates and after that the average cell growth for each day was calculated for 8 days. The standard deviation (Average±Standard deviation) was also displayed for the resulting bar graph as error bars. According to the results here, the best time to harvest the cells or carry ot seeding into scaffolds is between the 5th and 6th day.

4.3 Polyurethane scaffold comparison

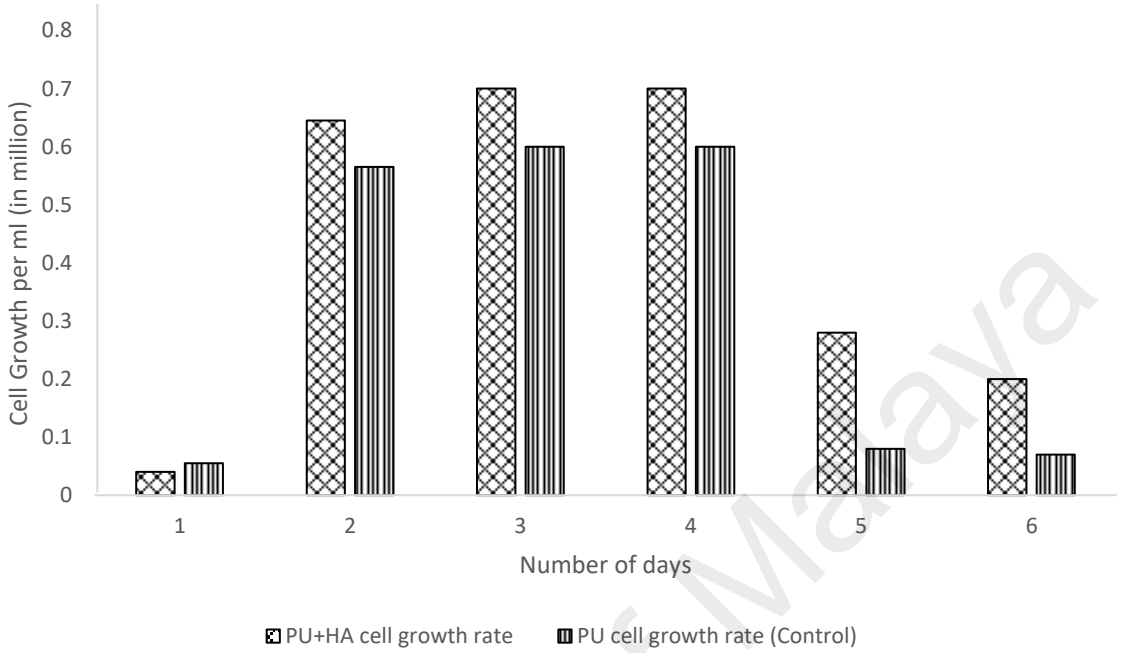

Figure 4.3 This graph compares the growth and attachment of cells to both polyurethane scaffolds over a period of 6 days. The PU scaffold with 0% HA is taken as the control.

This bar graph is obtained after measuring the number of cells attached to both types of scaffolds. It can be seen, that the PU scaffold with HA has better cell attachment throughout when compared to the PU with no HA. However, it is also noticeable that the overall cell growth on all days is very less with the maximum being 0.7 million cells per ml for the PU scaffold with HA on the 3rd and 4th days. The cell growth increases for both scaffolds in the first 3 days, remains constant on the fourth day and then decreases from the 4th to 6th day. There is a sharp decline in the cell growth for the PU scaffold with no HA.

5.0 CHAPTER 5: DISCUSSION

5.1 Cell growth profile analysis

The graph plot for the cell growth profile, is recorded to observe the pattern of growth for the cells and to see on what day do they reach maximum count before starting to die where the graph shows a decline in cell growth. According to the data obtained from the cell growth profile the fifth and sixth days are best for harvesting the cells.

5.2 Polyurethane scaffold analysis

From the previous cell growth profile, we concluded that the fifth or sixth day may be best for harvesting the cells. Hence, after cell revival we observe and count the cells as well as passage them until the fifth day. After that the they were seeded onto polyurethane scaffolds in a 6-well plate. Three wells contained samples of PU with 0% HA and parallel fibers and the other three wells contained samples of PU with 6.7% HA and random fibers.

Overall the results from this study shows that both scaffolds were not biocompatible with the HFOB cells even though previously according to the literature review it was expected that polyurethane scaffolds should be an ideal material for the growth and differentiation of osteoblast cells. Many studies, have claimed success in seeding SAOS-2 and HFOB cells in polyurethane and observed steady growth and expansion. This did not happen during our cell seeding experiment (Veiga and Branco 2015).

This could have occurred due to some of the limitations in the experimental study, The HFOB cells were already passage 6 and were passaged until passage 9 before being seeded into the scaffolds. The cells were already quite old and possibly inactive in division, resulting in less cell

attachment to both scaffolds. The use of geneticin as an antibiotic instead of pen-strep could also lead to better results with osteoblast cells. Furthermore, it may be the medium that caused the cells to become weak. The medium used was DMEM with phenol red, so in the next study it might be useful to use DMEM without phenol red as this stain causes mineral deformation for osteoblast cells.

It was observed however, that the cells grew and attached relatively more onto the PU with HA and this is because HA is an osteoconductive material. Despite its low mechanical properties, hydroxyapatite is a bioactive material that is good for bone tissue engineering. With regards to this there have been many bone tissue engineering studies in which composite scaffolds consisting of higher percentages of HA were used including polymer foams and electrospun fibers. It was often concluded that the scaffolds containing HA were more biocompatible with osteoblast cells (Zhihong Donga, a et al. 2009).

The reason for this may be that hydroxyapatite is a naturally occurring mineral that is found in teeth and bones in the form of crystalline calcium phosphate (Calcium apatite) making it a natural substitute for ECM in invitro cell seeding. In many researches, the use of natural materials to fabricate scaffolds has caused healthy cell proliferation and expansion. Materials such as collagen, hydroxyapatite, dermis and vascular grafts are more biocompatible as compared to synthetic polymers and metals. In the future, it may be a good idea to use polyurethane scaffolds with a higher percentage of HA, because HA on its own does not possess the mechanical strength and toughness of polymers and it's a brittle material. The combination of the two might produce a more durable scaffold. A biodegradable scaffold would also be better for tissue engineering applications, as it would disintegrate over time in the body leaving only the tissue (Grada, Kupcsika et al. 2003).

6.0 CHAPTER 6: CONCLUSION

To conclude this experimental study, new scaffolds were fabricated and used in cell culture in order to test their biocompatibility. However, the results that were recorded show that the cell attachment and growth for both scaffolds was quite low and further research must be conducted in order to fabricate scaffolds that are more biocompatible and practical for tissue engineering applications. The cell attachment to the PU scaffold containing HA was relatively better, therefore the percentage of HA in the scaffold could be increased in the future for further studies. There are many material combinations that could be explored and with different angle alignments to seed in cell culture, however due to time constraints the research could not be continued further.

It would have been interesting to conduct a study with HFOB cell culture where PU scaffolds with 0% HA and non-parallel fibers and PU with 6.7% HA and parallel fibers were used. The results could be recorded and then compared with the data that was obtained in this study. Furthermore, polyurethane scaffolds with a higher percentage of HA could also be explored as an option for HFOB cells. It would also be interesting to change some electrospinning parameters such as fiber diameter and the distance between the syringe and collector plate and design scaffolds with varying properties.

In the future, more studies on polyurethane scaffolds can be carried out in order to decide whether it is an ideal material for the growth of osteoblast cells and if it can form viable bone tissues that can be used in clinical applications and with patients.

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