Development of Biomimetic PHB and PHBV Scaffolds for a Three Dimensional (3-D) *In Vitro* Human Leukaemia Model

THESIS BY

SAIFUL IRWAN ZUBAIRI

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Department of Chemical Engineering
Imperial College London
South Kensington Campus
London, SW7 2AZ, U.K.
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DECLARATION

The work described in this thesis was carried out in the Department of Chemical Engineering, Imperial College London, United Kingdom between January 2009 and July 2012. Except where acknowledged, the material is the original work of the author and includes nothing which is the outcome of work in collaboration and no part of it has been submitted for a degree at any other university.
Leukaemia is defined as a group of haematological diseases (related to blood and blood-forming tissue) characterized by malignant proliferation of myeloblasts or lymphoblasts that replace normal bone marrow elements and infiltrate normal tissues. The study of leukaemia has been hindered by the lack of appropriate in vitro models, which can mimic this microenvironment. It is hypothesized that the fabrication of porous 3-D scaffolds for the biomimetic growth of leukaemic cells in vitro could facilitate the study of the disease in its simulated native 3-D niche. In this study, polyhydroxyalkanoate (PHA), in particular poly(hydroxybutyrate) (PHB) and poly(hydroxybutyrate-co-valerate) (PHBV) porous 3-D scaffolds with an improved thickness (in relative to the conventionally made PHA matrices) are utilized and investigated to model the abnormal 3-D leukaemic cellular growth system in the absence of exogenous cytokines. The polymeric porous 3-D scaffolds were fabricated using an ideal polymer concentration of 4% (w/v). The salt-leaching efficacy and the effect of salt residual on the cell growth media were carried out to validate the significant amount of salt remnant inside the porous materials. The physico-chemical characteristics of the porous 3-D scaffolds such as surface wetting, porosity, BET surface area and pore size distribution were studied by means of drop sessile analyzer (DSA), helium gas pycnometry, mercury intrusion porosimetry (MIP) and scanning electron microscopy (SEM). To increase probability of cellular attachment and proliferation, the polymeric scaffold surfaces were treated with O$_2$-rf-plasma (100 W at 10 min) and NaOH (0.6M). Next, in order to improve the in vitro 3-D leukaemic cell culture, two main bone marrow extracellular matrix (ECM) proteins which are collagen type I or fibronectin were immobilized via physical adsorption on the treated surfaces of the polymeric porous 3-D scaffolds. Meanwhile, the in vitro degradation studies were conducted on both polymeric scaffolds with the hydrolytic degradation media of phosphate buffered saline (PBS) and cell growth media. The scaffolds were analyzed and compared for mass loss, morphology and pH changed of the PBS and cell growth media throughout 45 weeks and 9 weeks of the study respectively. Overall, PHB and PHBV displayed a good seeding efficiency (24 h) and excellent leukaemic cellular growth for up to 6 weeks (protein-coated scaffolds), assessed by MTS assay and SEM. Once the abnormal hematopoietic 3-D model (cell lines) was established, a new model to culture human primary acute myeloid leukaemia mononuclear cells (AML MNCs) was studied, compared and validated. All leukaemic cells grew better in PHBV scaffolds coated with 62.5 µg/ml collagen type I and sustained cell growth in the absence of exogenous cytokines. As a result, it was concluded that PHBV-collagen scaffolds may provide and could be used, as a practical model with which to study the biology and treatment of primary AML in an in vitro mimicry without the use of 2-D culture system and animal models.
This thesis has resulted in the following papers and conference proceedings:

**Journal Papers**


**Selected refereed International Conferences**

1. Zubairi, S.I., Koutinas, A., Bismarck, A., Panoskaltsis, N., Mantalaris, A. “Porous three-dimensional scaffolds of poly(3-hydroxybutyric acid) (PHB) and poly(3-hydroxybutyric-co-3-hydroxyvaleric acid) (PHBV) with an improved thickness as cell growth supporting materials” at European Congress and Exhibition on Advanced Biomaterials and Processes (EUROMAT), 12 - 15 September 2011, Montpellier, France.

2. Zubairi, S.I., Koutinas, A., Bismarck, A., Panoskaltsis, N., Mantalaris, A. “Surface treatment of poly(3-hydroxybutyric acid) (PHB) and poly(3-hydroxybutyric-co-3-hydroxyvaleric acid) (PHBV) porous three-dimensional scaffolds with an improved thickness to enhance cell-biomaterial adhesion and interactions” at 24th European Conference on Biomaterials (ESB), 4 - 8 September 2011, Dublin, Ireland.
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<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
</tr>
<tr>
<td>AMU</td>
<td>Atomic Mass Unit</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Ar</td>
<td>Argon</td>
</tr>
<tr>
<td>ATC</td>
<td>Automatic temperature compensation</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BET</td>
<td>Stephen Brunauer, Paul Hugh Emmett and Edward Teller</td>
</tr>
<tr>
<td>BOD</td>
<td>Biochemical oxygen demand</td>
</tr>
<tr>
<td>CPO</td>
<td>Crude palm oil</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
</tr>
<tr>
<td>CH₂I₂</td>
<td>Di-iodomethane</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer-aided design</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>dp</td>
<td>Pore diameter</td>
</tr>
<tr>
<td>d</td>
<td>Thickness</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DIW</td>
<td>Deionized water</td>
</tr>
<tr>
<td>E</td>
<td>Compression modulus</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EKA</td>
<td>Electrokinetic analyzer</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ETO</td>
<td>Ethylene oxide</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>Eₐdh</td>
<td>Total energy change of separating liquid and solid adhesion</td>
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<tr>
<td>FFB</td>
<td>Fresh fruit bunches</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>f</td>
<td>Fraction of fluid area in contact with the material surface</td>
</tr>
<tr>
<td>GF</td>
<td>Growth factor</td>
</tr>
<tr>
<td>g</td>
<td>Gravity</td>
</tr>
<tr>
<td>h</td>
<td>Height</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin &amp; Eosin</td>
</tr>
<tr>
<td>HL-60</td>
<td>Designations of acute myeloid leukaemia cell line</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HM</td>
<td>Haematopoietic microenvironment</td>
</tr>
<tr>
<td>HSCs</td>
<td>Haematopoietic stem cells</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HV</td>
<td>Hydroxyvaleric acid</td>
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<tr>
<td>iep</td>
<td>Isoelectric point</td>
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<tr>
<td>JeKo-1</td>
<td>Designations of chronic lymphocytic leukaemia cell line</td>
</tr>
<tr>
<td>κ</td>
<td>Conductivity</td>
</tr>
<tr>
<td>K</td>
<td>Kelvin</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>kgf</td>
<td>Kilogram-force</td>
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<tr>
<td>L</td>
<td>Length</td>
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</tbody>
</table>
LCD  Liquid crystal display
M    Mass
M_w  Molecular weight
MNC  Mononuclear cell
MRI  Magnetic resonance imaging
M    Molarity
mcl- Medium chain length
m    Backbone of the molecular structure
MTS  (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NaOH Sodium hydroxide
NHL  Non-Hodgkin's lymphoma
N    Number of monomers
NS   Not significant
N    Newton
NaCl Sodium chloride
N_2  Nitrogen
NH_3 Ammonia
IMDM Iscove's modified Dulbecco's medium
O&G  Oil and grease
O_2  Oxygen
P    Porosity
p    Pressure
PASW Predictive Analytics Software
PHBHHx Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
Pen/Strep Penicillin-streptomycin
PB   Peripheral blood
PE   Polyethylene
PMMA Poly(methyl-methacrylate)
PDLLA Poly(D,L-lactate)
PLGA Poly(L-lactic-co-glycolic acid)
PS   Polystyrene
PCL  Poly(caprolactone)
PU   Polyurethane
PLA  Polylactic acid
PGA  Polyglycolic acid
PF   Polyfumarates
PCL  Polycaprolactones
PHB  Poly(hydroxybutyrate)
PHBV Poly(hydroxybutyrate-co-valerate)
POME Palm oil mill effluent
PSD  Pore size distribution
PBS  Phosphate buffered saline
RP   Rapid prototyping
Q    Volumetric gas flow rate
rf   Radio frequency
r    Surface roughness ratio
RL   Designations of chronic lymphocytic leukaemia cell line
R    Variable that determine types of homo-polymers (side chain)
RGD  Argenine (Arg) - Glycine (G) - Asparagine (Asp)
RPM  Revolutions per minute
RCF  Relative centrifugal force
RTU  Remote titration unit
RFGD Radio frequency glow discharged
scl- Short chain length
SED  Solvent emission directive  
SFE  Surface free energy  
scl-  Short chain length  
sccm  standard cubic centimetres per minute  
SFF  Solid freeform fabrication  
SCPL  Solvent-casting particulate-leaching  
SBR  Sequential batch reactor  
sd  Standard deviation  
SEM  Scanning electron microscopy  
TCP  Tricalcium phosphate  
TOC  Total organic carbon  
T-PLL  T-prolymphocytic leukaemia  
t  Time  
T\textsubscript{g}  Glass transition temperature  
TIPS  Thermal induced phase separation  
USD  United States dollar  
UV  Ultra violet  
UK  United Kingdom  
V  Volume of sample  
VOCs  Volatile organic compounds  
VFAs  Volatile fatty acids  
V\textsubscript{suction}  Volumetric gas suction flow rate  
V\textsubscript{p}  Specific pore volume  
W\textsubscript{SL}  Work of adhesion at solid-liquid interfaces  
W\textsubscript{LL}  Work of cohesion of water  
YIGSR  Tyr - Ile - Gly - Ser - Arg  
\zeta  Zeta potential  
\zeta\textsubscript{plateau}  Zeta potential plateau value  
\gamma\textsubscript{LV}  Liquid-solid interfacial tension  
\gamma\textsubscript{SL}  Solid-liquid interfacial tension  
\gamma\textsubscript{SV}  Solid-vapour interfacial tension  
\gamma\textsubscript{p}  Solid surface free energy  
\gamma\textsubscript{d}  Solid surface free energy of dispersive component (non-polar)  
\gamma\textsubscript{p\textsubscript{b}}  Solid surface free energy of polar component  
\gamma\textsubscript{s}  Liquid surface tension  
\gamma\textsubscript{p\textsubscript{d}}  Liquid surface tension of dispersive component (non-polar)  
\gamma\textsubscript{p\textsubscript{L}}  Liquid surface tension of polar component  
\theta  Contact angle  
\phi  Diameter  
\rho  Density  
\rho\textsubscript{b}  Bulk density  
\Omega  Ohm  
\sim  Approximation  
\rho\textsubscript{e}  Envelope density  
2-D  Two-dimensional  
3-D  Three-dimensional
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CHAPTER 1 Introduction

Tissue engineering can be defined as a process that (1) combines living cells with biomaterials, (2) utilizes living cells as cell therapy or diagnostic reagents, (3) generates tissues \textit{in vitro} for therapeutic implantation and (4) provides materials or technology to enable any of these approaches [1]. Tissue engineering has emerged as a multi-disciplinary field combining biology, materials science and surgical reconstruction in order to provide living tissue products that restore, maintain or improve tissue functionality [2, 3]. The need for this approach has been a primary option because of a lack of donor organs and tissues, painful procedures of secondary surgery and highly invasive procedures. This option is undeniably offered a dramatic ability to facilitate, repair, regenerate and replace damaged or diseased tissues. To be more specific, biomaterials (any matter, surface or construct that interacts with biological systems) have gained significant importance in the field of tissue engineering. Often these biomaterials (derived either from nature or chemically synthesized) are used for tissue regeneration when the surrounding defective tissue exhibits the natural potential for tissue regeneration. However, in situations where the tissue lacks this ability of regeneration, relevant cells as well as growth factors have been used to accelerate tissue regeneration. In addition, these biomaterials have also been combined with drugs and used as drug delivery systems, thereby reducing the microbial infections while maximizing tissue regeneration [4]. In general, there are three distinct approaches currently being used in the tissue engineering and regenerative medicine applications. These are (1) infusion of isolated autologous cells or cell substitutes, (2) implantation of tissue-inducing acellular (no containing cells) scaffold materials \textit{in vivo} (e.g., natural polymers and inorganic materials) by allowing the patient’s cells to repair the tissue guided by the scaffold and (3) implantation of expanded \textit{in vitro} autologous cells seeded in scaffolds [3].

In this thesis the investigation has focussed on the study of developing three-dimensional porous scaffolds from biopolymers (mainly from PHAs, particularly PHB and PHBV). The rationale of choosing this material is to promote the usage of palm oil mill effluent (POME) in producing poly(\(\beta\)-hydroxyalkanoates) via microbial fermentation process as an added value material for tissue engineering applications. Malaysia is one of the main exporters of palm oil in the world after Indonesia, Thailand, Nigeria and Colombia [5]. The up-stream processing of fresh palm fruit bunches to extract palm oil has generated an enormous amount of organic carbon content of liquid effluent (POME). In fact, this concentrated POME can be used as main carbon sources of microbial fermentation to produce PHA. In this study, two commercial PHAs from Sigma-Aldrich\textsuperscript{TM} which is PHB and PHBV were used to fabricate 3-D porous scaffolds with an improved thickness greater than 1 mm (in relative to the conventionally made PHA matrices) by using solvent-casting particulate-leaching (SCPL) method. Then, this fabricated
3-D porous scaffold were coated with extracellular matrix (ECM) proteins to produce a 3-D bone marrow biomimicry by providing an ideal microenvironment for the growth of leukaemia cell lines and primary sources (e.g., leukaemic cells taken from patient and normal stem cells). The 3-D model generated from this study will be used to explain the molecular determinants of leukaemogenesis, the cellular and microenvironmental elements that enhance leukaemia growth and protect the leukaemic stem cells from chemotherapy. Moreover, this 3-D bone marrow biomimicry scaffold has the potential to be used in the tissue engineering application by replacing the abnormal bone marrow consisting of leukaemic stem cells with normal stem cells via in vivo implantation. For that reason one particular class of the stem cells which are the hematopoietic stem cells (HSCs) were studied. Hematopoiesis is regulated in the bone marrow, a complex 3-D tissue composed by a heterogeneous population of haematopoietic and non-haematopoietic stromal cells. The hematopoietic stem cells and their microenvironment (HM) are unique and perform several tasks that no other cells can accomplish. They are indefinitely housed in the bone marrow in spaces called niches which control their properties, support HSCs self-renewal and prevent them from differentiating. Therefore the dysregulation of the niches, either in structure or function, results in the pathogenesis of disease states of the bone marrow, such as leukaemia which was studied in detail in this thesis. The different types of leukaemia are grouped by the cell affected, myeloid or lymphoid and by the rate of the cell growth, acute or chronic.

1. **Acute leukaemia** involves an overgrowth of very immature blood cells. This condition is life-threatening because there are not enough mature blood cells to prevent anaemia, infection and bleeding. A diagnosis of acute leukaemia is made when there are 20 percent or more blasts or immature cells in the bone marrow.

2. **Myelodysplastic Syndrome (MDS) or preleukaemia** is a condition in which the bone marrow does not function normally and therefore does not produce enough normal blood cells. The blood cells affected are white blood cells, red blood cells and platelets. Some cases of MDS may, over time, progress to acute leukaemia.

3. **Chronic leukaemia** involves an overgrowth of mature blood cells. Usually, people with chronic leukaemia have enough mature blood cells to prevent serious bleeding and infection.

The necessity of identifying the pathways and stimuli involved in the self-renewal and differentiation processes of both normal and leukaemic stem cells is required in order to understand the events that lead to the leukaemogenesis but at the moment it is unknown which are the abnormalities required for leukaemic transformation. It was decided to study the indicated problem and the results obtained from this study would perhaps elucidate a definite
answer in future. The results are presented here in this thesis. The specific scientific objectives for this thesis are explained thoroughly in Chapters 5, 6, 7 and 8.

The leukaemic cells are non-anchorage dependent cells which mean that they need to be immobilized onto a specialized 3-D structure for cell culturing or the production of cell products. The reasons why these type of cells need to be immobilized on 3-D porous structure is as follows: (1) to mimic a 3-D microenvironment of native bone marrow niches that produces leukaemic stem cells, (2) the conventional 2-D cultures cannot support long-term primary leukaemic cell growth without the support of stromal cells and the used of high concentration (µg/ml) of exogenous cytokines. These cytokine concentrations do not correspond to those found naturally in the BM, in the range of pg/ml [6, 7] and (3) to increase and stabilize the growth of leukaemic cells for a long period of time (up to 4 weeks) by providing biosignalling recognition template on the material surface and to give a mechanical support between the cell and material. To do so, the receptor-ligand interaction through the physical adsorption of certain ECM proteins onto the biomaterial surface is essential. For that reason, an ideal environment of protein-coated 3-D scaffold is needed to mimic the microenvironment of bone marrow niches \textit{in vitro}. The scaffolds were coated with two main bone marrow extracellular matrix proteins (e.g., collagen type I or fibronectin) to support all the cell functionalities (e.g., attachment, proliferation and differentiation).

In Chapter 2, a review of current research and background information is presented and separated into several main sections: biopolymers, properties of PHB and PHBV, sustainable production/economic feasibility, scaffolds for tissue engineering and leukaemias (acute myeloid leukaemia and chronic lymphocytic leukaemia). Chapter 3 introduces the aims and objectives together with the hypothesis generated at the beginning of the study. Materials and methodologies used in the investigation are presented in Chapter 4, which includes the technique of protein coating on the 3-D scaffolds for making an ideal environment for cellular growth. In Chapter 5, the two main polymers initially selected are introduced and fabrication and material/structural characterisation methods to produce and analyze them are detailed. Polymers selected in this study are microbial PHB and PHBV copolymers with hydroxyvaleric acid (HV) contents of 12% which are herein described as PHB and PHBV (12%). PHB and PHBV are the most widely used of the poly(β-hydroxyalkanoates) (PHA) family of thermoplastic aliphatic polyester. Initially, the preliminary cell-biomaterial affinity study on the porous 3-D scaffolds with an improved thickness greater than 1 mm (in relative to the conventionally made PHA matrices) is tested with AML cell line (HL-60) for 2 weeks culture period. Chapter 6 describes on the physico-chemical modifications (surface treatment) using the alkaline and rf-O$_2$ plasma treatment to increase the surface hydrophilicity. The selection of suitable treatment to affect on the cell-biomaterial affinity study of the chronic lymphocytic leukaemia (CLL) cell line (RL) for 2 weeks culture period is presented. Chapter 7 offers the
comparison between the 2 treated polymeric scaffolds coated with 2 main bone marrow ECM proteins (collagen type I or fibronectin), untreated and unmodified scaffolds seeded with CLL cell lines (RL and JeKo-1) for 6 weeks culture period. The results of the most suitable protein-coated scaffolds are presented. Chapter 8 includes the generation of a valid 3-D culture system using human primary leukaemia cells and the expansion of acute myeloid leukaemia blood mononuclear cells (MNCs) without the addition of exogenous cytokines. Finally, the main conclusions of the thesis are drawn in Chapter 9 and future work is identified.
2.1 Biomaterials for Tissue Engineering Applications

A biomaterial is a “material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body” [8]. Over the past fifty years, biocompatible materials have been used widespread in biomedical and tissue engineering applications and can now be considered “third-generation biomaterials” as it helps the body heal itself [9]. Initially, biomaterials were chosen because of their biological inertness, the goal was to minimize the body’s immune response to the foreign material (i.e., metallic implants were used for skeletal injuries) [10]. Though this goal is still valid today, scientists have come to understand that complete biological inertness is a synonym to non-recognition by the body. This lack of biological recognition is often accompanied by fibrous tissue encapsulation and chronic inflammation which in turn compromise the mechanical performance and long-term biocompatibility of the prosthesis. Thus, second-generation biomaterials were developed seeking to tailor or enhance biological recognition in an attempt to improve the biomaterial-body interface.

Second generation biomaterials used bioactive components that were specifically designed for use within the human body and promoted specific beneficial responses by the surrounding tissues. Two very typical examples of these components are synthetic hydroxyapatite and Bioglass®. They were used as porous scaffolds (synthetic hydroxyapatite), coatings or powders, and by the mid-1990s these new bioactive materials had attained clinical use for various dental and orthopaedic applications. The biomaterial-body interface problem was also addressed by exploiting resorbable materials and thus could eliminate such problem. For that reason, another class of second-generation biomaterials was introduced which is resorbable polymers. Resorbable polymers are the main example of these resorbable materials, namely polylactic, polyglycolic acid and polyhydroxyalkanoates which decompose hydrolytically into biological accepted molecules, H₂O and CO₂. They are used as sutures, screws in orthopaedics and in controlled-release drug-delivery systems. In these devices, cytostatic agents, cytotoxic agents, antithrombotic agents and/or anti-inflammatory agents are incorporated within resorbable polymeric matrices [10]. Therefore, the creation of third-generation biomaterials is to promote or inhibit specific cell activities through the combination properties of bioactive and bioresorbable [11]. Currently, biomaterial research efforts involve the development of materials that promote an ‘appropriate host response for a given application’ [12]. For example, artificial tissues being fabricated by placing cells within scaffold materials which help guide cell proliferation and differentiation. Typically, biomaterials can be divided into: polymers, metals and ceramics. Polymers classify as the largest class of biomaterials. The polymers used in
biomedical and tissue engineering scaffold fabrication can be divided into broad categories of synthetic, biodegradable synthetic and naturally derived polymers (biopolymers) [13], with a middle ground of semi-synthetic materials rapidly emerging [14]. Most of the biocompatible materials commonly in use in tissue engineering are adapted from other surgical implantation/uses, such as sutures, haemostatic agents and wound dressings [15]. Meanwhile, naturally derived polymers (biopolymers) can be of both plant and animal origin. Some examples of natural polymers derived from plants are lignocellulosic (biofibres), sodium alginate or natural rubber. Examples of those derived from animals are collagen, chitosan or gelatin. Natural polymers offer the advantage of biological recognition, which reduces problems such as antigenicity, cytotoxicity and indiscriminate protein adsorption. The disadvantages of using the natural polymers are they often require chemical or physical pre-treatment to enhance their material properties (e.g., resistance to enzymatic degradation) so that degradation rate could be reduced by providing sufficient time to the cell to regenerate a complete tissue architecture guided by the scaffold. These treatments however may have the toxic effects and affect cell growth (i.e., cross-linking with glutaraldehyde). Moreover, natural polymers may also include pathogenic impurities, variations in batch [16] and in general offer low reproducibility. Biodegradable synthetic polymers, on the other hand, offer high reproducibility and the possibility of large-scale production, as well as controlled mechanical and biodegradability properties through the usage of inorganic salts [13]. They lack, however, biological activity (cell-biomaterials recognition) and may be very hydrophobic. Despite of that, this type of polymer offers an advantage over naturally derived polymers in that they can be tailored to give a wide range of properties and which are more predictable [17]. In particular, many investigations have concentrated on synthetic biodegradable polymers that are already approved by the food and drug administration (FDA). The most common biodegradable synthetic polymers being used or studied include polyhydroxyalkanoates (PHAs), polylactic acid (PLA), polyglycolic acid (PGA), polyanhydrides, polyfumarates (PF), polyorthoesters, polycaprolactones (PCL) and polycarbonates (PC) [18]. One of the materials mentioned above used in this thesis are PHAs, in particular PHB and PHBV. This polymeric biomaterial has then been shaped and processed into three-dimensional (3-D) porous scaffolds to mimic the natural extracellular environment of bone marrow, enabling the study of abnormal haematopoiesis and providing a potential platform for drug discovery and therapeutic applications in vitro. This polymeric material will be described in detail in the following sections.

2.2 Polymer of Microbial Origin Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are a class of natural polyesters that are produced by numerous organisms as an internal carbon and energy storage, as part of their survival
mechanism [19]. Poly(β-hydroxybutyrate) (PHB), synthesized from *Bacillus megaterium*, was first mentioned in the scientific literature in 1925 by the French scientist Lemoigne [20]. He reported this bacterium can literally accumulate an intracellular homopolymer that consisted of 3-hydroxybutyric acids which were linked through ester bonds between the 3-hydroxyl group and the carboxylic group of the next monomer. Bacterially synthesized polyhydroxyalkanoates (PHAs) have attracted much attention because they can be produced from a variety of renewable resources, truly biodegradable and highly biocompatible thermoplastic materials [20]. Although a great variety of materials of this family can be produced, the use of PHAs in tissue engineering has been mainly restricted to PHB and poly(hydroxybutyrate-co-valerate) (PHBV) [2, 21]. To date, only several PHA (Figure 2.1) including poly(3-hydroxybutyrate) (PHB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), poly(4-hydroxybutyrate) (P4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) and poly(3-hydroxyoctanoate) (PHO) are available in sufficient quantity for application research [22].

Often the production of PHAs in general is found in combination to a shortage in a non-carborneous nutrient, e.g., nitrogen. Since the discovery of PHB more than 90 genera of archae (extremophiles: halophiles (salt lovers), thermophiles (heat lovers), and acidophiles (acid lovers)) and eubacteria (gram+ and gram−) have been detected in aerobic and anaerobic habitats, they are able to produce PHAs in wide range of molecular weight [23]. Moreover, types of bacterium and growth conditions determine the chemical composition of PHAs and the molecular weight which typically ranges from $2 \times 10^5$ to $3 \times 10^6$ Da [24, 25]. Today, PHAs are separated into three classes: short chain length PHA (scl-PHA, $C_3$ to $C_5$), medium chain length PHA (mcl-PHA, $C_6$ to $C_{14}$), and long chain length PHA (lcl-PHA, $> C_{14}$) [26].

![Figure 2.1: General molecular structures of polyhydroxyalkanoates.](image)

**Figure 2.1:** General molecular structures of polyhydroxyalkanoates. $m = 1, 2, 3$, yet $m = 1$ is most common, $n$ can range from 100 to several thousands. $R$ is variable (side chain). When $m = 1$, $R = CH_3$, the monomer structure is 3-hydroxybutyrate, while $m = 1$ and $R = C_3H_7$, it is a 3-hydroxyhexanoate monomer [21].

Research over the past 20 years focused on the substrate specificity of PHA polymerases. It was found that the supply of cells with a particular fatty acid is frequently reflected in the monomeric composition of the PHA. To date, more than 100 different monomers have been reported as PHA constituents [27, 28] but only few of these PHAs were produced in large quantities [29]. As a consequence little is known about the chemical and mechanical properties of the polymers. To date, PHA monomers with straight, branched, saturated, unsaturated, and also aromatic monomers were found. Of special interest are functionalized groups in the side
chain that allow further chemical modification, e.g., halogens, carboxyl, hydroxyl, epoxy, phenoxy, cyanophenoxy, nitrophenoxo, thiophenoxy and methylester groups \[28, 30\]. The length of the side chain (R) and its functional group considerably influence the properties of the bioplastic, e.g., melting point, glass transition temperature and crystallinity (stiffness/flexibility). Also the average molecular weight and the molecular weight distribution are dependent on the carbon source \[28\].

### 2.2.1 In Vitro Degradation of Polyhydroxyalkanoates (PHAs)

Degradation properties of a scaffold are of crucial importance in the long-term success of a tissue-engineered cell/polymer construct. The rate of degradation may affect many cellular processes including cell growth, tissue regeneration, and host response \[31\]. For that reason, *in vitro* degradation of the polymers is carried out to understand how these polymers degrade and how their physical and chemical properties change with hydrolytic degradation. In general, the *in vitro* degradation of PHAs usually happens in multistage process \[32\]. In the first stage, spanning a few weeks, the amorphous phase of the polymer begins to degrade. This was because the crystalline regions of the polymer were impermeable to water. Therefore, a hydrolysis process is only restricted to the amorphous regions of the polymer and to the fringes of the crystalline region \[33\]. Next, the crystalline part of the polymer begins to degrade; this resulted in the formation of monomers, dimers, and tetramers. Simultaneously, the molecular mass also decreased. As time progresses, the degradation process developed, and the polymer lost its mass. This hydrolytic degradation of PHAs has been known to be a slow process when compared to the enzymatic hydrolysis of PHAs \[32, 34\]. This is because of the relatively high crystallinity of PHAs, which leads to an increased impermeability of water into the crystalline regions \[35\] and is also due to the hydrophobic nature of long alkyl pendant chains \[36\].

Moreover, besides crystallinity and hydrophobic nature, there are other factors that affect the biodegradability of PHAs such as stereoregularity, molecular mass, monomeric composition and sample thickness \[32\]. Studies have been carried out by Mochizuki and Hirami \[37\] and Tokiwa and Calabia \[38\] to show that the biodegradation of PHAs was influenced by the chemical structure (i.e., the presence of functional groups in the polymer chain, hydrophilicity/hydrophobicity balance) and the presence of ordered structures, such as crystallinity, orientation, and morphological properties. Generally, the degradation of the polymer decreases with the increase of highly ordered structure, that is, increased crystallinity.

In addition, form of the fabricated polymer also has an effect to the degradability. Study carried out by Grizzi *et al.* \[39\] have shown that with different thickness of plates (2 mm thick), films (0.3 mm thick), microspheres (0.125 to 0.25 mm diameter) and beads (0.5 to 1.0 mm diameter), the plates degraded the fastest. Thick samples of these polyester polymers can lead to
a heterogeneous degradation which is a combination of surface erosion and bulk degradation. Heterogeneous degradation may be ascribed to two phenomena [13]: (1) Random hydrolytic ester cleavage (de-esterification process) either on the outer surface or inside the thick scaffolds; (2) Weight loss through the diffusion of oligometric species from the bulk material into solvent media. The oligometric compounds from the outer surface are easy to diffuse into the solvent as compared from the inside of the thick material surface. However, in central, autocatalytic effect will make the degradation even faster than the outer surface which could leads to a premature structural failure due to the accumulation of carboxylic acid functional groups.

2.3 Sustainable Production of Polyhydroxyalkanoates (PHAs)

2.3.1 Introduction

Malaysia is one of the world leaders in the production and export of crude palm oil. In Malaysia, the oil palm industry has contributed vastly towards the country’s economic well being. During the economic crisis in the late 1990s, the industry helped to cushion the impact of the economic downturn through its export-oriented activities which provided the much needed foreign exchange for the country. Crude palm oil (CPO) production has increased from only 1.3 million tonnes in 1975, to 4.1 million tonnes in 1985 and 7.8 million tonnes in 1995 to 17.56 million tonnes in 2009 [40].

The Malaysian palm oil industry has created various products as a consequence of the cultivation of oil palm and the production of the main product (palm oil) and secondary products (palm kernel oil and cake). The oil palm products are employed in numerous food and non-food applications [41]. In 2009, there were 418 crude palm oil mills, 59 refineries, 57 downstream industries and 18 oleochemical plants. The Malaysian Palm Oil Board’s long-term programme is to establish a biodiesel plant that will produce methyl ester (biodiesel) which can be used to replace petroleum diesel. There were 28 biodiesel plants with a production of 2.7 million tonnes per year methyl ester, respectively [40]. Another potential revenue generator is to convert the large quantity of biomass (13.2 million tonnes dry weight) into added value products [42]. However, this important economic activity generates an enormous amount of liquid effluent from the milling processes [43]. Palm oil mills with the wet milling (grinding) process accounted for the major production of waste [44]. Hence, the increase in number of mills will generate more environmental problems. In general, the palm oil milling process can be categorized into a dry and a wet (standard) process. The wet process of palm oil milling is the most common and typical way of extracting palm oil, especially in Malaysia. According to the industrial standard, the milling process produces wastewater in the range 0.44 to 1.18 m³/tonne fresh fruit bunches (FFB) with the average figure of 0.87 m³/tonne FFB. It is estimated
that for each tonne of CPO that is produced, 5 to 7.5 tonnes of water are required, and more than 50% of this water ends up as palm oil mill effluent (POME) [45]. It has been reported that for every tonne of the CPO produced, about 3.5 m$^3$ of POME is generated, which indicates that with some 500 palm oil mills, more than 17.5 million tonnes of CPO is produced annually. It is estimated that about 61.25 million m$^3$ of POME is generated from the palm oil industry annually. POME is an oily wastewater generated by palm oil processing mills and consists of various suspended components. On average, for each tonne of FFB processed, a standard palm oil mill generated about 1 tonne of liquid waste with biochemical oxygen demand (BOD) 27 kg, chemical oxygen demand (COD) 62 kg, suspended solids (SS) 35 kg and oil and grease (O&G) 6 kg [46]. The composition and characteristic of the raw POME obtained from a local palm oil mill factory is summarized in Table 2.1.

### Table 2.1: Composition and chemical properties of raw POME.

<table>
<thead>
<tr>
<th>Composition [47]</th>
<th>% content</th>
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<tbody>
<tr>
<td>Water</td>
<td>94.2 to 95.3</td>
</tr>
<tr>
<td>O&amp;G (oil and grease)</td>
<td>0.7 to 0.8</td>
</tr>
<tr>
<td>Total suspended solids</td>
<td>4 to 5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameters [46]</th>
<th>Units</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>-</td>
<td>4.8 ± 0.21</td>
</tr>
<tr>
<td>Total suspended solids</td>
<td>mg/L</td>
<td>35,000 ± 200</td>
</tr>
<tr>
<td>Turbidity (cloudiness/haziness)$^\Psi$</td>
<td>NTU</td>
<td>21,000 ± 300</td>
</tr>
<tr>
<td>COD</td>
<td>mg/L</td>
<td>65,000 ± 800</td>
</tr>
<tr>
<td>BOD</td>
<td>mg/L</td>
<td>27,000 ± 800</td>
</tr>
<tr>
<td>O&amp;G (oil and grease)</td>
<td>mg/L</td>
<td>8,000 ± 300</td>
</tr>
<tr>
<td>TOC (total organic carbon)</td>
<td>mg/L</td>
<td>12,300 ± 570</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>mg/L</td>
<td>142 ± 19</td>
</tr>
<tr>
<td>Ammonia</td>
<td>mg/L</td>
<td>62 ± 10</td>
</tr>
</tbody>
</table>

*Note: values represent means of triplicate determination.

$^\Psi$ A nephelometer, measures how much light is scattered/ reflected by suspended particles in the water. The greater the scattering, the higher the turbidity.

### 2.3.2 The Science of Biopolymers

The usage of conventional plastics has resulted in environmental degradation [48]. The production of biodegradable polymers is seen to be a viable alternative with the increasing environmental pressure to replace conventional plastics. The problem that is faced by industry is the high production costs of biopolymers. Biodegradable polymers derived from polyhydroxyalkanoates (PHAs) are considered to be good candidates for biodegradable plastics due to their large range application and capability of being produced from renewable resources [49, 50]. These materials have attracted interest because of their potential use as biodegradable alternatives to petroleum-based synthetic plastics such as polypropylene (PP) and polyethylene (PE). Polyhydroxyalkanoates (PHAs) are mainly produced by microbial fermentation processes,
and a major challenge is to reduce their production costs [49]. A feasibility study using fermentative volatile fatty acids (VFAs) as carbon source to synthesise PHA by activated sludge (treating sewage and industrial waste waters using air and microorganisms to reduce the organic content of the sewage) was carried out to simultaneously reduce the production cost of PHAs and disposal amount of organic wastes [51]. Several efforts have been investigated to produce PHAs by microbial fermentation on organic waste (palm oil mill effluent (POME), olive oil and kitchen waste). The production of biodegradable polymers from oil palm industry can be seen as beneficial to the environment as well as contributing to sustainable development [52, 53]. Until recently, the remaining 90% discharged (empty fruit bunches, fibres, fronds, trunks, kernels, POME) was considered as waste and either burned in the open air or left to settle in waste ponds [51, 54]. By utilizing the POME and empty palm oil fibre bunch (EPFB) as carbon source and support matrix, the disposal of POME that needed further treatment could be reduced. Although POME consists of high organic acids and is suitable to be used as a carbon source, POME is usually present in a complex form that cannot directly be utilised by PHA producing bacterial species for PHA synthesis. Typically, raw POME is difficult to degrade because it contains significant amounts of oil (tryacylglycerols) and degradative products such as diacylglycerols and monoacylglycerols and fatty acids [52, 55]. The fatty acids composition (C_{12} to C_{20}) of each of this fraction are different from one another and contribute to a high value of pollution load in POME. Therefore, anaerobic treatment has been proposed to reduce the POME characteristics. It is one of the naturally occurring processes involving decomposition and decay, in which complex organic matters are broken down into their chemical constituents. Hydrolysis and acidogenesis are the first step to convert the wastes to short-chain VFAs (i.e., acetic, butyric and propionic acids) as shown in Table 2.2. After that, the VFAs will be utilised by PHA-producers for PHA production [51, 56].

### Table 2.2: VFAs composition (%) of fermented POME in anaerobic reactor [46].

<table>
<thead>
<tr>
<th>Systematic name</th>
<th>Generic/trivial name</th>
<th>Compound</th>
<th>% constituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanoic Acid</td>
<td>Acetic acid</td>
<td>CH₃COOH</td>
<td>57</td>
</tr>
<tr>
<td>Butanoic Acid</td>
<td>Butyric acid</td>
<td>C₃H₇COOH</td>
<td>33</td>
</tr>
<tr>
<td>Propanoic Acid</td>
<td>Propionic acid</td>
<td>C₃H₅COOH</td>
<td>6</td>
</tr>
<tr>
<td>Pentanoic Acid</td>
<td>Valeric acid</td>
<td>C₅H₉COOH</td>
<td>4</td>
</tr>
</tbody>
</table>

#### 2.3.3 Biopolymer Production using POME

The price of PHAs is mainly dependent on substrate costs, accounting for about 40% of the total production cost [57]. In the last decade, a variety of low cost carbon substrates (e.g., starch, tapioca hydrolysate, whey and molasses) have been tested for PHA production to reduce the production cost. POME can be considered as an alternative to the above mentioned carbon substrates with no cost of using it for PHA production. The production of VFA and PHA using
POME as substrates are listed in Table 2.3. According to Ali et al. [58], with a content of 50% PHA in the dried cells and 2% dissolved in the chloroform, the calculated minimum cost for obtaining PHA from POME is below USD2/kg. By increasing the PHA content in the cell from 50% to 80%, the unit cost of PHA could be slightly reduced; whereas an increase in the amount of PHA dissolved in chloroform from 2% to 5% would result in a remarkable reduction of the PHA cost to less than USD1/kg. The above mentioned price was in line with the latest market price which is quoted at about €1.50/kg (~USD2/kg) produced by one of the largest volume manufacturers (Mirel™) [49]. This price was much higher than that of starch polymers and other bio-based polyesters due to high raw material costs (30% to 40% of total cost), small production volumes and high processing costs (particularly for purification) [49].

Nevertheless, POME is usually presented in complicated forms that cannot be directly reused by PHA-producing species such as *Ralstonia eutropha*, a representative bacterium for PHA synthesis [52]. It was proposed that an anaerobic treatment of POME could be coupled with PHA production using heterotrophic bacteria (bacteria that use organic (carbon-containing) compounds as a source of energy and carbon) to reduce PHA production costs [46]. According to Ali et al. [59], it was critical to maintain the pH at 7 in the anaerobic treatment of POME sludge in the first stage of the process, in order for only acetic and propionic acid to be produced and not formic acid and biogas. With increasing concentrations of formic acid (for a pH maintained below 4), the PHA yield and content in *Rhodobacter sphaeroides* IFO 12203 dropped from 0.50 g/g and 67% to 0.21 g/g and 18%, respectively. Hassan et al. [60] later found that the presence of sludge in the anaerobically treated POME inhibited PHA accumulation by *R. sphaeroides* IFO 12203. Based on the studies, it seems that the PHAs are being produced in a POME without sludge as opposed to a treated POME with sludge. Moreover, a low concentration of ammonium would accelerate the PHA production in a synthetic waste with an organic acid profile which was observed during POME treatment. However, Hassan et al. [60] found that addition of ammonium and phosphate to anaerobically treated POME was required to maintain the cell activity and production of PHA since neither ammonium nor phosphate was present in the anaerobically treated POME. In total, the organic acid concentrations obtained from anaerobically treated POME were too low [51, 59] for it to be reused as raw material in the production of PHA on an industrial scale. The underlying reason was that this would require a production reactor with a much larger size than that of a reactor for normal bioplastic production (lab scale anaerobic reactor (organic acid production): 19 L; lab scale aerobic reactor (PHA production): 6 L) [46]. The organic acids in the anaerobically digested POME could be concentrated by evaporation for use as substrates in the fed-batch non-sterile PHA fermentation system using *R. eutropha* ATCC 17699. Although the proposed overall zero emission system appeared to be practical, major drawbacks were found, including the rather low yield and productivity of PHA by *R. eutropha* when the concentrated organic acids from POME were
used as compared to synthetic organic acids. This could be due to the high presence of ammonium (1.5 g/L) or other compounds in the anaerobically digested POME concentrate [61].

Md. Din et al. [5] proposed the suitability of using mixed cultures (mixed with different bacteria strains) to produce PHA in POME since most prokaryotes are capable of PHA production. The study noted that by using mixed cultures and POME, different types of PHA-constituents could be obtained. The harvesting of these PHA-constituents was more reliable for use as biodegradable plastics material as opposed to a single PHA-constituent.

Table 2.3: Various products in bioprocesses using POME as substrates.

<table>
<thead>
<tr>
<th>Product</th>
<th>Microorganism, fermentation substrate and fermentation conditions</th>
<th>Maximum production</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>VFA</td>
<td>Mixed cultures, POME + palm oil sludge. 30 °C, pH was controlled at 7, SBR (sequenced batch reactor), 24 h</td>
<td>7.8 g/L</td>
<td>Ali et al. [59]</td>
</tr>
<tr>
<td>VFA</td>
<td>Mixed cultures, POME + palm oil sludge with a ratio of 1:1, 300 rpm, 30 °C, pH was controlled at 7, stirred tank bioreactor fermentation, 84 h</td>
<td>10 - 14 g/L</td>
<td>Yee et al. [62]</td>
</tr>
<tr>
<td>VFA</td>
<td>Mixed cultures, POME + palm oil sludge, pH was controlled at 7, SBR, 96 h</td>
<td>10.27 g/L</td>
<td>Cheong et al. [63]</td>
</tr>
<tr>
<td>Single constituent PHA</td>
<td>Rhodobacter sphaeroides IFO 12203, Synthetic waste based on organic acid profiles obtained during POME treatment. 30 °C, pH was controlled at 7, photobioreactor fermentation, ≈ 200 h</td>
<td>≈ 4.0 g/L</td>
<td>Ali et al. [59]</td>
</tr>
<tr>
<td>Single constituent PHA</td>
<td>Rhodobacter sphaeroides IFO 12203, anaerobically digested POME, 30 °C, pH was controlled at 7, photobioreactor fermentation</td>
<td>&gt; 2.0 g/L</td>
<td>Ali et al. [58]</td>
</tr>
<tr>
<td>Single constituent PHA</td>
<td>Alcaligenes eutrophus H16(ATCC 17699), standard medium with feeding of acetic acid obtained from anaerobically digested POME (single VFA), 400 rpm with aeration rate of 0.75 L/min, 30 °C, pH was controlled at 7, stirred tank bioreactor fermentation, 17 h</td>
<td>1.8 g/L</td>
<td>Hassan et al. [60]</td>
</tr>
<tr>
<td>Single constituent PHA</td>
<td>Ralstonia eutropha ATCC 17699, concentrated organic acid from anaerobically digested POME (100 g/L of total acids with acetic:propionic = 3:1), 400 rpm with aeration rate of 0.75 L/min, 30 °C, pH was controlled at 7, bioreactor fermentation, ≈ 65 h</td>
<td>≈ 6.25 g/L</td>
<td>Hassan et al. [61]</td>
</tr>
<tr>
<td>Multiple constituents PHA</td>
<td>Mixed cultures (mixed with different strains), high concentration of POME with 490 COD/N ratio (g COD/g N) and 160 COD/P ratio (g COD/g P), 1000 rpm with aeration rate of 1.5 L/min, 30 °C, pH was controlled at 7, SBR</td>
<td>24.24 g/L</td>
<td>Md. Din et al. [5]</td>
</tr>
</tbody>
</table>
A type of mixed culture was maintained in a SBR and a high concentration of POME proposed for this system in order to generate autotrophic (an organism that produces complex organic compounds (such as carbohydrates, fats and proteins) from simple substances present in its surroundings (light, inorganic (CO₂) and organic matters)) rather than heterotrophic bacteria (depending only on organic matters as an energy source) in the production of PHA. However, the average PHA production by using POME could only reach 44% of the CDW, indicating that an optimisation of the PHA sludge content must be carried out by varying the oxygen rate, feeding regime or transient conditions (passing with time before changing to other condition).

As we have discussed in detail on how POME could come out with a promising biopolymer (PHAs) for many applications, the justification on why this material was used is to promote the usage of POME in producing PHAs via microbial fermentation process as added value materials for the study of leukaemia in a 3-D cell culture system. This will be discussed in detail in the next chapter onwards. The added value here is a rough estimation based on the above mentioned information and current market price. The rough estimation of PHAs revenue per annum that could be procured is shown in Table 2.4.

<table>
<thead>
<tr>
<th>Table 2.4: Rough estimation of PHAs revenue per annum on utilizing POME.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw POME generated annually [45]</td>
</tr>
<tr>
<td>Concentrated POME (90% H₂O removal)</td>
</tr>
<tr>
<td>Maximum PHA production (24.24 g/L) [5]</td>
</tr>
<tr>
<td>Current market price (Mirel™)</td>
</tr>
<tr>
<td>Value added of PHAs production (revenue)</td>
</tr>
</tbody>
</table>

2.4 Polymers for Tissue Engineering Applications: Polyhydroxyalkanoates (PHAs)

With all the advantages and disadvantages comprising from both naturally derived and synthetic biodegradable polymers as described in the earlier section, one of the synthetic biodegradable polymers that could compromise with all of the major requirements on fabricating three-dimensional (3-D) porous scaffold (i.e., porosity, mechanical strength, surface properties, biocompatible, etc.) is the naturally synthesized polyhydroxyalkanoates (PHAs). In this study, PHAs are used for tissue engineering of 3-D cell culture system due to its acceptable biodegradability [64], biocompatibility [25] and resemble as polypropylene (PP) in which offering a wide range of mechanical properties [2]. PHAs have already been studied to some extent for tissue engineering applications mainly for scaffold materials in combination with ceramic materials [65] as a vehicle for drug delivery [66] and also as a material for cardiac
tissue engineering [21]. The potential use of PHAs in tissue engineering is illustrated in Figure 2.2.

Figure 2.2: Role of PHAs in Tissue Engineering [2].

To be useful as tissue engineering scaffolds, PHAs must possess the five key properties. They must be (1) biocompatible, (2) support cell growth, (3) guide and organize the cells, (4) allow tissue ingrowth and (5) ultimately, be able to degrade to non-toxic products [2]. If these key properties are met by the PHAs, then the additional benefits of using PHAs in tissue engineering are huge. For example, unlike many other degradable polymers, the properties of PHAs can be tailored with a wide range of building blocks, various treatments can be used to attach bioactive factors, alter surface and mechanical properties and a number of methods can be used to provide a range of degradation rates [2]. For instance, the brittleness of PHB was improved through copolymerization process of  \( \beta \)-hydroxybutyrate with  \( \beta \)-hydroxyvalerate [20] which was first commercialized in 1990. The copolymerization produces less crystalline, more flexible and more readily processable materials than pure PHB. Depending on the requirements of different applications, PHA can be either blended, surface modified or combined with other polymers, enzymes or even inorganic materials such as hydroxylapatite to further adjust their mechanical properties or biocompatibility [21]. In addition, tissue engineers have determined that the surface structure is also an important factor. Porous surfaces can be produced by the leaching technique, which is done by blending of PHA with a salt that can be washed out with
water. The surface of PHA materials can be rendered more hydrophilic as was shown by the treatment of P(HB-co-HV) with allyl alcohol gas plasma that led to an increase of wettability [67].

Apart from tailoring its physico-chemical properties, the biocompatibility of two PHA polymers, namely, PHB and PHBV have been studied by a number of different research groups. For instance, the polymers have been reported not to induce any prolonged acute inflammatory responses when implanted in vivo (subcutaneously into the neck folds of mice) for up to 5 weeks in the form of tablet [68]. Separately, it has also been reported that when a highly porous well-interconnected PHBV structure was seeded with fibroblasts, it sustained a cell proliferation rate similar to that observed in collagen sponges for 35 days, with a maximum cell density being observed on day 28 [69]. An interesting aspect of PHA scaffolds is the fact that the tissue-engineered cells can be implanted together with the supporting scaffold [2]. This approach was exemplified by Sodian and coworkers [70] who used scl-PHA for the fabrication of a tri-leaflet heart valve scaffold. A porous surface was achieved with the salt leaching technique resulting in pore sizes between 80 and 200 μm. The scaffold was seeded with vascular cells from ovine carotid artery and subsequently tested in a pulsatile flow bioreactor. The cells formed a confluent layer on the leaflets. The authors pointed out that the whole scaffold was molded completely and that a simple melting process was used to fix the leaflets to a model conduit.

2.5 Scaffolds Requirement for Tissue Engineering Applications

In tissue engineering, as in many other engineering fields, the design of the artificial structural may be as crucial as the material it is made of. As was mentioned earlier, the scaffolds, cells and signals are the main building blocks of tissue engineering. Moreover, scaffolds must provide the cells with the appropriate physical support (i.e., porosity, pores interconnectivity and pores size distribution), chemical signals (i.e., biosignaling components from ECM proteins) and mechanical strength to allow them to generate specific cells and tissues. Scaffold design and development is mainly an engineering challenge and is in fact one of the main goals of this study. In scaffold-based TE strategies, a key component of the scaffold that serves as a template for cells interactions and for the formation of the extracellular matrix (ECM) is providing structural support to the newly formed tissue. A temporary 3-D scaffold mimicking the physiological functions of the ECM is critical to preserve cells ability to differentiate into their native phenotypes and to constitute a structural template to fill the tissue lesion. Tissue engineering scaffolds are meant to be colonized by cells and should transmit the chemical and physical signal necessary to ensure adequate cells and tissues growth. An ideal tissue engineering scaffold should fulfill a series of requirements which are as follows [2, 71]:
1. The scaffold should be non-immunogenic, non-toxic, biocompatible and easily manufactured.

2. The scaffold material has the ability to biodegrade. Its degradation products should not be toxic and should be easily eliminated from the implantation site by the body (i.e., secretion via urine or respiratory system; \( \text{H}_2\text{O} \) and \( \text{CO}_2 \)).

3. Macrostructure: Macro and micro-structural properties affect not only cells survival, signaling, growth, propagation and reorganization but play also a major role in modelling cell shape and gene expressions, both related to cell growth and preservation of native phenotypes [72, 73].

4. Porosity and pore interconnectivity: Scaffolds should possess interconnected open pore geometry and spread porosity (usually exceeding 90%) with a highly porous surface and microstructure. This would allow in vitro cell adhesion, ingrowth and reorganization and would provide the necessary space for neovascularization in vivo [74]. Pore interconnectivity directly influences the diffusion of physiological nutrients and gases to cells as well as the removal of metabolic waste and by-products from cells [72]. The diverse nature of tissue architecture requires different microenvironments for regeneration, including the employment of scaffolds with optimal pore sizes. In addition, since the mechanical strength decreases by increasing the porosity, the void volume in scaffolds for load-bearing tissues must be tuned to allow both the accommodation of the large number of cells and the maintenance of the structural strength required [75].

5. Pore size and surface area: Moreover, a good distribution of the large number of cells requires high internal surface area to volume ratios [72]. Since pore size and internal surface area are related linearly, a compromise between both properties has to be determined depending on the scaffold's application. When the pore diameter is too small, cells may provoke pore occlusion (blockage of pore) and prevent cellular penetration within the scaffold.

6. Surface properties: Scaffold surface properties such as morphology, hydrophilicity, surface energy and charge are factors controlling in vitro cell adhesion, migration, phenotype maintenance and intracellular signaling as well as in vivo cell recruitment and healing at the tissue-scaffold interface [76-78]. Cell response to the biomaterial is not mediated by a direct contact but rather through an interfacial layer formed on material surface once it is in contact with a physiological environment. Such a layer is created as result of non-specific adsorption of ECM proteins. Polymer-surface engineering is a useful tool to improve scaffold multifunctionality and to design biomimetic materials able to interact with the surrounding environment by biomolecular recognition [79]. Moreover, surface modification is critical to elicit specific cellular responses and direct new tissue formation. Bioactive ligands, such as peptides and polysaccharides, may either be physically adsorbed (non-covalent interactions)
or covalently grafted onto the surface (chemically immobilized) or included in the bulk (during the fabrication process) to promote specific cell adhesion. Usually, the bio-molecular recognition of the material by the cells is achieved by incorporating cell-binding peptides in the form of native long chain of ECM proteins such as fibronectin, laminin and vitronectin [80] or more frequently, in the form of short peptide sequences derived from ECM proteins, such as arginine-glycine-aspartic acid (RGD) peptides [81]. The employment of the long chain proteins during adsorption onto the biomaterial surface is the most advantageous thanks to its stability, straightforward synthesis and absence of random folding [81].

7. Mechanical properties: The scaffold should have sufficient mechanical strength during in vitrō culturing to maintain the spaces required for cell ingrowth and matrix formation. Moreover, it must provide sufficient temporary mechanical support, matching the mechanical properties of the host tissue as closely as possible, to bear in vivō stresses and loading. Therefore, the materials must be selected and/or designed with degradation and resorption rate and mechanical properties, such that sufficient structural integrity of the scaffold is retained until the newly grown tissue is able to support loads and stresses and can assure its structural role [82].

2.6 Techniques for Polymeric Scaffolds Preparation

Many fabrication methods as shown in Figure 2.3 have been developed in order to attain these requirements; these methods are briefly described as below.

2.6.1 Solvent-Casting and Particulate-Leaching (SCPL)

The solvent casting and particulate leaching method was developed by Mikos et al. [83] amongst others for polylactic acid (PLA) and polyglycolic acid (PGA) polymers, and several authors have used the method to manufacture composite scaffolds [84, 85]. It consists of dissolving a polymer in a solvent and then adding particles of a leachable porogen (i.e., salt particles, glucose, paraffin spheres, etc.). The mixture forms a thick paste which is left to dry in air or under vacuum until the solvent has evaporated completely. The porogen is then leached out and leaves behind a network of interconnected pores. In the case of composites, the second phase is added with the porogen and remains within the structure after the porogen is leached out. Additionally, a thermal treatment can be used to modulate the crystallinity of the polymer by melting the polymer and controlling the cooling rate. The advantages of the solvent casting method are that it is a simple and fairly reproducible method which does not require sophisticated apparatus. The disadvantages include thickness limitations intrinsic to the particle leaching process, limited mechanical properties and some authors question the homogeneity and
interconnection of the pores in the scaffolds, as well as the presence of residual porogen and solvent [86].

Figure 2.3: Schematic of commonly used techniques for scaffold production: (a) Solvent casting-particle leaching process. (b) Freeze drying process (TIPS - below freezing point < 0°). (c) Supercritical fluid technology. (d) Phase separation technology (TIPS - above freezing point > 0°). (e) Wet spinning process for the production of 3-D polymer scaffold. (f) Electrospinning process. (g) Melt molding technique. A mould filled with polymer powder and porogen component is heated above the polymer glass-transition temperature (Tg) and a pressure (F) is applied to the mixture. Then the porogen is leached out leaving a porous structure. (h) Layout of Solid Free Form (SFF) process. A 3-D computer model, directly designed in CAD software (simplified CAD model) or obtained from a medical imaging technique (bio-mimetic model) is expressed as a series of cross-sectional layers. From the data, the SFF machine fabricates the physical model (Adapted from [74]).

2.6.2 Thermally Induced Phase Separation (TIPS)

Thermally induced phase separation was first applied to PLA scaffolds by Schugens et al. [87] and several authors have applied this technique to composite scaffolds [88, 89]. It consists of inducing a solid-liquid or liquid-liquid of polymer-solvent phase separation. This is done by dissolving the polymer in a solvent and quenching the solution at a certain temperature. The quenching induces a phase separation into a polymer-rich phase and a polymer-poor phase. As the solvent crystallizes, the polymer follows accordingly to the crystallized solvent microstructural template. The solvent must then be removed from the phase separated solutions either by freeze-drying (sublimation process) or by solvent extraction [90]. The solvent leaves behind a microstructural foam of polymer as the solvent is sublimed during the freeze drying
process. The main advantage of the phase separation method is that pore morphology and orientation can be tailored by altering the thermodynamic (quenching temperature) and kinetic parameters of the processing (time of phase separation). Its disadvantages include the use of potentially toxic solvents and a high degree of anisotropy (not isotropic; not in the same direction) of the porosity.

2.6.3 Gas-Foaming (Super Critical Fluid Technology)

The gas foaming process is used to fabricate highly porous foams without the use of organic solvents [91]. Organic solvents may leave residues behind which can have toxic effects in vitro and may cause inflammation in vivo. This high pressure CO₂ gas technique can avoid the use of toxic solvents in scaffold fabrication. The process consists of saturating the polymer mix with gas at high temperatures and pressures. Then, a thermodynamic instability is created by quickly decreasing the temperature and pressure which stimulates the nucleation and growth of pores of gas within the polymer. Gas-foaming yields high porosities (up to 93%) and varying the temperature, pressure and rates of parameter reductions can modulate the pore sizes. The main disadvantage is the resultant scaffolds contain a nonporous surface film with mixed open and closed cell structures (poor interconnectivity) which is not suitable for application as tissue engineering scaffolds [17].

2.6.4 Fibre Bonding

The fibre bonding method was first developed by Cima et al. [92] who produced scaffolds made of polyglycolic acid (PGA) polymer. They took advantage of the fact that PGA was available as sutures and thus in the shape of long fibres. In fibre bonding, two fibre materials can be attached to each other via ‘heat fuse’ or ‘embed’ methods and then one material is dissolved in a selective solvent to obtain the fibre scaffold [93]. Mikos et al. [94] improved the structural stability of the constructs developing a fibre bonding technique in which the PGA fibres are joined at their cross-linking (interconnected) points by ‘sintering’ (bonding via heating) above their melting point temperature. The main advantage of the fibre bonding technique is the very high surface area-to-volume (m⁻¹) ratio which makes them ideal for tissue engineering applications. Although the fibre bonding technique can produce highly porous scaffolds with interconnected pores that are suitable for tissue regeneration, this method involves the use of solvents that could be toxic to cells if not completely removed [95] which could reduce the ability of cells to form new tissue in vivo.
2.6.5 Electrospinning

The electrospinning process uses an electric field to control the formation and deposition of polymer fibres on a substrate. Sheets and cylindrical shapes can be fabricated with this technique. Electrospinning method is reported capable to fabricate polymer fibres range from a few nanometers to hundreds of microns [96]. The ability to co-spin polymers with various additives offers the possibility of functional fibres. PGA and PLA fibres are two commonly used biopolymers used with this technique [97]. They have been used either alone or combined with other biomaterials such as collagen to enhance their biocompatibility.

2.6.6 Solid Free Form (SFF)

Solid free form fabrication (SFF) is a developing technology that enables the fabrication of custom made devices directly from computer data such as computer aided design (CAD), computed tomography (CT) and magnetic resonance imaging (MRI) data [98]. The digital information is then converted to a machine specific cross-sectional format, expressing the model as a series of layers. The file is then implemented on the SFF machine, which builds customer designed 3-D objects by layered manufacturing strategy whereby 3-D objects are fabricated with layer-by-layer building via the processing of solid sheet, liquid or powder material stocks [74]. For example, Chiang et al. [99] have used this technique to improve the in-vitro response of osteoblast cells with porous scaffolds of triblock PEG-PCL-PEG copolymer with predetermined pore size of 400 µm to 420 µm and porosity of 79%. These techniques allow obtaining complex customized scaffolds design, minimal manpower requirement, highly accurate and consistent pore morphology, anisotropic structure, diverse range of processing conditions. Shortcomings include dependence on the processing technique, high temperature, lack of mechanical strength (but much better than salt-leached technique), limited material range and pores are usually huge (suitable for bone tissue growth) [72].

2.7 Surface Engineering: Surface Modifications of Polymeric Porous 3-D Scaffolds

Several surface modifications techniques have been developed to improve wetting, adhesion, and printing of polymer surfaces by introducing a variety of polar groups (by introducing a random and non-specific groups surface rendering), with little attention to functional group specificity. However, when surface modification is a precursor to attaching a bioactive compound (i.e., drug delivery application), these techniques must be tailored to introduce a specific functional group.
2.7.1 Physico-Chemical Modifications (Surface Treatments)

2.7.1.1 Wet Chemical Method

In wet chemical surface modification, a material is treated with liquid reagents to generate reactive functional groups on the surface. This classical approach to surface modification does not require specialized equipment and thus can be conducted in most laboratories. It is also more capable of penetrating porous three-dimensional substrates than plasma and other energy source surface modification techniques [100] and allows for in situ surface functionalization of microfluidic devices. For instance, concentrated sodium hydroxide and sulfuric acid have been used to generate carboxylic acid groups by base and acid hydrolysis of PMMA [101, 102]. Specifically, a 16 h treatment in 10M sodium hydroxide at 40 °C was reported to produce 0.66 nmol/cm² carboxylic acids on PMMA [101]. Unfortunately, these wet chemical methods are non-specific, producing a range of oxygen-containing functional groups. In addition, those which target modification of polymer side chains (as in PMMA ester modification) depend on the side chain surface orientation. The degree of surface functionalization may therefore not be repeatable between polymers of different molecular weight, crystallinity or tacticity [103]. These wet chemical methods also generate hazardous chemical waste and can lead to irregular surface etching [104]. Many of these techniques also require some extended treatment in concentrated corrosive solutions. For these reasons, while useful in the laboratory environment, these wet chemical processes may not be suitable for larger scale (e.g., industrial applications).

2.7.1.2 Ionized Gas Treatments

There are several types of ionized gas treatments (e.g., corona discharge, UV irradiation and flame treatment) but plasma is the most practical one. Plasma is a high energy state of matter, in which a gas is partially ionized into charged particles, electrons, and neutral molecules [103]. Plasma can provide modification of the top nanometer of a polymer surface without using solvents or generating chemical waste and with less degradation and roughening of the material than many wet chemical treatments [104, 105]. The type of functionalization imparted can be varied by selection of plasma gas (Ar, N₂, O₂, H₂O, CO₂, NH₃) and operating parameters (pressure, power, time, gas flow rate) [106]. Oxygen plasma is often used to impart oxygen containing functional groups to polymer surfaces such as PCL [107], PE [108] and PET [109]. In addition to oxygen, carbon dioxide plasma has been used to introduce carboxyl groups on PP and PE [110], PS [111] and air plasma has been used to oxidize PMMA [112]. Ammonia and nitrogen plasmas have been used to impart amine groups to the surface of PTFE [113] and PS [111], respectively. Inert gases (e.g., Argon) can be used to introduce radical sites on the polymer surface for subsequent graft copolymerization. However, with the exception of a recent
development of an atmospheric plasma system condition [114], plasma generation requires a vacuum to empty the chamber of latent gases (e.g., atmospheric gases), which presents complications for continuous operation in a large scale industrial setting. Also, results are difficult to repeat between laboratories as there are many parameters involved to optimize conditions, including time, temperature, power, gas composition/flow/pressure, orientation of reactor and distance of substrate from plasma source [103]. It should be noted that in addition to the monomers and gases intentionally introduced to the plasma chamber, latent (hidden) chemicals from prior users may be present thus posing a risk of contamination. The plasma chamber should therefore be adequately cleaned, for example by oxygen plasma, before introducing polymers for surface modification.

2.7.2 Biological Surface Modifications (Biological Surface Coating)

2.7.2.1 Protein-Surface Interactions

Protein adsorption on polymer surfaces has significant importance in biomedical applications both in vitro and in vivo [103]. The first event that usually occurs when a synthetic material in contact with a cell culture medium containing dissolved proteins is the adsorption of the protein to the surface. Other responses, such as the attachment of cells are secondary and depend on the nature of the adsorbed protein layer. Controlling adsorption from a single protein solution as well as more complex mixtures requires an understanding of (a) protein structure and property, in terms of stability, conformational dynamics and the tendency to aggregate; (b) the polymer surface that allows the introduction of functional groups at the surface to enhance biocompatibility with the biological surface; and (c) analytical techniques that can quantify the protein adsorption. The concept of biological surface modification consists of surface functionalization by imparting polar-containing functional groups through physico-chemical treatment. Next, the treated-surfaces are immobilized with bio-active compounds (e.g., peptide, oligonucleotide, enzyme and antibody) to provide biosignalling recognition surface template for increasing cell-material affinity. The concept is illustrated in Figure 2.4.

![Figure 2.4: The concept of biological surface modification (Adapted from [103]).](image-url)
Much published work on protein adsorption at the solid-liquid interfaces has mainly focused on the amount of protein adsorbed, with the little attention on the protein conformation, orientation and structural changes in the adsorbed layer. The major parameters that may influence protein-surface interactions include surface charge (electrostatic interactions) [115, 116], hydrophobicity/hydrophilicity of the surface [103] or possibly a combination of both parameters. Figure 2.5 illustrates two types of chemical properties that affect protein adhesion. The chemical properties include surface charge (electrostatic interactions) and polarity (hydrophobicity/hydrophilicity) which actually affect (1) the amount of the adsorbed proteins and type of protein adsorbed, (2) protein-binding strength and (3) the adsorbed protein conformational and/or orientation. The above mentioned effects will eventually contribute to the promotion/inhibition of cell growth [103].

![Mechanisms of protein adhesion](image)

**Figure 2.5: Mechanisms of protein adhesion (Adapted from [103]).**

Other factors include electrostatic forces between adsorbed molecules (e.g., electrostatic forces between other adjacent absorbed protein molecules), solvent-solute interactions and the morphology (e.g., surface roughness, pore size and porosity) and chemistry of the solid surfaces (e.g., surface free energy). The adsorption of proteins at the solid/liquid interface is a complex phenomenon that involves the following sequence of steps [117]:

1. Transport of proteins towards the interface.
2. Attachment at the interface.
3. Final molecular structural rearrangement in the adsorbed position.
4. Detachment from the interface.
5. Transport away from the interface.

In protein-surface interaction, the governing factors are determined both by physical state of the material surface in contact with the protein solution, the molecular structure of the protein and the environment of the solution. Work in literature has highlighted a number of biologically active coatings on engineered material surface either from serum-supplemented media [118],
monocomponent ECM proteins in solution [103, 119] and the covalent attachment (grafting/bioconjugations) of the cell-adhesive peptide region of the ECM protein, Arg-Gly-Asp (RGD) [103, 120] where such modified surfaces have applications in bioartificial organs, medical devices and disposable apparatus [103].

The primary aim of coating solid surfaces with adhesion proteins and peptides mentioned above is to provide a biosignaling recognition template on the material surface for cell attachment through receptor-ligand interaction and to provide a strong mechanical contact between the cells and the surface [6, 120]. Coating surfaces with adhesive proteins derived from ECM such as fibronectin, vitronectin and collagen provides an ‘adhesive interface’ between the scaffold materials and the cells that resemble the native cellular milieu (environment), whose organization and production modulates and enhances cell adhesion through transmembrane integrin receptors [103]. The advantage of complete ECM proteins to peptide sequence is that they not only provide the cell-binding sequence for cell adhesion, but also provide secondary interactions with other ECM proteins and growth factors strengthening cell adhesion which results in enhanced cell growth and maturation. Furthermore, ECM proteins being the natural ligands found in in vivo do not cause any harmful side effects. Whilst, short-chain peptides (frequently found in numerous adhesive proteins) have lower binding to cells and selective activity for distinct integrin subtypes and are easily cleaved by enzymes [121]. However, short peptides in particular arginine-glycine-aspartic acid (RGD), do offer some advantages of enhanced stability during immobilization, increased likelihood of retaining proper orientation of binding domains after immobilization and the economic advantage of laboratory synthesis [103]. Native ECM protein tends to be randomly folded upon adsorption to the biomaterial surface such that the receptor binding domains are not always stERICally (related to spatial arrangement of atoms in a molecule) available [81]. There are three major functions for the ECM:

1. They provide structural support to the tissue.
2. They provide substrate for cell adhesion and cell migration.
3. The ECM regulates cellular differentiation and metabolic function, for example by modulating the cell growth through the binding of growth factors.

Meanwhile, the in vivo ECM encompasses three major classes of biomolecules, which provide the complex structural entity surrounding and supporting the cells. These are:

1. Structural proteins (e.g., collagen and elastin).
2. Specialized proteins (e.g., fibronectin and laminin).
3. Proteoglycans (e.g., hydrophilic heteropolysaccharides, glycosaminoglycans (GAGs) -

They form extremely complex high molecular weight components of the ECM.
The natural, ECM-derived polymers have biological properties (e.g., biosignaling mechanism) that synthetic polymers lack, whilst, synthetic polymers are cost-effective, exhibit less batch to batch variability and have physico-chemical properties which are readily modified to suit specific applications. Work in the literature on the 3-D ECM substrates for tissue engineering applications investigated a number of scaffolds that mimic the 3-D structures prepared from natural ECM-derived biopolymers, such as collagen, composite bioabsorbable (e.g., PLA and PGLA) with bioactive ceramic materials (e.g., hydroxyapatite, tri-calcium phosphate and wollastonite) or hybrid combination of natural and synthetic polymers [74]. For instance, the use of nanofibrous scaffolds (obtained by electrospinning) of combined poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and type-I collagen for tissue-specific cells development in 3-D organization. The use of the above mentioned composite scaffolds resulted in acceleration of the adhesion and growth of NIH3T3 cells more effectively than the PHBV nanofibrous scaffold, thus making the former a good scaffold for tissue engineering [122]. Collagen is not the only ECM protein that has been extensively studied or applied in the hybrid biomaterials. Functional biomaterials have been designed utilizing fibronectin adsorption to poly(D,L-lactade) (PDLLA), poly(L-lactic-co-glycolic acid) PLGA, polystyrene (PS), poly(methyl-methacrylate) (PMMA), poly(caprolactone) (PCL) and polyurethane (PU) 3-D scaffolds to elicit cellular response and AML’s leukaemic cell lines adhesion strength as well as assist in the development of the in vitro 3-D leukaemia model [6].

2.7.2.2 ECM Proteins: Levels of Structure and Functions

2.7.2.2.1 Collagen Type 1

Collagen is the major protein comprising the ECM. There are at least 12 types of collagen with type I, II and III being the most abundant protein in mammals, making up about 25% to 35% of the whole-body protein content which form fibrils of similar structure [123]. Collagens are predominantly synthesized by fibroblast. However, epithelial cells are also synthesized these proteins. Collagen type I is found in high concentration in tendon, skin, bone and fascia (a connective tissue that surrounds muscles, groups of muscles, blood vessels and nerves - closely packed bundles of collagen fibres) which are consequently convenient and abundant sources for isolation of the natural polymers. This like rod proteins is 300 nm long, 1.5 nm in diameter and consists of three coiled polypeptide composed of two \( \alpha \)1 chains and one \( \alpha \)2 chain. Each chain consists of 1050 amino acids twisted around each other in a characteristic right-handed triple helix [123]. There are three amino acids per turn in the helix and every third amino acid is a glycine (Gly). Collagens are also rich in proline (Pro makes up about 17% of collagen) and hydroxyproline (Hyp) [124]. The triple helix gives collagen a rigid structure. It
maintains the mechanical integrity of tissues. The 3-D structure of collagen fibres also provides flexibility and strength to the ECM.

### 2.7.2.2 Fibronectin

Fibronectin is a high molecular weight, adhesive, glycoprotein found in both blood plasma (formerly called ‘cold-insoluble globulin’ (CIg) or soluble plasma fibronectin) [125] and the ECM (insoluble cellular fibronectin secreted by various cells, primarily fibroblasts) [126]. The role of fibronectin is to attach cells to a variety of ECM. Fibronectin attaches cells to all matrices except collagen type IV that involves laminin as the adhesive molecules [124]. Fibronectin is a dimer of two peptide chains with a molecular weight of approximately 275 kDa which are linked through two interchain disulphide bonds at the COOH-terminal end of the molecule. Each chain is 60 nm to 70 nm long and 2 nm to 3 nm thick. Fibronectin contains at least 6 tightly folded domains each with a high affinity for a different substrate such as heparin sulphate, collagen (types I, II and III), fibrin and cell-surface receptors.

### 2.8 Leukaemias

The leukaemias are a group of diseases characterized by the clonal proliferation of malignant white blood cell precursors [127]. They can also be defined as a group of neoplasms (any abnormal growth of new tissue, benign or malignant and also called tumour) characterized by malignant proliferation of myeloblasts or lymphoblasts that replace normal bone marrow elements and infiltrate normal tissues [128]. They differ in the lineage and degrees of differentiation of the cells involved and are divided into two main groups: lymphoid and myeloid. These two groups are further subdivided into acute and chronic forms, where in the former relatively immature cells are produced and the untreated disease progresses rapidly (acute), and in the latter more mature cells are produced and the disease pursues a more indolent course (chronic).

#### 2.8.1 Acute Myleoid Leukaemia (AML)

##### 2.8.1.1 Definition

AML is a morphological heterogenous disease. Since several haematopoietic cell lines may be involved, granulopoietic cells, monocytes, erythroblasts and even megakaryocytes are frequently found together [129]. In AML, the bone marrow makes many unformed cells called blasts. Blasts normally develop into white blood cells that fight infection. However, the blasts are abnormal in AML. They do not develop and cannot fight infections. The bone marrow may
also make abnormal red blood cells and platelets. They crowd out the normal red blood cells, white blood cells and platelets the body needs which lose the ability to differentiate normally and to respond to normal regulators of proliferation. This loss leads to fatal infection, bleeding or organ infiltration, typically in the absence of treatment within 1 year of diagnosis [130].

2.8.1.2 Incidence

AML is the most common type of acute leukaemia. The incidence of AML in the United States was reported approximately 2.4 per 100,000 and it increases progressively with age to a peak of 12.6 per 100,000 adult each year, mostly in older adults with the age of 65 years and older [131]. Moreover, leukaemia accounts for 3% of all cancers in the United States and for 4% of total cancer-related mortality [128]. Prevalence of AML is around 3 to 8 cases per 100,000 rising to 17 to 19 cases per 100,000 adults aged 65 years and older [130].

2.8.1.3 Risk Factors

As reported by Carol [128], the etiology (the study of origin) of leukaemia is unknown, although a number of risk factors have been identified that may contribute to the development of this disease. The first and arguably most important of these risk factors are genetic mutations. Secondly is the ionizing radiation which has been well established as a risk factor in the development of acute leukaemia (e.g., exposed to ionizing radiation after the detonation of the atomic bombs at Hiroshima and Nagasaki [132] and the power plant accident at Chernobyl). Other risk factors as reported in the report is the exposure to certain chemicals and drugs (e.g., benzene in shoe making, pesticide in farming, painting, smoking [133] and hair dye).

2.8.2 Chronic Lymphocytic Leukaemia (CLL)

2.8.2.1 Definition

Chronic lymphocytic leukaemia (CLL) is a disease characterized by the relentless accumulation of CD5+ B lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs (lymph nodes and spleen) [134, 135]. As a leukaemia, it is usually characterized by an increase in the lymphocyte count in the peripheral blood > $5 \times 10^9$ L$^{-1}$. The entity formerly defined as CLL of T lymphocytes is now renamed as T-prolymphocytic leukaemia (T-PLL) [136].

At present, no chemotherapeutic regimens can be considered curative and all patients will die with (or because of) their disease [136]. Moreover, at a certain stage if the cancerous lymphocytic cells are too severe, it could turn into advanced CLL which is known as lymphoma.
(a cancer of lymphatic system). About 5% of CLL cases will turn into lymphoma. They can be classified into two types: (1) Hodgkin’s lymphoma and (2) Non-Hodgkin’s lymphoma (NHL). These two types of lymphomas behave, spread and respond to treatment differently. The main difference between Hodgkin's and non-Hodgkin's lymphoma is in the specific lymphocyte each involves. A physician can tell the difference between Hodgkin's and non-Hodgkin's lymphoma by examining the cancer cells under a microscope. If in examining the cells, there is a presence of a specific type of abnormal cell called a Reed-Sternberg cell (usually B-cell), the lymphoma is classified as Hodgkin's. If the Reed-Sternberg cell is not present, the lymphoma is classified as non-Hodgkin's (could be B- or T-cell). Both types of lymphoma can start almost anywhere because of the lymphatic tissue can be found in many parts of the body (e.g., lymph nodes, lymph, tonsils, stomach, skins, etc.). Meanwhile, non-Hodgkin's lymphoma is actually a heterogeneous group of over 30 types of cancers with differences in the microscopic appearance and biological characterization of the malignant lymphocytes [137].

2.8.2.2 Incidence and Prevalence

CLL is the most common leukaemia in adults, in the western countries, representing about 25% to 30% of all leukaemias [135]. About 15,000 new cases are diagnosed in adults in the USA each year [138, 139]. CLL is more common in men than women, with a sex ratio of about 1.5 to 2:1 [135]. The incidence rate is about 2 to 6 new cases every 100,000 individuals per year and increases with age, reaching 12.8/100,000/year at 65 years of age that is the mean age at diagnosis [136]. Prevalence of CLL, that is how commonly a disease or condition occurs in a population, is around 30 to 50:100,000 (0.03% to 0.05%), based on the estimated incidence and the long median survival of the disease [136].

2.8.2.3 Risk Factors

The occurrence of CLL is not due to any known environmental factor as ionizing radiations, chemical compounds and it is not more frequent in patients affected by immunodeficiencies [135]. In contrast, a genetic and familial predisposition appears to be relevant in the pathogenesis of the disease as supported by two series of evidences. First, CLL is very rare in China and Korea and virtually absent in Japan. The low incidence rate is maintained within the Japanese emigrants and their progeny (descendants from a mixed marriages), thereby excluding an environmental modifier of such genetic predisposition [140]. Secondly, epidemiological (studies on how often the disease occur) evidences indicate that in 5% to 10% of cases there is a familial susceptibility to CLL (and other lymphoid malignancies) [141] with two or more individuals affected in the same family. The overall risk to develop such diseases is between
two and seven times higher in first relatives of CLL patients as compared to the general population [142]. This familial predisposition is accompanied by the so-called anticipation phenomenon (symptoms of a genetic disorder become apparent at an earlier age as it is passed on to the next generation) [141, 143, 144].
CHAPTER 3 Goals and Objectives

The in vitro cultivation of leukaemic cells in two dimensions is extremely difficult without the addition of exogenous cytokines to the culture. Survival of the cell blasts is enhanced if they are co-cultured with stroma or fibroblasts, suggesting that they are dependent on the extracellular matrix (ECM), adhesion proteins and cytokine expression of their bone marrow microenvironment for survival. The study of the impact of these physico-chemical influences on the leukaemic bone marrow and the process of leukaemogenesis is not only limited by the requirement of exogenous cytokines for the maintenance of such cells in culture, but also by the lack of a three dimensional growth micro-environment, which is not supported by 2-D cultures.

The proposed study focuses on the development and optimization of a 3-D culture system for the culture of human primary leukaemic cells in the absence of exogenous cytokines. Since the nature of the scaffold affects cell attachment, adhesion, growth and differentiation, preliminary studies on fabrication, physical and material characteristics, surface treatment and biological surface coating were conducted with respect to cellular response of AML (e.g., HL-60) and CLL (e.g., JeKo-1 and RL) cell lines to ensure that the human primary leukaemic cells were not wasted. Although the leukaemic cell lines are already cytokine-independent in 2-D static cultures, this main study of the cell lines in 3-D focusing on the material and physical culture characteristics required by cells wherein at the same time clarify the environmental needs of the human primary leukaemic cells as well.

Since leukaemic cell (e.g., AML or CLL) is a heterogeneous disease, the system is optimized for the culture of different leukaemic cell lines prior to the evaluation of human primary leukaemic cells. The possibility of mimicking the 3-D bone marrow structure seemed a solution to overcome the problem of culturing leukaemic cells in vitro. By recreating similar microenvironment it is hypothesized that cells would survive for long periods of time (up to 4 weeks proliferation period) and they could be investigated in their natural environment without the addition of external growth factors and at the same time maintaining their self-renewal potential.

The specific objectives of this thesis are outlined as follows:

1. To fabricate a novel PHB and PHBV porous 3-D scaffolds with an improved thickness greater than 1 mm (in relative to the conventionally made PHA matrices) by using the solvent-casting particulate-leaching (SCPL) technique.
2. To analyze the structural, material and physico-chemical characteristics (e.g., porosity, pore size, BET surface area, hydrophilicity/hydrophobicity and zeta-potential measurement) of PHB and PHBV porous 3-D scaffolds.
3. To study the *in vitro* degradation of polymeric porous 3-D scaffolds in the hydrolytic degradation media (phosphate buffered saline) and cell growth media by means of gravimetric analysis, pH change and structural morphology.

4. To identify the suitable surface treatment of PHB and PHBV porous 3-D scaffolds by means of alkaline treatment and rf-O$_2$ plasma treatment which could enhance cellular growth of CLL cell line (RL).

5. To optimize the cellular growth of CLL cell lines (RL and JeKo-1) by immobilizing two main bone marrow ECM proteins (e.g., collagen type I and fibronectin) on the surface of PHB and PHBV porous 3-D scaffolds by means of a physical adsorption (non-covalent interactions).

6. To generate the cellular growth of abnormal 3-D culture system using human primary AML mononuclear cells (MNCs) without adding exogenous cytokines.
CHAPTER 4 Materials and Methods

4.1 Materials

Poly(3-hydroxybutyric acid) (PHB; $M_w = 300,000$ g/mol; CAS No. 29435-48-1), poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV; $M_w = 680,000$ g/mol with 12% (w/w) polyhydroxyvalerate (PHV) content; CAS No. 80181-31-3) [145, 146] and cell culture grade sodium chloride (particles size: 212 $\mu$m to 850 $\mu$m) were purchased from Sigma-Aldrich® (Dorset-United Kingdom). Chloroform 99.9% in purity (AnalaR®) was obtained from VWR International (Leicestershire, United Kingdom). GIBCO® Iscove's Modified Dulbecco's Medium (IMDM), GIBCO® Phosphate Buffered Saline (PBS) and Foetal Bovine Serum (FBS heat inactivated) were purchased from Invitrogen™ Ltd., United Kingdom. One well-characterized human leukaemia cell line which is HL-60 (from a patient with acute promyelocytic leukaemia) and two human chronic lymphocytic leukaemia cell lines (RL and JeKo-1) were purchased from ATCC®, UKCCL-240. MTS-based CellTiter® 96 AQueous Assay was purchased from Promega (Madison, United States of America).

4.2 Fabrication of an Improved Thickness of PHB and PHBV Porous 3-D Scaffolds using the Solvent-Casting Particulate-Leaching (SCPL) Technique

4.2.1 Studies of Polymer Concentrations with Respect to Homogenization Time

Polymer concentrations of 1%, 2%, 3%, 4% and 5% (w/v) PHB and PHBV (containing 12% by weight of polyhydroxyvalerate (PHV)) in powder and pellet forms respectively were prepared in 60 ml of boiled reflux chloroform (99.9% purity) at 60 °C by using 500 ml SCHOTT® Duran bottle. Polymer solutions were considered as a homogenous solution if there were no apparent residues of glutinous materials in the solution or remains at the bottom of the bottle. Five replications were made on each polymer at a different concentration for data collecting ($n = 5$). All measurements were expressed as mean ± standard deviation (sd) and statistical analyses were computed for statistically differences between polymer concentrations as a function of time.
4.2.2 Studies of Polymer Concentrations with Respect to Polymeric Porous 3-D Scaffolds

Thickness

Polymer concentrations of 1%, 2%, 3% and 4% (w/v) for PHB and PHBV (containing 12% by weight of polyhydroxyvalerate (PHV)) were prepared in 60 ml of boiled reflux chloroform (99.9% purity) at 60 °C for 8.2 ± 1.48 min and 17.8 ± 2.28 min respectively in a fume cupboard (V_{suction} = 1.24 ± 0.20 m/s; T = 20 ± 1 °C). The polymer solutions were cooled down for 10 min prior to the mixing with sodium chloride. Once cooled, the polymer solutions were then added evenly over a coated aluminum foil glass Petri-dish (internal diameter, 8.8 cm × height, 1.8 cm). Sodium chloride crystals (particles size: 212 µm to 850 µm) with a salt weight fraction of 99% (w/w) were then poured and stirred evenly into the polymer solutions with continuous stirring until the polymer-solvent solution became pasty, thick and packed (Table 4.1). The fully filled pasty Petri-dish was tapped a few times so that the content became compact and even. This procedure was carried out in the fume cupboard. Subsequently, the packed-in pasty were put immediately inside the lyophilization flask (to minimize rapid phase separation, which could result in an etched surface) and air-dried for 2 days for complete solvent evaporation. The dried cast polymers were peeled carefully from the aluminum foils and then dialyzed with 10 liters of cold deionized water (DIW) at 20 ± 1 °C for 2 days to remove all sodium chloride crystals (2 dried casts/10 liters DIW). The deionized water was changed twice a day. The wet porous materials were put on top of the tissue paper for several times until they became partially dry. They were frozen at -70 °C for 3 h and then transferred into a cryostat bath containing ethylene glycol (-15 °C) to lyophilize the remaining deionized water and chloroform via the sublimation process under vacuum pressure of 2 mbar for 2 days. The thickness of these porous 3-D scaffolds was measured by using 150 mm digital LCD Vernier caliper. Ten replications were made for each polymers at different concentration (n = 10). Porous 3-D scaffolds from each replicate were cut into 10 sections, 5 of which were randomly selected for thickness measurements (expressed as mean ± standard deviation (sd) computed for differences between the polymer concentrations as a function of thickness). The schematic of the solvent-casting particulate-leaching (SCPL) process is illustrated in Figure 4.1.

Table 4.1: Preparation condition of polymeric porous 3-D scaffolds.

<table>
<thead>
<tr>
<th>PHB/PHBV mass (g)[a]</th>
<th>NaCl mass (g)[b]</th>
<th>NaCl weight fraction (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6 (1%, w/v)</td>
<td>172.3 ± 6.1</td>
<td>99</td>
</tr>
<tr>
<td>1.2 (2%, w/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 (3%, w/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.4 (4%, w/v)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[a] Polymer solution was prepared in 60 ml of hot chloroform. [b] The amount of NaCl that was used to make a fully filled pasty Petri dish without spilling out the polymer solution.
Figure 4.1: Schematic of SCPL process. The process comprises (1) mixing of polymer solution with porogen; (2) adding the polymer solution with porogen into a Petri-dish and then incubated in the lyophilization flask to avoid development of etching surfaces; (3) evaporation of solvent for 48 h in the fume cupboard. The solvent evaporation is complied with the United Kingdom Solvent Emission Directive (SED), 2002 for Halogenated VOCs: < 20 mg/m³ (< \( \leq 12 \text{ kg of CHCl}_3 \)); (4) leaching out porogen from dried cast polymer + porogen by using 10 liters of deionized water for 48 h (changed twice/day) at 20 ± 1°C; (5) lyophilized porous 3-D scaffolds with the thickness greater than 1 mm; (6) Cuboids with the size of \( \sim 10 \text{ mm} \times \sim 10 \text{ mm} \times \sim 5 \text{ mm} \) were excised prior to the physico-chemical analysis, \textit{in vitro} degradation measurement and cellular proliferation studies.

4.2.3 Fabrication of Solvent-Cast Thin Film

Fabrication of PHB and PHBV thin films were prepared as described in previous studies [145, 147-154] and used to compare in terms of its wetting behaviour with porous 3-D scaffolds fabricated using SCPL. A solvent-cast film was made by pouring a polymer solution of 4% (w/v) into a casting block on a clean polypropylene (PP) sheet with the size of 30 cm × 30 cm. Then, the casting block was immediately moved upwards towards the end of the sheet so that the polymer solution can be smeared evenly. The sheet coated polymeric thin film was left for a day in a fume cupboard at 20 ± 1°C for complete solvent evaporation prior to the wetting analysis.

4.3 Determination of Salt-Leaching Efficacy Measured Via Ion Conductivity and Gravimetric Analysis

Polymer concentrations of 4% (w/v) for PHB and PHBV (containing 12% by weight of polyhydroxyvalerate (PHV)) were used for preparing dried cast polymers as described in the above protocol. The conductivities (\( \kappa \)) of standard sodium chloride solution (for standard calibration curve) and solution with leached sodium chloride from the dried cast polymers were measured by using OAKTON™ Con 11 conductivity meter. The Automatic Temperature
Compensation (ATC) function was activated to compensate the reference temperature of 25 °C. The sodium chloride solutions was first prepared on the serial concentration (5 mg/ml, 10 mg/ml, 20 mg/ml, 30 mg/ml) and the conductivities (mS/cm) at 20 ± 1 °C was plotted against its respective concentration to establish a standard calibration curve. The amounts of sodium chloride that have been leached out from the dried cast polymers were determined in 10 replications (n = 10). The conductivity was measured twice a day (10 liters of the deionized water were changed twice a day) until it reached down to 2.73 µS/cm (conductivity of deionized water at 20 ± 1 °C). Then, the total concentration and mass of sodium chloride were determined from the standard curve (Appendix A) based on the sums of conductivities. Finally, to determine any potential of polymer weight loss during the salt-leaching process, a gravimetric analysis was conducted after the lyophilization process. The efficacy (%) of salt-leaching measured via ion conductivity and gravimetric analysis after the lyophilization process was determined based on the Eq. 4.1, Eq. 4.2 and Eq. 4.3 respectively.

\[
\text{Efficacy of salt-leaching measured via ion conductivity (\%) = } \frac{M_{\text{NaCl in the dried cast}} - M_{\text{NaCl dissolved in the DIW}}}{M_{\text{NaCl in the dried cast}}} \times 100% \tag{4.1}
\]

\[
\text{Efficacy of salt-leaching via gravimetric analysis (\%) = } \frac{(M_{\text{NaCl in the dried cast}} - M_{\text{NaCl remains inside the lyophilized scaffolds}})}{M_{\text{NaCl in the dried cast}}} \times 100% \tag{4.2}
\]

\[
\text{Weight of NaCl remains inside the lyophilized 3-D scaffolds (g) = } M_{\text{Lyophilized porous 3-D scaffolds}} - 2.4 g \text{ (weight of polymer at 4\%, w/v)} \tag{4.3}
\]

4.4 Effect of Sodium Chloride Residual in Polymeric Porous 3-D Scaffolds on Cell Growth Media

Polymer concentrations of 4\% (w/v) for PHB and PHBV (containing 12\% by weight of polyhydroxyvalerate (PHV)) were used for preparing polymeric porous 3-D scaffolds as described in the above protocol. The porous 3-D scaffolds were put into 50 ml centrifuge tubes containing cell growth media of 90\% (w/v) Iscove’s Modified Dulbecco’s Medium (IMDM) + 10\% (w/v) Foetal Bovine Serum (FBS) + 1\% (w/v) Penicillin-Streptomycin (PS) (V = 30 ml, pH 7.2) and incubated at 37 °C for 7 days. The scaffolds were taken out from the incubator and the conductivity was measured 5 min after the removal from the incubator. The conductivity (κ) of cell growth media in the presence of scaffolds was recorded daily at 20 ± 1 °C (room temperature). Three replications were prepared for each polymer (n = 3). All measurements were expressed as mean ± standard deviation (sd) and statistical analyses were computed for
differences between conductivity ($\kappa$) of cell growth media in the presence of scaffolds and control (cell growth media without a scaffold).

4.5 Surface Treatment of Polymeric Porous 3-D Scaffolds

4.5.1 Alkaline Treatment

To increase the biocompatibility of the polyesters used in this study, surface hydrolysis by NaOH was used to break the ester-linkage of the polymers and also to functionalize by the insertion of polar groups [155]. In fact, the hydrolyzation process will generate more hydroxyl groups which lead to increased surface hydrophilicity thus improving the capacity for the cells to adhere to the polymer surfaces [156]. PHB and PHBV (4%, w/v) porous 3-D scaffolds in a 6-well tissue culture plate were soaked in 10 ml of NaOH solution with serial concentrations (0.2 mol/L, 0.4 mol/L, 0.6 mol/L, 0.8 mol/L, 1.0 mol/L) for 60 min at 20 ± 1 °C for surface treatment. The optimal treatment time was taken from the previous study [155]. The treated porous 3-D scaffolds were taken out and rinsed extensively with deionized water until the pH of the rinsing water became neutral. They were then air dried at 37 °C overnight prior to the water contact angle measurement and cell proliferation studies. Three replications were made on each polymer at different NaOH concentrations ($n = 4$).

4.5.2 Oxygen Plasma Surface Treatment

For the comparison of the different surface treatments with respect to cell attachment and proliferation, another type of surface treatment was employed. This was using rf-oxygen plasma. The surface treatment was conducted as described in previous studies [150, 151]. The surfaces of the polymeric porous 3-D scaffolds were treated with rf-plasma (Diener Electronic GmbH, Nagold, Germany) using oxygen gas to make their surfaces more hydrophilic prior to the water contact angle measurement and cell proliferation studies. The main characteristics of the rf-plasma and treatment parameters used are summarized in Table 4.2. In brief, the porous 3-D scaffolds with an approximate size of 10 mm × 10 mm × 5 mm placed into the 24-well cell culture plate were positioned on the sample stage in the reaction chamber. Initially, the reaction chamber was evacuated to about 0.1 mbar. Oxygen gas was introduced into the reactor at a rate of 30 standard cm$^3$/min, and the plasma was ignited. The plasma treatment was performed at a 13.56 MHz pulse frequency and power of 100 W. The temperature in the chamber was maintained at 17 °C. The scaffolds were treated for 10 min. The modified scaffolds were stored in deionized water in a refrigerator at 4 °C until use.
Table 4.2: Main characteristics and treatment parameters of the O\textsubscript{2} rf-plasma reactor for polymeric porous 3-D scaffolds surface treatment.

<table>
<thead>
<tr>
<th>Main characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma reactor manufacturer</td>
</tr>
<tr>
<td>The plasma model</td>
</tr>
<tr>
<td>Plasma generator</td>
</tr>
<tr>
<td>Threshold volumetric flow rate (Q)</td>
</tr>
<tr>
<td>Vacuum pump</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power (P)</td>
</tr>
<tr>
<td>Exposure time (t)</td>
</tr>
<tr>
<td>Volumetric gas flow rate (Q)</td>
</tr>
</tbody>
</table>

[a] Gas conversion unit: 1 sccm = 7.43583 × 10\textsuperscript{-4} M\textsubscript{a} (mg/s) [M\textsubscript{a} = Atomic Mass Unit (AMU)]; O\textsubscript{2} = 15.9994 AMU.

4.5.3 Weight Loss after Surface Treatment

The weight loss of the polymeric porous 3-D scaffolds, after treatment, was calculated through the following formula:

\[
\text{Weight loss} \% = \left( \frac{M_0 - M_1}{M_0} \right) \times 100\% \quad (4.4)
\]

where M\textsubscript{0} and M\textsubscript{1} are the weights of dried untreated and treated polymeric porous 3-D scaffolds, respectively.

4.6 Effect of Sterilization on Surface Properties of Polymeric Porous 3-D Scaffolds

Sterilization was carried out using a combination of 8 min under UV light (230 V, 50 Hz, 0.14 A, Kendro Laboratory Products, United Kingdom) and immersion for 2 h in Ethanol (70% v/v). Subsequently, the polymeric porous 3-D scaffolds were washed several times (at least 3 times) for 5 min in PBS as described in the previous study [6]. Lastly, both polymeric porous 3-D scaffolds were vacuum dried for 24 h at 20 ± 1 °C prior to the ζ-potential and water contact angle measurement.

4.7 Characterization of the Polymeric Porous 3-D Scaffolds

4.7.1 Structural/Morphological Analysis: Scanning Electron Microscopy (SEM)

The vertical cross-section through PHB and PHBV (4%, w/v) porous 3-D scaffolds were all analyzed by using SEM (JEOL JSM-5610LV, JEOL Ltd., Welwyn Garden City, United Kingdom) to observe the internal pore structure. The specimens were sectioned, mounted on aluminum stumps, followed by coating with gold in a sputtering device in an argon atmosphere.
for 2 min at an acceleration current of 20 mA prior to the examination by SEM at an acceleration voltage of 20 kV.

4.7.2 Structural Properties of the Polymeric 3-D Scaffolds

4.7.2.1 Material Characterization of Polymeric Porous 3-D Scaffolds: Physical Properties

Parameters such as apparent density (skeletal density) obtained by using helium displacement pycnometry (AccuPyc 1330 V3.00, Micromeritics) and geometrical bulk density (mass of scaffold/geometric volume of scaffold) by using gravimetric analysis were used to calculate % porosity of the scaffolds [157]. Four porous 3-D scaffold samples (an approximate size of 10 mm × 5 mm × 5 mm) were analyzed to obtain statistically relevant data (n = 4). Percent porosity is determined based on the given equation below (Eq. 4.5):

Porosity (φ), % = 1 - [(Geometrical bulk density/Skeletal density)] × 100% \hspace{1cm} (4.5)

4.7.2.2 Structural/Morphological Analysis: Pore Size Distribution

A mercury intrusion porosimetry (AutoPore IV 9500 MIP; Micromeritics® Instrument Corporation, United States of America) was used to determine the pore size distribution of the PHB and PHBV (4%, w/v) porous 3-D scaffolds. A penetrometer with 6 ml bulb volume was used with sample weight in the range 0.09 to 0.12 g. The intrusion chamber was then filled with mercury at a pressure of 1.52 kPa and the samples were penetrated with mercury until a maximal pressure of 675.50 kPa at which the total intrusion volume reached a plateau [83]. As for the pore size distribution, the Laplace-Washburn equation (Eq. 4.6) was used to calculate the pore diameter $d_p$ in relation to the applied external pressure, $p$ needed to force mercury into the pores. The mercury surface tension $\gamma$ is (480 mN/m) and the contact angle ($\theta$) between mercury and polymer is assumed to be 130°. Details concerning the experimental and theoretical procedures for the measurements performed are reported elsewhere [158].

$d_p \times p = 4 \times \gamma \cos \theta$ \hspace{1cm} (4.6)

where $p$ is pressure needed to force mercury into the pores, $d_p$ is the diameter of the pore with respect to the applied external pressure, $\gamma$ is the surface tension of the mercury (480 mN/m), and $\theta$ is the contact angle of the mercury on the solid surface (130°).
4.7.2.3 Material Characterization of Polymeric Porous 3-D Scaffolds: BET Surface Area

The surface area of the polymeric porous 3-D scaffolds was determined using nitrogen adsorption isotherms at 77 K utilizing surface area analyzer gas absorption BET (ASAP® 2010; Micromeritics® Instrument Corporation, United States of America) [157, 159]. The sample holder was filled with polymeric porous 3-D scaffolds pieces. Before performing the gas sorption experiments, surface contaminants were removed via a ‘degassing’ step. The samples were dried inside the measuring cell at temperature below their glass-transition ($T_g$) temperature under vacuum overnight. For the analysis part, nitrogen (the adsorbate) was admitted into the evacuated sample chamber. The detailed explanations of the experimental and theoretical procedures for the measurements performed are reported elsewhere [160].

4.7.2.4 Mechanical Testing of Polymeric Porous 3-D Scaffolds

Compressive tests were carried out using a Lloyds universal testing machine (Lloyds EZ50, Lloyds Instruments Ltd., Fareham, United Kingdom) to determine the compressive modulus (E) and compressive strength of the scaffolds. The samples (an approximate size of 10 mm × 10 mm × 7 mm to 9 mm) were compressed at a loading rate of 0.6 mm/min by means of a compression plate with a load cell of 5 kg ± 0.1 g (49.03325 N), until a compression of 85% of the initial test piece thickness is attained (Table 4.3). Afterward, the unloading curve was measured at the same rate until the separation between the compression plate and the base plate is equal to the initial test piece thickness. Two different type of polymeric scaffolds in 3 replicates ($n = 3$) were analyzed to obtain statistically relevant data.

Table 4.3: Main characteristics and operational parameters of the mechanical testing of polymeric porous 3-D scaffolds.

<table>
<thead>
<tr>
<th>Main characteristics</th>
<th>Operational Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Lloyds universal testing machine</td>
</tr>
<tr>
<td>Instrument model</td>
<td>Lloyds EZ50, Lloyds Instruments Ltd.</td>
</tr>
<tr>
<td>Load cell</td>
<td>5 kg ± 0.1 g (49.03325 N)</td>
</tr>
<tr>
<td>Loading rate</td>
<td>0.01 mm/sec (0.6 mm/min)</td>
</tr>
<tr>
<td>Temperature</td>
<td>20 ± 1°C</td>
</tr>
<tr>
<td>Sample thickness</td>
<td>PHBV ~7 mm; PHB ~9 mm</td>
</tr>
</tbody>
</table>

4.7.3 Surface Properties of Polymeric Porous 3-D Scaffolds

4.7.3.1 Physico-Chemical Analysis: Water Contact Angle and Surface Free Energy

Static water contact angle ($\theta_{\text{H}_2\text{O}}$) [161, 162] on the air surface of PHB and PHBV (4%, w/v) porous 3-D scaffolds and the corresponding solvent-cast thin films were measured using a Drop
Shape Analyzer (DSA30E, KRÜSS GmbH, Germany) [163]. Re-distilled water of approximately 10 µl was gently plated on the vertical cross section and on top of the surfaces of the porous 3-D scaffold and thin film respectively (10 µl was used to prevent gravitational distortion of the spherical profile) [148]. At least ten readings on different parts of the porous 3-D scaffold and thin film were averaged for data collecting (n = 10). The capture was triggered as soon as the drop reached on the surface and the measurement time was less than 5 s. All measurements were conducted at 20 ± 1 °C. Meanwhile, Fowkes equation was chosen as the equation of state (EOS) to determine the surface free energy of the solvent-cast thin films (γₖ). In this case, an adhesion energy/work of adhesion is calculated wherein it is a sum of two partial contributions, each of which is expressed as the geometric mean polar of two adhesion parameters, γₗ and γₛ, the first of which characterizes the ‘adhesive power’ of the liquid, and the second, of the solid (Eq. 4.7). The surface free energy was determined by contact angles of two types of reference liquids which are water (H₂O) and di-iodomethane (CH₂I₂) [164]. In addition, several calculations were made to verify the surface hydrophilicity/hydrophobicity of the thin films by means of the surface free energy (γₛ), the work of adhesive (Wₘₜ) and spreading coefficient at solid-liquid interfaces [165] (Eq. 4.10). All calculations were based on Young (Eq. 4.8), Dupree (Eq. 4.9) and Fowkes equations (Eq. 4.7) and expressed as follows:

\[
\gamma_L(1 + \cos \theta) = 2[(\gamma_{Ld}^d \gamma_{Sd}^d)\frac{1}{2} + (\gamma_{LP}^d \gamma_{PS}^d)\frac{1}{2}] \quad (4.7)
\]

\[
(\cos \theta)\gamma_L = \gamma_L - \gamma_{LS} \quad (4.8)
\]

\[
W_{SL} = \Delta G = E_{adh} = \gamma_L + \gamma_L - \gamma_{LS} \quad (4.9)
\]

\[
(S_{H₂O/thin \, film}) = W_{SL} - W_{LL} (W_{LL} = \Delta G = 2 \times \gamma_L = 72.8 \times 2 = 145.6 \text{ mN/m}) \quad (4.10)
\]

where γᵣₗ and γᵣₛ are the dispersive component, and polar component, respectively; θ is the contact angles to water and to di-iodomethane (CH₂I₂), respectively. For water, γₗ = 72.8 mN/m, γᵣₗ = 22.8 mN/m and γᵣₛ = 51.0 mN/m. For di-iodomethane (CH₂I₂), γₗ = 50.8 mN/m, γᵣₗ = 50.8 mN/m and γᵣₛ = 0 mN/m.

4.7.3.2 Surface Chemistry: ζ-Potential Measurements

To determine the ζ-potential of both polymeric scaffolds the Electrok inetic Analyser (EKA, Anton Paar KG, Graz, Austria) was used. This is based on the streaming potential method as described in the previous study [166]. Time- and pH-dependent ζ-potential measurements were performed in 1 × 10⁻³M KCl supporting electrolyte solutions. In order to determine the effect of
pH on the ζ-potential ($\zeta = f(pH)$), the initial starting point (i.e., constant ζ-potential), was fixed by the long time measurement at a constant ionic strength (1 mM KCl). This was to discard the possible effects of hydrophobic recovery and the polymer swelling in water. For pH-dependent ζ-potential measurements the pH solution was varied with either 0.1M HCl (pH 1) or 0.1M KOH (pH 13) using a remote titration unit (RTU, Anton Paar KG, Graz, Austria) at a constant temperature of 20 ± 1 °C. A cylindrical cell compartment was used for polymeric porous 3-D scaffolds.

### 4.8 In Vitro Degradation Studies of Polymeric Porous 3-D Scaffolds

For the *in vitro* degradation studies, cuboids of PHBV and PHB (4%, w/v) porous 3-D scaffolds with an approximate size of 10 mm × 10 mm × 5 mm were weighed to measure their initial weight. The samples were put into 50 ml centrifuge tubes 1) containing Invitrogen™ GIBCO® phosphate buffered saline (PBS) (V = 30 ml; product pH: 7.2 ± 0.05; measured pH 7.14) and incubated for 12 weeks (short-term); 2) cell growth media of GIBCO® RPMI 1640 (V = 30 ml; product pH: 7.13; measured pH 7.14) and incubated for 8 weeks and 3) Invitrogen™ GIBCO® phosphate buffered saline (PBS) (V = 30 ml; product pH: 7.2 ± 0.05; measured pH 7.14) and incubated for 40 weeks (long-term) at 37 °C. At the end of the period (on a weekly basis), samples were removed from the PBS/cell growth media, gently washed with deionized water and vacuum dried (2 mbar) for 48 h prior to weighing. At the same time, pH in the supernatant was recorded to observe any acidic degradation. Residual weight of the porous 3-D scaffolds as opposed to its initial weight was calculated after each incubation period as shown in Eq. 4.11. All measurements were expressed as mean ± standard deviation (sd) relative to the initial values.

\[
\% \text{ Residual weight of the scaffolds} = \frac{[M_{t=1 \text{ to } 40 \text{ weeks}} - M_{t=0 \text{ week}}]}{M_{t=0 \text{ week}}} \times 100\% \quad (4.11)
\]

### 4.9 Cell Culture on Porous 3-D Scaffolds

#### 4.9.1 Leukaemic Cell Lines Seeding and Culture

One well-characterized human leukaemia cell line which is HL-60 (from a patient with acute promyelocytic leukaemia) and two human chronic lymphocytic leukaemia cell lines (RL and JeKo-1) were studied for the *in vitro* cell-biomaterials interaction study. The cells were cultivated in standard polystyrene non-pyrogenic tissue culture flasks (T-flask, Corning® Inc., New York, United States of America) and in a humidified incubator at 37 °C, 5% CO₂ using protocol recommended by the cell line provider. For static seeding of porous 3-D scaffold
cultures, 100 µl of cell suspension (3.7 x 10^5 cells/scaffold) were seeded onto the inner side of the sterile porous 3-D scaffolds. Prior to that, sterilization was carried out using a combination of 8 min under UV light (230v, 50Hz, 0.14A, Kendro Laboratory Products United Kingdom) and immersion for 2 h in Ethanol (70%). Then, the scaffolds were washed twice for 5 min in PBS before adding the media and placing in a humidified incubator for 2 days at 37 °C and 5% CO₂. Next, it was placed into the 24-well tissue culture plate (Costar®, Corning® Inc., New York, United States of America) and incubated for 15 min at 37 °C and 5% CO₂ before adding 1.5 ml of a complete culture media in order not to wash the cells out and allow time for settling down. If this is not done, the cells will float in the medium and might not attach to the surface of porous 3-D scaffolds. Scaffold cultures seeded with cells underwent a half-medium exchange every two to three days (depending on the media colour changes) which is in the same way as cell cultures treated in the flasks.

4.9.2 AML Blood MNCs Separation and Handling

Human primary acute myeloid leukaemia blood was obtained in accordance with regulatory and ethical policies (Brent Research Ethics Committee 10/H0717/3). The MNCs were isolated using Ficoll-Paque (GE Healthcare, Sweden) density gradient centrifugation. The frozen human aspirated primary AML blood cells were gently aspirated from the vial using a 10 ml syringe and 18G needle. The aspirated blood was placed drop-wise into 50 ml centrifuge tube containing 30% (v/v) foetal bovine serum (FBS; GIBCO Invitrogen), 70% (v/v) Iscove’s Modified Dulbecco’s Medium (IMDM; GIBCO Invitrogen) and 1% Penicillin/Streptomycin (Gibco, Invitrogen Ltd., United Kingdom) by adjusting the total volume to 20 ml. Then, 10 ml of Ficoll-Paque (GE Healthcare, Sweden) was gently injected into the blood and centrifuged at 199.19 g (RPM = 936 (200 RCF); radius of rotation = 203 mm) for 35 min. The middle buffy coat layer which contains the mononuclear fraction was gently removed using a 10 ml syringe and 18G needle. The supernatant was collected into a single 50 ml centrifuge tube and the cell count was conducted using a standard hemocytometer. The supernatant containing MNCs were centrifuged again at 199.19 g for 10 min to remove any excess Ficoll-Paque (GE Healthcare, Sweden) and plasma. The remaining cells (precipitate) were re-suspended in 30% (v/v) foetal bovine serum (FBS; GIBCO Invitrogen), 70% (v/v) Iscove’s Modified Dulbecco’s Medium (IMDM; GIBCO Invitrogen) and 1% Penicillin/Streptomycin (Gibco, Invitrogen Ltd., United Kingdom) and adjusted to the final concentration of 20 x 10^6 cells/ml.
4.9.3 AML Blood MNCs Seeding and Culture

The AML blood MNCs were cultivated on scaffolds as follows: 100 µl of MNCs suspension (seeding density = 2.0 x 10^6 cells/scaffold) were seeded onto the sterile PHBV scaffolds (both protein and non protein-coated), placed in 24 tissue culture plates (Costar®, Corning® Inc., New York, United States of America) and incubated for 15 min at 37 °C and 5% CO₂ before adding 1.5 ml of Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco, Invitrogen Ltd., United Kingdom) with 30% foetal bovine serum (FBS, heat-inactivated, Invitrogen Ltd., United Kingdom) and 1% Penicillin/Streptomycin (Gibco, Invitrogen Ltd., United Kingdom). Half-medium exchange was carried out every other day. Cytokines were not added at any stage of the cell culture. 2-D control cell culture was performed by seeding AML blood MNCs onto sterile standard 24-well tissue culture plates (Costar®, Corning® Inc, New York, United States of America) at a concentration of 2.0 × 10^6 cells/well in 1.5 ml IMDM (Gibco, Invitrogen Ltd., United Kingdom) with 30% FBS heat-inactivated (Invitrogen Ltd.) and 1% Penicillin/Streptomycin (Gibco, Invitrogen Ltd.). Half-medium exchange was carried out every other day.

4.9.4 Collagen Type I and Fibronectin Coating

The polymeric porous 3-D scaffolds were coated with collagen type I from calf skin and/or fibronectin from bovine plasma. The treated porous 3-D scaffolds were immersed into different protein concentrations inside the centrifuge tube (50 ml) containing 25 ml of protein solutions: 125 µg/ml or 62.5 µg/ml of collagen type I soluble in 0.1M of acetic acid (Fisher Scientific, United Kingdom) and dissolved in deionized water from a NANOpure (Barnstead, Duque, IA, conductivity 1.8 mΩ/cm). Alternatively, 25 µg/ml or 50 µg/ml of fibronectin were as well diluted in deionized water (each protein is based on the lowest and the highest concentration used up to now) [6]. Throughout the immersion process, the protein solutions were forced into the porous structure by using a vacuum pressure of 2 mbar for 10 min for homogeneous protein coating. In order to unblock the pores window, remove the unbound proteins and to allow the cells seeded to penetrate deeper into the scaffold, the scaffolds were again immersed and washed thoroughly in the PBS under vacuum pressure (2 mbar) for 10 min. The protein-coated scaffolds were placed in a 24-well tissue culture plate at 20 ± 1 °C (room temperature) prior to the sterilization process. The vacuumed immersion surface coating process with ECM proteins is illustrated in Figure 4.2.
Figure 4.2: Schematic representation of the biological surface coating of two main bone marrow ECM proteins (collagen type I and fibronectin) on the PHB and PHBV porous 3-D scaffolds via physical adsorption method. The scaffolds were immersed with proteins solution under vacuum pressure to produce homogeneous surface coating. *4 porous 3-D scaffolds/polymer (n = 4).

4.9.5 Characterization Methods of 3-D Scaffolds during Cell Culture

4.9.5.1 Cell Seeding Efficiency of 3-D Scaffolds

The cell-seeded 3-D scaffolds were allowed to proliferate for 24 h and subsequently the cell seeding efficiency was calculated after 24 h as given by the equation below.

Number of viable cells/ml = Average number of viable cells for both chamber x 10^4/ml (volume of one square chamber: 1 mm x 1 mm x 0.1 mm) x 2 (dilution factor) \[ \text{(4.12)} \]

Seeding efficiency after 24 h (%) = \[ \frac{\text{[(Number of cell seeded onto the 3-D scaffolds} - \text{Number of cell remaining after 24 h)]}}{\text{Number of cell seeded onto the 3-D scaffolds}} \times 100\% \] \[ \text{(4.13)} \]

The number of cells remaining in the old medium was counted using the haemocytometer with a magnification of x20. To perform the cell count, 20 µl of the cell suspension was added to 20 µl of Erythrosin B (ATCC®, Manassas, VA) with the dilution factor of 2 (i.e., DF = 2 = 20
µl cell/20 µl cell + 20 µl Erythrosin B) in the 96-well plate. 10 µl of the solution was used to fill both chambers of the haemocytometer making sure that there was no air bubbles trapped inside the chambers. Using a light microscope (Leica DM microscope, Milton Keynes, United Kingdom) the number of viable (unstained) and non-viable (stained red) cells were counted in five 4 x 4 squares (or 0.1 cm² area). After 24 h in culture, the seeded 3-D scaffolds were transferred to a new well plate with a new culture medium ready for a short-term (7 and 14 days) and long-term cell proliferation studies (up to 6 weeks). The MTS assays were carried out on a proliferation period on a weekly basis.

4.9.5.2 MTS-based CellTiter96® Aqueous Assay-Cell Proliferation

Cell proliferation was quantitatively assessed by changes in the number of metabolically active cells using the tetrazolium compound CellTiter96® Nonradioactive Cell Proliferation Assay (MTS Assay) (Promega, United States of America) [167]. The reaction solution (100 µl containing 95.2 µl MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium] and 4.8 µl phenazine methosulfate (PMS)) [168] were added with cell seeded 3-D scaffolds with the ratio of 1 ml to 200 µl (growth media to MTS solution). The MTS cell proliferation is a calorimetric assay that measures the reduction of the tetrazolium compound by the cells into a coloured formazan product that was soluble in tissue culture media. The amount of formazan produced, as a measured by the absorbance, is directly proportional to the number of living cells in the culture. During the analysis, the seeded 3-D scaffolds were transferred to a new 24-well tissue culture plate. Then 1.5 ml of the culture media (pre-warmed at 37 °C) was added to each of the cell seeded 3-D scaffolds. 300 µl of MTS solution, thawed for 10 min in a water bath at 37 °C, were added to each of the well. The cell seeded 3-D scaffolds with the MTS solution were incubated over 3 h at 37 °C and 5% CO₂. Eventually, the intense purple solution became visible. Then, 100 µl of the purple solution was taken out and transferred into the ELISA 96-well plate. It was then diluted 3x with the cell growth media prior to the absorbance measurement (100 µl of growth media in 3 wells each). 100 µl of the diluted cell solutions was transferred into the new ELISA 96-well plate in 2 replicates with 2 replicates of control (empty scaffold with no cell). The absorbencies of the produced formazan were recorded at 490 nm using a 96-well plate reader (Elx 808, Ultra Microplate Reader, Bio-TEK Instruments, Inc., Bath, United Kingdom).

4.9.5.3 Assessing Cell Attachment and Spreading using SEM

Following the cultivation of the AML cell line (HL-60), CLL (RL and JeKo-1) and human primary AML MNCs on the scaffolds for 7, 14 and 28 days, the scaffolds were examined by
SEM (JEOL JSM-5610LV, JEOL Ltd., Welwyn Garden City, United Kingdom). Briefly, the scaffolds were fixed with 2.5% (v/v) PBS-buffered glutaraldehyde solution (Fluka BioChemika, Switzerland) for 40 min at 4 °C and washed twice with PBS. Serial dehydration in ethanol (50%, 70%, 90%, 95%, and 100%) followed, each for 10 min, after which the samples were dried in an aseptic environment for 4 h. The specimens were cut with the Amati® POLITRAFOR hot-wired polystyrene foam cutter (4.5 V battery powered) to enable examination of longitudinal and transverse sections. The specimens were sputter-coated with gold in argon atmosphere for 2 min prior to the examination by SEM at an acceleration voltage of 20 kV.

4.9.5.4 Cell Viability Measurement

The cells were manually extracted from the scaffolds using a tweezer (to hold the scaffolds) and pipette. The extracted cell solution was transferred into the eppendorf™ tube (1.5 ml) prior to the measurement. The cell viability was measured using a standard hemocytometer and erythrosine B stain solution (ATCC®) at days 0, 7, 14 and 28; number of repetitions (n) = 2. Using a light microscope (Leica DM microscope, Milton Keynes, United Kingdom) the number of viable (unstained) and non-viable (stained red) cells were counted in five 4 x 4 squares (or 0.1 cm² area).

5.0 Statistical Analysis

Data were presented as means ± standard deviation (sd) of mean. Statistical comparisons were performed using Students t-test (PASW version 17.0 IBM Co.) for the thickness, salt-leaching efficacy and physico-chemical analysis. Statistical analysis of data for polymer concentrations, sodium chloride residual effect, in vitro degradation and the cellular proliferation studies were carried out by one-way analysis of variance (ANOVA) to determine the presence of any significant difference among sample means of the groups, followed by Tukey’s test (PASW version 17.0 IBM Co.) for multiple comparisons to determine the values that were significantly different. The p < 0.05 were considered statistically significant.
5.1 Introduction

Polyhydroxyalkanoates (PHA) are aliphatic polyesters that are synthesized by microorganisms under unbalanced growth conditions [169, 170]. They are generally biocompatible, biodegradable (via microbial degradation and enzymatic hydrolysis) and thermoprocessable, making them attractive as biomaterials for applications in medical devices and tissue engineering and regenerative medicine (TERM). Over the past years, PHA, particularly poly-3-hydroxybutyrate (PHB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), poly-4-hydroxybutyrate (P4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) and poly-3-hydroxyoctanoate (PHO) were demonstrated to be suitable for tissue engineering and are reviewed thoroughly in many literature-cited publications [2, 21, 66, 147, 171-173]. The most common type is PHB, which comes from the polymerization of 3-hydroxybutyrate monomer. The physico-chemical features of PHB in addition to the mechanical properties are similar to those of polypropylene (PP) and polylactic-co-glycolic acids (PLGA), except for reduced properties of stiffness [21, 170]. Besides PHB, its copolymers with diverse ratios of hydroxyvalerate (HV) which are known as PHBV is also extensively used. These copolymers are less crystalline, more flexible and more readily processable than PHB itself [174-179].

Apart from that, scaffold design and fabrication are one of the major areas of biomaterial research, and they are also important subjects for tissue engineering and regenerative medicine research. Briefly, the principal function of a scaffold is to manoeuvre cell behaviour such as migration, proliferation, differentiation, maintenance of phenotype and apoptosis by facilitating, sensing and responding to the environment via cell-matrix and cell-cell communications [180, 181]. Furthermore, the scaffold provides the necessary support for cells to proliferate and maintains their differentiated functions and its architecture defines the ultimate shape of a new organ [182]. Therefore, many methods have been developed to prepare porous 3-D scaffolds in tissue engineering including gas foaming, fibre extrusion and bonding, 3-D printing, phase separation, emulsion freeze-drying and porogen leaching [17, 83, 145, 183-188]. One of the prominent methods of making a porous 3-D scaffold is the solvent-casting particulate-leaching (SCPL). This method was first developed in the early 1990s by Mikos et al. [83] among other things for polylactic acid (PLA) and polyglycolic acid (PGA) polymers and several authors have used the method to manufacture composite scaffolds [167, 183]. Besides that, according to Chen et al. [90] and Mooney et al. [189] the extensive use of solvents (some of which are hazardous and essentially toxic) also presents an intricacy, as any residuals of the solvent would
hinder the cell attachment and proliferation onto the scaffold. On the contrary, Cao et al. and his colleagues [187] have shown that the low toxicity of solvents is detected from the use of SCPL can be brought down to acceptable levels for tissue engineering application. On the other hand, many studies have been reported using PHB and PHBV as biomaterials fabricated by both solvent casting and SCPL technique for in vitro and in vivo studies with various cell lines (i.e., osteoblasts, fibroblasts, epithelial cells and ovine chondrocytes (obtained from cartilage)) [21, 146, 148, 149, 174, 190]. For instance, Rivard et al. [69] demonstrated that PHBV matrices (9% (w/w) 3-hydroxyvalerate) sustained a fibroblast cell proliferation rate similar to that observed in collagen sponges for up to 35 days. Köse et al. [150, 151] used the macroporous PHBV matrices for bone tissue engineering and the results showed that the growth of osteoblasts on matrices was observed after 29 and 60 days of incubation. For cartilage tissue engineering, Deng et al. [145] investigated the proliferation of rabbit articular cartilage-derived chondrocytes on the PHBHHx and PHB 3-D scaffolds fabricated from SCPL. The result showed that the chondrocytes proliferated on the 3-D scaffolds and preserved their phenotype for up to 28 days. However, as far as we know, among many other studies conducted using the PHB and PHBV as biomaterials, only two studies (Misra et al. [191] and Huang et al. [192]) have successfully used porous 3-D scaffolds with a substantial level of thickness greater than 1 mm with most of the rest in thin films formation of less than 1 mm. However, none of those studies used any hematopoietic cell origin (i.e., myeloblast and lymphoblast) for cell-biomaterials interaction study.

### 5.2 Aim and Hypothesis

This investigation was based on the hypothesis that the usage of solvent-casting particulate-leaching (SCPL) process for PHB and PHBV will create a porous 3-D scaffold with an improved thickness greater than 1 mm (in relative to the conventionally made PHA matrices). These scaffolds will have interconnected pores to allow cell penetration and spread and would be able to mimic the 3-D microenvironment of bone marrow niches. Moreover, the 3-D growth configuration for AML (HL-60) expansion will replicate the in vivo bone marrow microenvironment more faithfully than 2-D culture, resulting in an enhanced in vitro culture without the need to add exogenous cytokines.

### 5.3 Approach of Study

In this study, an optimal polymer concentration, the 3-D scaffold thickness, the efficacy of the salt-leaching process and effect of sodium chloride remnants on cell growth media electrolytes balance were described thoroughly. Briefly, this work showed major improvement
in fabricating porous 3-D scaffolds using SCPL with a satisfactory thickness greater than 1 mm. Apart from that, the sodium chloride residual inside polymeric porous 3-D scaffolds showed irrelevant conductivity (κ) changes on cell growth media electrolytes content. These preliminary results have signified the suitability of that material for in vitro cellular proliferation. Then, physico-chemical characterizations (i.e., porosity, pore size distribution, BET surface area and hydrophobicity/hydrophilicity), short-term in vitro degradation in hydrolytic media (PBS) and mechanical testing (compressive test) were carried out. Finally, a cell-biomaterial affinity study was carried out by using a human acute myeloid leukaemia cell line (HL-60) (Table 5.1). Both polymeric porous 3-D scaffolds were cultured in vitro for 1, 7 and 14 days to observe cellular attachment and proliferation.

### Table 5.1: Acute myeloid leukaemia (AML) cell line properties. The table summarizes the main properties of the HL-60 cell line.

<table>
<thead>
<tr>
<th>Cell properties</th>
<th>Cell line HL-60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell phenotype</td>
<td>Myelocytic cell</td>
</tr>
<tr>
<td>Original disease of patient</td>
<td>AML, M2</td>
</tr>
<tr>
<td>Patient data</td>
<td>36-year-old Caucasian woman</td>
</tr>
<tr>
<td>Source of material</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>Year of establishment</td>
<td>1976</td>
</tr>
<tr>
<td>Culture Medium</td>
<td>90% IMDM + 10% FBS + 1% PS</td>
</tr>
<tr>
<td>Sub-cultivation routine</td>
<td>Maintain at 0.1 to 1 x 10^6 cells/ml. Renew media every 2 to 3 days</td>
</tr>
<tr>
<td>Doubling time</td>
<td>36 to 48 hrs</td>
</tr>
<tr>
<td>In situ morphology (cell size)</td>
<td>8 µm</td>
</tr>
</tbody>
</table>

#### 5.4 Results

##### 5.4.1 Polymer Concentrations with Respect to Homogenization Time

The polymer concentrations in chloroform were selected based on the previous studies [145, 147-153] in the range of 1% to 5% (w/v). The complete homogenization time was measured at those selected concentrations range for PHB and PHBV in powder and cylindrical pellet forms. The polymer solutions of PHB and PHBV were considered as homogeneous solutions in which it became a translucent and white milky solution with no apparent particles or glutinous substances in the solution. Based on the results (Figure 5.1), both polymers were able to dissolve 4-times higher than its initial mass of 0.6 g (1% w/v) in 8.2 ± 2.28 min (PHB) and 17.8 ± 1.48 min (PHBV). Then, as the mass increases from 2.4 g (4%, w/v) to 3.0 g (5%, w/v), the solution became inhomogeneous. However, after 41 ± 4.18 min (PHB) and 56 ± 8.22 min (PHBV) of continuous heating, the colour and the appearance of the solution was observed to change noticeably by turning from white milky solution into dark brown to indicate the
deterioration of a solution due to the thermal decomposition. On the other hand, the solubility rate of PHB was observed to be higher than that of PHBV (Figure 5.1; \( p < 0.05 \)) as expected due to the 2.3-fold lower molecular weight (\( M_w \)) of PHB (300,000 g mol\(^{-1}\)) compared with that of PHBV (680,000 g mol\(^{-1}\)). The results were consistent with Sperling and Nicholson [193, 194] where the lower molecular weight (\( M_w \)) of polymer, the faster it dissolves into the bulk solution although the crystallinity (%) of PHBV was by far lowered than PHB (Table 5.2). Hence, the polymer molecular weight (\( M_w \)) was considered the prominent factors that put in order of which polymer solubilized first. Meanwhile, based on the visual observation, polymer concentrations of 1% to 4% (w/v) and 5% (w/v) for both polymers were considered as homogeneous and inhomogeneous solutions respectively. In fact, the visible accumulation of glutinous semi-solid substances was observed in both polymer solutions of 5% (w/v). Thus, it was suggested that the optimal polymer concentration (saturation point) for PHB and PHBV should be between of 4% to 5% (w/v). Furthermore, a statistical approach of using design of experiments (DOE) was suggested to determine the exact optimal concentration for both polymers.

**Figure 5.1:** Kinetics of the PHB and PHBV homogenization process in chloroform with respect to different concentrations, % (w/v). (A): Inhomogeneous polymer solutions occurred with the appearances of glutinous polymer materials at the bottom of the SCHOTT\textsuperscript{\textregistered} Duran bottle. Results shown were means (±sd) of 10 experiments/concentration (\( n = 10 \)). *Significant difference with \( p < 0.05 \) for the value changed as compared to the previous value. \( ^{\Psi} \) \( p < 0.05 \) for solubility rate of PHB vs. PHBV.
Table 5.2: Melting temperature, heat of fusion at melting point and crystallinity of PHB and PHBV.

<table>
<thead>
<tr>
<th>Polymer[a][b][d]</th>
<th>Melting temperature</th>
<th>Heat of fusion at melting ($\Delta H_m$)</th>
<th>Crystalinity (%)[c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB</td>
<td>179.9 °C</td>
<td>104,974 J/kg</td>
<td>71.9</td>
</tr>
<tr>
<td>PHBV (12% PHV)</td>
<td>152.1 °C</td>
<td>72,708 J/kg</td>
<td>49.8</td>
</tr>
</tbody>
</table>

[a] Thermal analysis data was provided by the Sigma-Aldrich®. [b] Thermal analyses of PHB and PHBV was measured using a model DSC-7 differential scanning calorimeter (Perkin Elmer, USA) under a nitrogen atmosphere, at a heating rate of 10 °C/min. [c] Crystallinity was determined using the following heat of fusion values for 100% crystalline materials: $\Delta H_0$, PHB = 146,000 J/kg. The $\Delta H_0$ for PHBV was assumed to be the same as that for PHB [195]. [d] The degree of crystallinity, $H^*(\%)$; of the polymer could thus be estimated by using the following equation: $H^*(\%) = \Delta H_m/\Delta H_0 \times 100\%$.

5.4.2 Polymer Concentrations with Respect to Polymeric Porous 3-D Scaffolds Thickness

The thickness of porous 3-D scaffolds was considered as one of the important physical characteristics to produce a 3-D bone marrow biomimicry in vitro, which supports cellular proliferation. As far as we know, no studies have demonstrated the formation of PHB and PHBV porous 3-D scaffolds with a thickness greater than 1 mm. Most of the studies [70, 147, 152, 153, 177] have shown that PHB and PHBV can only be fabricated as thin films with a thickness of less than 1 mm irrespective of which methods were implemented. Based on the above mentioned results, the polymer concentrations of 1% to 4% (w/v) were selected for fabricating the porous 3-D scaffolds. During the fabrication process of SCPL, both polymers with concentrations of 1% and 2% (w/v) produced thin films with a thickness of 514 ± 4 µm. In fact, both polymers were exemplified brittle thin films with no apparent porous structure. As for both polymers with concentration of 3% and 4% (w/v), a significant increase of thickness was observed ($p < 0.01$) (3% (w/v): 1.80 ± 0.79 mm (PHB), 1.60 ± 0.79 mm (PHBV) and 4% (w/v): 5.25 ± 0.36 mm (PHB), 4.40 ± 0.52 mm (PHBV)). Even though both polymers with a concentration of 3% (w/v) produced a substantial level of thickness, it also developed a brittle thin film on top of the porous 3-D scaffolds due to the rapid phase separation (etching effect). However, for both polymers with a concentration of 4% (w/v), there were no apparent irregularities of the thickness, no etching surfaces observed and the formation of porous 3-D scaffolds appeared to be ideal with a thickness greater than 1 mm (Appendix B). Based on the above mentioned findings, it can be concluded that a polymer concentration of 4% (w/v) was an ideal concentration in fabricating a good representative of porous 3-D scaffolds with an improved thickness greater than 1 mm (in relative to the conventionally made PHA matrices). These polymeric porous 3-D scaffolds of 4% (w/v) were further utilized to determine the efficacy of salt leaching process and the effect of sodium chloride residual over cell growth
media electrolytes balance via conductivity measurement. Morphology of the scaffolds prepared using SCPL on different polymeric concentrations are shown in Figure 5.2.

![Figure 5.2](image)

**Figure 5.2:** Morphology of the polymeric porous 3-D scaffolds prepared using SCPL technique: (a) Aerial view of the PHB 5% (w/v) 3-D scaffold with scattered etching brittle thin films; (b) Aerial view of the PHBV 5% (w/v) porous 3-D scaffold with several ruptured spots due to the salt-leaching process; (c) Solid brittle thin film with apparent heterogeneous surfaces; (d) Aerial view of the PHB and PHBV 3% (w/v) which produced a solid etching brittle thin films on top of the porous 3-D scaffolds.

### 5.4.3 Efficacy of SCPL Measured Via Conductivity and Gravimetric Analysis

One of the disadvantages using SCPL is the existence of porogen residual. Until now, none of the previous studies who used SCPL in fabricating porous 3-D scaffolds have explained the salt-leaching efficacy and the amount of porogen remnants left inside the fabricated porous 3-D scaffolds. Only a few studies have shown some concern about the porogen residual [83, 174, 176] but none of them have ever conducted any independent experimental work to validate such claims. It was observed that the salt-leaching efficacy of PHB porous 3-D scaffolds (92.95 ± 2.39%) was significantly higher than PHBV (82.20 ± 4.87%) ($p < 0.05$). This indicates that salt-leaching process in PHB porous 3-D scaffolds was more efficient than in PHBV. Moreover, it is expected that water will leach NaCl more efficient than PHBV because PHB is more hydrophilic than PHBV (Table 5.4) as it allows water to penetrate deep inside the porous
structure. On the other hand, both polymers produced high efficacy of salt removal after the lyophilization process measured via gravimetric analysis (PHB: 99.66 ± 0.15%; PHBV: 99.96 ± 0.02%). The results showed that there was no significant loss of polymer weight throughout the whole process. In fact, a visible observation was carried out to proof the above mentioned report wherein there was no apparent detachable material the scaffolds floating on the water during the salt-leaching process. However, there was approximately less than 0.2 g of NaCl extra weight (based on the gravimetric analysis) in the dried cylindrical shapes of porous 3-D scaffolds, which indicate that the salt remnants were still trapped inside the porous structure. Overall, the gravimetric analysis is considered the best method to determine salt leaching efficacy than the conductivity measurement. The insensitivity testing probe, inaccuracy of the automatic temperature compensation system, fluctuations of ambient temperature, inaccurate concentration of prepared standard salt solutions and unstirred salt solution during the conductivity measurement could be some of the reasons of a big difference between the gravimetric results.

5.4.4 Effect of Sodium Chloride Residual in Polymeric Porous 3-D Scaffolds on Cell Growth Media

Relating to the above mentioned findings, both polymeric porous 3-D scaffolds were subjected to 7 days incubations at 37 °C and 5% CO₂ in cell growth media to observe conductivity (κ) changes due to the salt remnants inside the scaffolds (Figure 5.3). No media changes were conducted until day 7. The cell growth media were prepared according to the recommendation of the cell provider and the initial pH was adjusted to 7.2. Thus, the conductivity (κ) of cell growth media without a scaffold (control) was measured with a value of 20.77 mS/cm at 20 ± 1 °C. It was observed that as the time of incubation progresses up to 7 days, there were insignificant differences of conductivity (κ) between both polymers in comparison with the control (p > 0.05). This indicates that the amount of NaCl left inside the porous 3-D scaffolds was irrelevant in affecting the electrolytes balance of cell growth media. In fact, the amount of sodium chloride left inside the porous 3-D scaffolds might be considered as too small to produce any adverse effect to cell growth due to the imbalance of electrolytes from the hypertonic media solution (i.e., excessive inorganic salts).
Figure 5.3: Conductivity (κ) of cell growth media as a function of time at 20 ± 1 °C. The polymeric porous 3-D scaffolds were submerged in cell growth media (90% IMDM + 10% FBS + 1% PS) and incubated at 37 °C, and 5% CO₂ for 7 days. NS indicates no significant difference as compared with the control (n = 3).

5.4.5 Structural Analysis: Mercury Intrusion Porosimetry (MIP)

For tissue engineering applications, the most essential parameter of the pore network is diameter of the interconnecting pore apertures. However, the optimal value of the pore size and interconnection size are still open to debate [196]. Generally, the acceptance interconnection size to enable blood vessel ingrowth, cell invasion and enhanced biological response is between 50 to 100 µm [197]. Values of ideal pore diameters have been reported between 100 to 500 µm [73, 198]. Figure 5.4 shows interconnected pore distributions obtained from mercury intrusion porosimetry (MIP). The vertical axis is a derivative of the volume of mercury intruded into the 3-D porous scaffold relative to the interconnect pore diameter [199]. Moreover, majority of the interconnected pore window diameter (D_{mode}) for both of the scaffolds were observed to be more than 100 µm (PHB = 100 µm to 200 µm; PHBV = 211 ± 5.7 µm). The pore size distributions for both polymers were observed to be in the range of −1 µm to 350 µm. According to Safinia et al. [157] although this method could give incorrect pore size distributions (PSDs) due to the severe compression, MIP could still provide a reasonable pore size distribution. Alternatively, a maximum bubble point test could be the best non-destructive method to examine the pore size distribution accurately as it measures based on the number of bubble streams, which relates to the number of pores going through the entire scaffold. From this pressure, the values for the corresponding pore diameters could be eventually calculated.
Figure 5.4: Interconnected pore distributions of PHBV and PHB (4%, w/v) porous 3-D scaffolds determined by using mercury intrusion porosimetry (MIP). ($\Psi$) $p < 0.05$ for the volume of mercury intruded increase/decrease as compared to the previous value ($n = 3$).

5.4.6 Material Characterization: Physical Characterization of the Polymeric Porous 3-D Scaffolds

The physical properties of the porous 3-D scaffolds (i.e., geometrical bulk density, skeletal density and porosity) were summarized in Table 5.3. The geometrical bulk density, skeletal density and porosity of the PHBV were observed to be significantly higher than PHB ($p < 0.05$). In fact, both polymers have a total porosity in the range of 80% to 90%. However, the high porosity of PHBV might be due to the low crystallinity, high molecular weight [73] and more flexibility than PHB as both polymers used the same salt weight fraction (%, w/w) to develop a highly porous structure. As for the BET surface area, PHBV was exhibited to be significantly higher than PHB ($p < 0.05$), which is a clear indication that pore sizes decrease due to the high porosity of the scaffold (high surface area-to-volume ratio). Therefore, parameters such as anaverage apparent density (skeletal density), porosity and BET surface area do not provide any relevant information about the structural properties of the polymer scaffolds. Although the physical properties were irrelevant in describing structural behaviour, the properties (i.e., porosity and skeletal density) would possibly provide a glimpse on the cellular response. As to guarantee the entrance of large numbers of cells and allow the formation of cellular associations, a scaffold should possess high porosity with a complex network of channels and interconnected pores of appropriate size and shape [200]. Hence, PHBV is expected to allow
better cellular attachment and growth inside the porous structure than PHB which is in fact in good agreement with the cellular proliferation result on day 14.

**Table 5.3:** Physical properties of PHB and PHBV (4%, w/v) porous 3-D scaffolds. The table summarizes the principal physical properties of two polymeric porous 3-D scaffolds prior to the *in vitro* cell proliferation studies.

<table>
<thead>
<tr>
<th>Structural properties</th>
<th>Polymeric porous 3-D scaffolds</th>
<th>PHB (4%, w/v)</th>
<th>PHBV (4%, w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaffold thickness (mm)</td>
<td>5.25 ± 0.36</td>
<td>4.40 ± 0.52**</td>
<td></td>
</tr>
<tr>
<td>Pore size distributions (µm)</td>
<td>Pore sizes in the range: −1 to 350</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modal $D$ (µm)</td>
<td>100 to 200</td>
<td>211 ± 5.7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Physical properties</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BET surface area, $A_s$ (m²/g)[a]</td>
<td>0.70 ± 0.02</td>
<td>0.82 ± 0.03*</td>
<td></td>
</tr>
<tr>
<td>Geometrical bulk density (g/cm³)</td>
<td>0.084 ± 0.15</td>
<td>0.072 ± 0.28*</td>
<td></td>
</tr>
<tr>
<td>Skeletal density (g/cm³)[b][c]</td>
<td>0.47 ± 0.52</td>
<td>0.92 ± 0.14*</td>
<td></td>
</tr>
<tr>
<td>Porosity (%)</td>
<td>82.12 ± 0.18</td>
<td>92.17 ± 0.73*</td>
<td></td>
</tr>
</tbody>
</table>

***(p < 0.01) - Results were considered statistically significant (n = 10) as compared with PHB.**

**(p < 0.05) - Results were considered statistically significant (n = 4) as compared with PHB.**

$D = \text{interconnected pore window diameter (} D_{\text{mode}}: \text{one of a range of values that has the highest frequency). [a] BET surface area (m²/g) = total skeletal surface area (m²)/skeletal mass (g). [b] } \rho_s \text{ is the skeletal density of the crushed scaffolds, which was determined from helium pycnometry. [c] The higher pore volume (the higher the amount of absorbate intruded), the lower the skeletal volume.}

**5.4.7 Morphology of Porous Structure Using Scanning Electron Microscopy (SEM)**

The morphology of internal PHB and PHBV (4%, w/v) porous 3-D scaffolds were observed using SEM. A rectangular cube of porous 3-D scaffold was sectioned vertically to permit examination of their micro- and macro-pore structure. It was observed that both fabricated polymeric porous 3-D scaffolds were displayed with quite irregular pore morphology with an angular shape, as shown by the scanning electron micrographs of longitudinal cross-sections (Figure 5.5e and f). In addition, both polymers produced large amounts of closed pores than open pores with low availability of interconnected pores. In theory, the closely packed adjacent porogens after the porogen-leaching process will leave a hole between two closely-tight face-to-face contact porogens [201]. However, the two closely tight porogens might have been in the position of either side contact or point contact configuration which resulted in either no pores interconnectivity or small puncture inside the pores respectively (Figure 5.6) [202]. Therefore, based on the above mentioned reports, it was confirmed that both polymeric porous 3-D scaffolds demonstrate pore sizes in the range of −1 µm to 350 µm and availability of dispersed distribution of both open and closed pores with lower pore interconnectivity.
Figure 5.5: Morphology and SEM of polymeric porous 3-D scaffolds in cuboids with an approximate size of 10 mm × 10 mm × 5 mm: (a) Aerial view of PHB (4%, w/v), (b) Side view of PHB (4%, w/v), (c) Aerial view of PHBV (4%, w/v), (d) Side view of PHBV (4%, w/v), (e and f) vertical cross-sections of PHB (4%, w/v), magnifications 35x (e) and 100x of the boxed area (f), (g and h) vertical cross-sections of PHBV (4%, w/v), magnifications 35x (g) and 100x of the boxed area (h).

Figure 5.6: Influence of particle shape on the neck characteristics.

5.4.8 Physico-Chemical Analysis: Water Contact Angle and Surface Free Energy

Measuring the water contact angle ($\theta_{H2O}$) is a standard method used to study the hydrophilicity of a biomaterial surface and can help to evaluate the possible effect of a biomaterial on cell adhesion and growth [203]. Evidently, both polymeric porous 3-D scaffolds exhibited a true water contact angle ($\theta_{porous}$) in the range of about 94° to 117°, whilst all of the thin film counterparts fabricated by using the solvent casting showed values in the range of about 66° to 79° (Table 5.4) (Figure 5.7). The huge differences could be attributed to the heterogeneous wetting condition of the Cassie-Baxter regime where corrections of the contact angle need to be applied [204]. On top of that, both polymeric porous 3-D scaffolds and the corresponding thin films were considered as hydrophobic materials as they possess high degree of water contact angle of more than 65° [205]. Hence, the highly observed surface hydrophobicity of the polymeric porous 3-D scaffold in comparison with that of solvent-cast thin films (in accordance with the Cassie-Baxter regime) was perhaps due to the 1) surface heterogeneity (e.g., availability of surface roughness which could lead to hysteresis, different phases or different materials conforming the whole topography); 2) air trapped inside the pore grooves (enhanced the surface hydrophobicity because the drop is then partially sitting on air) and 3) contaminants of salt on the pore surfaces (which could lead to a decrease of solid/vapour surface tension) [206]. Finally, to determine the amount of wetting that occurred on both polymeric solvent-cast thin films; surface free energy ($\gamma_{SL}$), work of adhesion ($W_{SL}$) and spreading coefficient at solid-liquid interfaces were calculated (Appendix F). The results showed that both polymers produced (+) and (-) signs of the work of adhesion and spreading
coefficient respectively (the signs were described in Table 5.4). The wettibility of both scaffolds is shown in Appendix C. Thus, with all of the analyses taken, it is proved that both polymers were considered as hydrophobic materials.

Table 5.4: Wetting and wettability of water on PHB and PHBV solvent-cast thin films surfaces.

<table>
<thead>
<tr>
<th>Surface physico-chemistry</th>
<th>Solvent-cast thin film[a][b]</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHB (4%, w/v)</td>
<td>PHBV (4%, w/v)</td>
<td></td>
</tr>
<tr>
<td>Contact angle, $\theta_{\text{apparent}}$ (deg.)</td>
<td>66.80 ± 0.2</td>
<td>79.24 ± 0.4*</td>
<td></td>
</tr>
<tr>
<td>Surface free energy, mN/m ($\gamma_s$)</td>
<td>54.13 ± 0.3</td>
<td>46.93 ± 0.2*</td>
<td></td>
</tr>
<tr>
<td>Work of adhesive, mN/m ($W_{\text{SL}}$)[c]</td>
<td>+109.42 ± 0.2</td>
<td>+97.41 ± 0.3*</td>
<td></td>
</tr>
<tr>
<td>Spreading coefficient (S_{\text{H2O/thin film}})[d]</td>
<td>-36.38 ± 0.3</td>
<td>-48.39 ± 0.2*</td>
<td></td>
</tr>
</tbody>
</table>

[a] Equilibrium contact angle on solvent-cast thin films on polypropylene sheet ($n = 10$). [b] Contact angle of polypropylene (PP) sheet without PHB and PHBV coating = 92.43 ± 0.3°. [c] (+) or (-) work of adhesive: A non-spontaneous or spontaneous process respectively. [d] (+) or (-) spreading coefficient: Water will spread or not spread over the surface respectively. *$p < 0.05$ as compared with PHB.

Figure 5.7: Morphology of solvent-cast thin polymeric films: (a) Aerial view of PHB (4%, w/v) and (b) Aerial view of PHBV (4%, w/v).

5.4.9 In Vitro Degradation of the Polymeric Porous 3-D Scaffolds

Natural polymers degrade rapidly in the biological medium when appropriate hydrolytic enzymes are available and used [9, 207]. Whilst for the synthetic polymers since they are produced via chemical synthesis, they are quite resistant and disappear slowly from the environment [151]. Irrespective of polymer categories, the rate and mode of degradation of the polymers influence their service life, mechanical properties and the response of the biological system towards them [207]. The 80 days of in vitro degradation study was selected based on the reference of Yoshiharu et al. [208] where no mass loss was recorded on PHB films in a buffer solution at 37 °C until 180 days. A decrease in molecular weight was observed after an induction period of about 80 days. Therefore, based on that finding, a benchmark was set so that
we can observe the polymers degradability in the form of porous scaffold for a period of 80 days (short-term observation). The results would perhaps to give an initial indicator for studying its stability on a long-term *in vitro* cell proliferation (up to 6 weeks). Through visible observation, both PHB and PHBV porous 3-D scaffolds still preserved their structural integrity and morphology until the end of the incubation period although accumulation of medium particulates was observed from day 7 to day 48 and day 7 to day 37 respectively (Figure 5.8). The accumulation of medium particulates for both polymers was consistent with the mass analysis results. It was observed that mass of PHB porous 3-D scaffolds was gradually decreased ($p < 0.01$) from day 7 to day 48 by $13 \pm 4.6\%$ of their initial weight (Figure 5.8). Meanwhile, the mass of PHBV porous 3-D scaffolds decreased rapidly at day 7 ($p < 0.01$) and gradually reduced from day 7 to day 37 by $14 \pm 3.7\%$ of its initial weight. However, PHB and PHBV foams started to alleviate from day 48 and 37 respectively until day 81. Moreover, the extent of weight loss from the gravimetric analysis was seemed to be very significant due to the surface erosion where unlike the pH analysis on the same period, there was no prominent pH dropped to indicate any accumulation of acidic degradation products due to the autocatalytic hydrolysis effect. Apart from the mass analysis, it was observed that the rate of PHBV *in vitro* degradation process was noticeably much faster than PHB until day 48 ($p < 0.01$). However, both polymers then remained approximately at the same rate until day 81 ($p > 0.05$). Table 5.2 summarizes the melting temperatures, the heat of fusion at melting point and the crystallinity of the polymers. PHBV showed the lowest crystallinity values and PHB, the highest as provided by the Sigma-Aldrich™. The *in vitro* degradation test showed that PHBV degrades more rapidly than PHB at the earlier phase (day 1 to day 25). The phenomenon could possibly due to the degree of open porosity which could easily uptake more water through the capillary action. However, the data on this was inconclusive. It is recommended to study its permeability to determine the ability of the porous scaffolds to absorb water. On top of that, the degree of crystallinity would have a significant effect on the water uptake of the porous scaffold. In theory, the lower the degree of the crystallinity, the higher the degradation rate of degradable polymers be likely to occur due to the higher amount of amorphous region which makes the solvent molecules (e.g., water) to easily absorb inside the polymer matrix (the absorption of solvent inside the highly crystallized polymer is hindered due to its nature plate-like structure) [209]. This phenomenon will eventually make the ester-linkages to weaken the molecular structure as a result of the hydrolyzed acidic degradation products accumulation (i.e., carboxylic acid groups). However, there was no correlation between the extent of degradation and the crystallinity of the polymers since PHBV, which had lower crystallinity than the PHB, also showed almost no severe degradation during the period of 25 to 81 days on both pH and weight examination.
Figure 5.8: Kinetics of *in vitro* degradation process for PHB and PHBV (4%, w/v) porous 3-D scaffolds were measured via mass analysis. The polymeric porous 3-D scaffolds were submerged in phosphate buffered saline (PBS) and incubated at 37 °C. Samples were periodically removed and dried under vacuum pressure prior to the analysis. (* p < 0.05 for percent decreased as compared to the previous value (n = 6). Ψ Significant difference with p < 0.01 between each polymers are highlighted by the line (n = 6).

5.4.10 Mechanical Properties of Polymeric Porous 3-D Scaffolds

The *in vivo* cancellous bone (containing soft marrow tissue) has porosity ranges 30% to 90% [210]. As we were mimicking the skeletal structure and microenvironment of the cancellous bone, the usage of compression testing on this porous 3-D scaffold is advantageous. Although the objective of this study is to produce a 3-D biomimicry *in vitro* model, this 3-D biomimicry scaffold has the potential to be used to treat leukaemia patient by replacing the abnormal cancellous bone with stem cells-loaded scaffolds via *in vivo* implantation.

Figure 5.9 shows the stress-strain behaviour of PHB and PHBV scaffolds under compressive loading. Table 5.5 shows the mechanical properties of the polymeric scaffolds. The results indicate that both polymeric scaffolds were not significantly different to their compression modulus (E) and ultimate compressive strength (p > 0.05). The insignificant differences in the compression modulus can be attributed to the fact that both PHB and PHBV scaffold possesses a fair distribution of the anisotropic pore size even though both of them contained different porosity. On top of that, under the compressive force, PHBV and PHB scaffolds underwent three main stages of deformation, which are commonly observed for porous structures (the so-called ‘cellular structures’ in solid mechanics) [211]. Under compression, the scaffolds exhibited a non-linear elasticity at low stresses known as a toe region (high deformation/low force characteristic) and then followed by the stiffness regime (the linear elasticity is controlled
by cell wall bending). The stress rose steeply as it entered the densification regime where further compaction led to the collapse of the cells (of the ‘cellular structure’) [212]. When the cells have almost completely collapsed, opposing cell walls touch, with further strain compressing the solid itself, giving the ultimate compression stress with the ruptured of the structure. Apart from that, it is evident from Figure 5.9 that both, PHB and PHBV scaffolds did not undergo elastic deformation during compression (no unloading curves were observed) due to the fact that both polymeric scaffolds were crushed, compacted and ultimately shattered into several fragments until a compression of about 87% to 90% of the initial test piece thickness is attained. However, if both polymeric scaffolds were compared with other porous materials (e.g., porous biodegradable composites, porous bioactive ceramics and porous biodegradable polymers with 75% porosity and mostly interconnected) and cancellous bone (elastic modulus: 0.1 to 0.5 GPa and compressive strength: 4 to 12 MPa) [13, 213], both polymers have a very low mechanical strength and perhaps unsuitable to be used for \textit{in vivo} implantation application, unlike for the \textit{in vitro} 3-D biomimicry culture that sustained structural integrity of the scaffold for up to 6 weeks in cell growth media. This phenomenon may well be explained by the fact that both polymers have high in porosity (> 80%) which resulted in fewer thick pore walls and apparently make them easier to bend and compress to a certain level [13].

\textbf{Figure 5.9:} Stress-strain behaviour of PHB and PHBV (4%, w/v) porous 3-D scaffolds under compressive loading.
Table 5.5: Mechanical properties of PHB and PHBV (4%, w/v) porous 3-D scaffolds.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Mechanical Properties</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Compressive modulus</td>
<td>Ultimate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(GPa)[a]</td>
<td>compressive</td>
<td>strength</td>
</tr>
<tr>
<td>PHB (4%, w/v)</td>
<td>0.00027 ± 0.15</td>
<td>1.97 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>PHBV (4%, w/v)</td>
<td>0.00019 ± 0.32</td>
<td>1.83 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

[a] Mean (±sd) slopes of the stiffness area (C) as shown in Figure 5.9. [b] Samples were crushed, compacted and eventually ruptured into several fragments (n = 3).

5.4.11 Cell Culture on Porous 3-D scaffolds: Acute Myeloid Leukaemia (AML)

5.4.11.1 Seeding Efficiency of Acute Myeloid Leukaemia Cell Line (HL-60) on Polymeric Porous 3-D Scaffolds

The ability to support the attachment and promote the proliferation of cultured cells is a prerequisite of a functional scaffold [82]. To evaluate a cellular behaviour on the PHB and PHBV porous 3-D scaffolds, a human cell line of acute myeloid leukaemia (HL-60) was seeded initially in the 24-sterile cell culture well plate for 24 h. The seeding efficiency shows the percentage of cells that remained in the scaffold after 24 h of seeding. It can be seen that the seeding efficiency for PHB and PHBV were not statistically different (p > 0.05) with 56.10 ± 20.73% (207,657 ± 76,869 cells) and 36.73 ± 11.21% (136,182 ± 41,574 cells) respectively (Figure 5.10). The results were consistent with earlier study [6] as most of the FDA-approved polymers (e.g., PDLLA, PLGA, PS, PMMA, PCL and PU) seeded with AML cell line (HL-60) have a seeding efficiency in the range of 40% to 70%.

Figure 5.10: Cell seeding efficiency of PHB and PHBV scaffolds seeded with HL-60 cell line. The cultures were performed in parallel on 2 separate occasions, each in quadruplicate.
5.4.11.2 Cell Proliferation Assay of Acute Myeloid Leukaemia Cell Line (HL-60) on Polymeric Porous 3-D Scaffolds

After the cell seeding efficiency on PHB and PHBV porous 3-D scaffolds has been determined, both polymers were then subjected to a short-term cell proliferation until day 14. The MTS assay was used to compare the cellular proliferation on two types of polymeric porous 3-D scaffolds via absorbance measurement ($\lambda = 490$ nm) as shown in Figure 5.11. Evidently, the cellular growth of HL-60 was significantly higher ($p < 0.05$) for both polymers from day 1 to day 7. Then, the cellular growth of HL-60 was significantly higher for PHBV ($p < 0.05$) from day 7 to day 14 whilst PHB remains unchanged. The probable explanation for this phenomenon might be due to the highly porous nature of PHBV 3-D scaffolds which has provided extra surfaces onto which the cells can propagate deep inside the scaffolds (Figure 5.13a to d). In fact, it also provides more structural space for the accommodation and attachment of cells and enables the efficient exchange of nutrients and metabolic waste. Apart from the MTS analysis, viability of the cells extracted from the scaffolds was assessed at days 0, 7 and 14 (Figure 5.12). The cells cultivated in the PHBV 3-D porous scaffolds had the best viability compared with those supported by the PHB scaffolds ($p < 0.05$). Overall, the low seeding efficiency, low viability of the cells and slow cellular growth from day 1 up to day 14 on both polymeric porous 3-D scaffolds were possibly due to the high surface hydrophobicity and availability of dispersed open pores with lower interconnectivity. The high surface hydrophobicity could lead to a low absorption of important adhesive ECM proteins (i.e., fibronectin and vitronectin) from the supplemented serum-containing culture media for modulating cell adhesion and proliferation. Whilst dispersed open pores with lower interconnectivity could produce insufficient mass transport across the porous 3-D scaffolds (i.e., nutrients and metabolites) or possibly cells clogged during the initial cell seeding due to the overcrowded cells inside the non-interconnected pore which could trigger necrosis.
Figure 5.11: Cellular growth of AML cell line (HL-60) on PHB and PHBV. The MTS assay was used as a measure of proliferation of leukaemic cell line (HL-60) on both polymers. (*) PHBV displayed a significantly higher ($p < 0.01$) absorbance in comparison to PHB at day 14. Results shown were means ($\pm$sd) of quadruplicate wells ($n = 4$) obtained by subtraction from cell-free equivalents, to eliminate $A_{490}$ produced by the scaffold + media (negative control) alone.

Figure 5.12: Viability of the HL-60 cells seeded onto the PHB and PHBV porous 3-D scaffolds during a period of 14 days. *$p < 0.05$, compared between groups. For all panels, results were means ($\pm$sd), $n = 2$ replicates per experiment, $N = 2$ separate experiments.
5.5 Discussion

Both natural and synthetic scaffolds for tissue engineering provide a matrix onto which cells may adhere and aid in shaping and defining \textit{in vivo} cell growth [146, 149]. In fact, the use of scaffolding materials for tissue engineering is an attempt to mimic a 3-D microenvironment of the extracellular matrix (soft or hard connective tissue) [187]. For that reason, to promote a tissue formation and other cellular proliferation mechanisms, an ideal scaffold should provide a highly biocompatible 3-D substrate to enable cell adhesion, migration, proliferation and differentiation function [181]. Most of the fabricated scaffolds made from PHB and PHBV using the SCPL method produced matrices, which was lesser than 1 mm in thickness. This could possibly due to several uncontrolled process parameters (i.e., polymer concentrations, polydispersity (M\textsubscript{w}/M\textsubscript{n}), rate of solvent evaporation, salt weight fraction, etc.) which have resulted in the formation of various scaffolds architecture (i.e., uneven surfaces, formation of etching brittle thin films, etc.). These problems were consistent as reported by Hutmacher [214].
and other studies [13, 158, 215] whereas this technique is very likely to produce a limitation to thin structures, residual particles in the polymer matrix and irregularly shaped pores. However, to the extent of our knowledge, no other researchers have investigated an ideal polymer concentration that could create a reasonable thickness of porous 3-D scaffolds specifically using SCPL technique. Up to now, there are two studies [191, 192] which have successfully fabricated the PHB and PHBV/apatite composite porous 3-D scaffold with a thickness greater than 1 mm prepared by using SCPL with a polymer concentration of 3% (w/v) and 10% (w/v) respectively. However, the study done by Huang et al. [192] was contrary to our findings that further incremental of polymer concentrations up to 5% (w/v) would eventually produce an inhomogeneous solution (Figure 5.1). In fact, the usage of 5% (w/v) onwards might have resulted in structural instability (i.e., the detachable part of the scaffolds), formation of etching surfaces and irregularities of shape and sizes. Moreover, it has been expected that a composite material would actually produce porous 3-D scaffolds with a thickness greater than 1 mm because any incorporating of reinforced materials (i.e., inorganic materials) might have increased the sturdiness and flexibility of the formed 3-D structure due to the reduced polymer crystallinity [216]. As for the study done by Misra et al. [191], they used sugar cubes as the porogen preform with thickness of 10 mm. The PHB solution in chloroform was impregnated into the sugar cubes until saturated, dried and leached using water. This ingenious foam replication method will eventually produce a

Besides the structural instability issues with respect to polymer concentration, another problem that could occur during the fabrication process is the formation of brittle thin film (known as the ‘etching effect’) on surface of the scaffolds. The formation of etching thin films for polymers concentration of 3% and 5% (w/v) were consistent with the reference of Hutmacher [214] where skin layers are formed during rapid evaporation of solvent and eventually resulted in agglomeration of salt particles underneath the skin layers. This agglomeration of salt makes the pore size difficult to control and detachable sections of polymer cast during salt leaching process were seems to be unavoidable [214]. Apart from that, irregularities of scaffold thickness were due to the difficulty in removing salt particles deep inside the matrix of polymer-porogen cast (during the salt-leaching process) [83]. This problem was consistent as reported by Thomson [217] wherein the thicker the scaffold produced from SCPL (i.e., greater than 1 mm), the harder the salt needed to be removed deep inside it. As the scaffold accumulated such amounts of salt, it can easily produce detachable sections during the salt-leaching process due to the unbearable extra weight content, which could have resulted in irregularities of scaffold thickness. Equally important, the author has also raised some doubt as to whether the technique was applicable in the reconstructive applications where scaffolds with several centimetres (cm) thick may be required for mimicking the thickness of extracellular matrix (ECM) and other parts of tissue or bone. However, we have proof that with a thickness
greater than 1 mm, the structural integrity of both polymeric porous 3-D scaffolds remained intact with no apparent weight losses. In fact, we have also proof that with the thickness greater than 1 mm, there were irrelevant electrolytes changed in cell growth media, although there was a significant amount of salt remnants left inside both polymeric porous 3-D scaffolds.

Furthermore, an in vitro degradation study was carried out to measure the stability of PHB and PHBV porous 3-D scaffolds in the hydrolytic media of phosphate buffer saline (PBS). The results showed a significant mass loss due to the surface erosion [218] (Figure 5.8). The above finding was consistent with the insignificant pH change’s result which indicate that there was no autocatalytic degradation (breakdown products produced in the initial phase accelerate the rate at which subsequent degradation proceeds) going on inside the porous 3-D scaffolds throughout the whole incubation period. In fact, the results were also in line with Zinn et al. [9] study wherein PHB was degraded by surface erosion at a slower rate than PHBV due to their high crystalline properties.

On the other hand, both polymeric porous 3-D scaffolds were considered high in surface hydrophobicity as compared to the corresponding solvent-cast thin films (Table 5.4). Thus, the huge differences in water contact angle (θH2O) were possibly due to the rough surfaces in which can be attributed to a surface heterogeneity (from the distribution of pore size), [161] air that may be trapped on the rough surface (the droplet is partially sitting on air), high crystallinity (compressed aggregate of plate-like chain packing of the outermost groups of atoms at the air/solid interface) which could hinder wetting through H-bonding due to the hidden polar functional groups [219] and possibility of salt remnants on the pore surfaces, which influence the interfacial behaviour between vapour, liquid and solid. In fact, one of the above mentioned influences was well in agreement with Ito et al. [220] wherein water contact angles of 3-D PHBV (2%, w/w) nanofibre mat were greater than that of the corresponding solution-cast thin films (i.e., approximately 110° versus 81° respectively). The observed hydrophobicity of the fibre mats in comparison with that of the films was due to the surface roughness that introduced multiple contact points on the surface of the water droplet such that the interface between the water droplet and the fibre mat surface was not exactly solid/liquid [221]. However, it was suggested that in order to alter the hydrophobicity of the surfaces, one possible method is to modify the surface chemistry by using several well known treatments (i.e., acids-bases, enzymes, gas plasma and other chemical reagents).

Finally, by analyzing the in vitro cell proliferation results, we found out that there were several physico-chemical characteristics that have significantly affected low density of cell attachment and proliferation. First, the availability of randomly open pores with fewer amounts of pore throats interconnectivity linking to individual pores in both polymeric scaffolds although there are many interconnected pores with diameters greater than 100 µm (Figure 5.4). This has led to a weak distribution of cells throughout the porous structure. This phenomenon
has resulted in less cell migrations, leading to greater cell death in the initial growth phase due to the increased overcrowding situation [222]. However, the cellular growth trend changed (an increase in absorbance) as time progresses due to the microenvironment changes inside the cell-loaded porous scaffolds. This is well in agreement with our in vitro degradation result suggesting that within 2 weeks of incubation, the internal porous structure of the scaffold has changed dramatically. This implies that the microenvironment of the cells whose number increase in time within the structure carrier is expected to become less restrictive as time progresses because the foam gets gradually more hollow making more volume available for cell proliferation [151]. For that reason, we absolutely agreed that a good arrangement of physical properties may promote high cell attachment and proliferation by providing more spaces for better gas, nutrients exchange or more serum protein adsorptions and adequate chemical signaling for migration, differentiation and apoptosis [6, 163].

Apart from the influence of pore interconnectivity, it seems that the surface chemistries of both polymers might also contribute to the low cell attachment and growth observed. Wang et al. [223, 224] have reported that the appropriate hydrophilic or hydrophobic area distribution was very important for cell attachment. The appropriate hydrophilicity/hydrophobicity of material has to do with the certain degree of surface free energy. This surface free energy might play a role in attracting particular proteins (i.e., collagen type I and fibronectin) to the surface of the scaffold and in turn improving the attraction (affinity) of the cells to the scaffolds. High surface free energy would also facilitate the serum-supplemented cell growth media to spread out further and enhance the adsorption of ECM proteins monolayer onto the polymeric surface which in turn, increased the cell adhesion, spreading and proliferation.

Third, in nature, leukaemic stem cells growth in a soft marrow tissue (a 3-D gel-like micro-environment) which consists of heterogeneous population of haematopoietic stem cells and non-haematopoietic stem cells. The 3-D gel-like microenvironment biomimicry scaffolds (e.g., 3-D hydrogel) would be the best environment for culturing leukaemic cells rather than on the porous 3-D scaffold as it can support high cell density and no need of any treatment to render its surface hydrophobicity. Vu et al. [225] have shown an increased in leukaemia cell viability and an improved 3-D cell proliferation (27-fold) by using a standard 3-D hydrogel compared to 2-D cultures (18-fold). Moreover, immobilization with RGD peptides promoted further cell growth (43-fold). The cells were packed in clusters form inside the alginate hydrogels and spontaneously differentiated into a more diverse myeloid population. However, the limited mechanical stability and fast bulk degradation of hydrogels in cell growth media have been a major drawback in cell culture work. For that reason, the best way to mimic the real microenvironment of bone marrow is to fill a porous 3-D scaffold structure with hydrogel which acts as a soft marrow tissue housed inside the 3-D skeletal structure. This infusion method could
possibly provide a great potential for studying extrinsic contributions inside the abnormal bone marrow by providing a robust and faithful 3-D platform.

5.6 Conclusion

In conclusion, a polymer concentration of 4% (w/v) for PHB and PHBV was an ideal concentration to promote a good representative of porous 3-D scaffolds with an improved thickness greater than 1 mm (in relative to the conventionally made PHA matrices). In fact, both polymers have produced stable structural 3-D porous scaffolds with no etching brittle thin films on top of the scaffolds, detachable sections and irregularities in its shape and size. Moreover, the insignificant differences of conductivity (κ) for both polymeric porous 3-D scaffolds immersed in cell growth media in comparison with the control (cell growth media with no scaffolds) demonstrated that the availability of salt remnants inside the porous structure were irrelevant in affecting the electrolytes balance which could lead to cellular damage. Overall, an improved thickness of PHBV (4%, w/v) porous 3-D scaffolds had shown better performances of HL-60 cellular attachment and proliferation than PHB. This result has demonstrated that either these artificial structures were fabricated as solvent-cast thin films or porous 3-D scaffolds, the low degree of cellular attachment and slow proliferation observed seem to be prominently affected by the highly hydrophobicity surfaces. Apart from that, the development of randomly open pores with lower interconnectivity for both polymeric scaffolds might also contribute to the observed problem. Therefore, it could be suggested that by modifying to a certain levels of surface modification (i.e., rf-O₂ plasma or alkaline treatment) and fabrication techniques (i.e., spherical porogen with compression), both polymeric porous 3-D scaffolds would perhaps to allow better cellular attachment and growth due to the well adsorption and distribution of the ECM proteins and dispersive interconnected pores. However, further evaluations are still required to confirm such preliminary results.
CHAPTER 6 Surface Treatments of the Polymeric Porous 3-D Scaffolds with an Improved Thickness to Enhance Cell-Biomaterial Adhesions and Interactions

6.1 Introduction

Polyhydroxyalkanoates (PHA) are a class of biodegradable polyesters, attracting research for the use in tissue engineering application due to its slowly degrading characteristics. These stereoregular polymers (having an ordered arrangement of pendant groups along the chain) are synthesized by numerous bacteria as an intracellular carbon and energy storage compound and accumulated in granules in the cell’s cytoplasm [226]. Most of the naturally occurring reserved polymer granules contain specifically poly(3-hydroxybutyrate) (PHB). Depending on the bacterial species and carbon substrates, copolymers with a variety of side-chains and corresponding properties can be produced by fermentation technologies [227]. Although a thermoplastic polyester with properties similar to polypropylene, PHB is considered to be too brittle for many applications. Copolymers and blends of -3HB with other monomers such as 3-hydroxyvalerate, 3-hydroxyhexanoate, and 4-hydroxybutyrate, provide superior mechanical characteristics to PHB and permit modulation of the degradation dynamics [21, 170, 228-230]. In contrast to 3-hydroxyvalerate (as PHBV), the incorporation of 4-hydroxybutyrate as [P(3HB-co-4HB)] has a much stronger influence on the polymer properties. Therefore, it has been considered as one of the most suitable PHA for implantable medical scaffolds due to its nontoxic degrading products which is occurring naturally in the human body [231].

Although it might be highly suitable for implantable medical material, cell culture experiments on that particular polyester suggested a different kind of story. It seems that mammalian cell line (e.g., human umbilical vein endothelial cells (HUVECs)) attached to PHB suffered from altered cell morphology, cytoskeletal characteristics and cell adhesion formation as compared to other surfaces such as glass or tissue culture polystyrene [227]. Despite the fact, there were some studies that relate to some alteration of the cell physico-chemical characteristics, there is a much bigger problem faced by this type of biodegradable polyester, which is a low cell affinity due to its highly hydrophobic material. As a hydrophobic polymer material, the poor hydrophilic property of PHA has hindered the extensive usage in the field of medical application [232]. Thus, it is necessary to improve the hydrophilic properties of PHA to meet the rigorous tissue engineering requirements. A possible and effective way is through surface modification. In order to trigger the cell-matrix adhesion on PHA surfaces, several surface treatment techniques [2] have been recently applied including alkaline hydrolysis, [233, 234] ion implantation, [235] ammonia plasma treatment [236, 237] or rf-oxygen plasma treatment [151] followed by chemical grafting [238, 239] and lipase treatment [156]. However, in some cases of the surface chemical grafting (e.g., immobilization of bio-molecules), the
interaction between bulk polymer and the bioactive molecules is usually very weak (non-covalent interactions) and thus the surface is quite unstable [240]. In this study, we are looking at the rf-oxygen plasma and alkaline treatment since both methods have been used extensively in surface modification. Yet, both have their advantages and disadvantages. One of the problems of plasma treatment is that the effect of surface treatment is not permanent since the surface tends to revert to the untreated state in a short time (potential aging) [241]. However, it is likely depending on how it is stored or preserved prior to the usage (e.g., storage in low temperatures of 0 °C to 4 °C) [242, 243]. In comparison, alkali treatment has been proven to be an effective method to improve the wetting and adhesion property of polymer surface [227].

However, the impact of the surface modifications on the adsorption and displacement patterns of proteins and the subsequent cell response are hardly understood so far [227]. For instance, Rouxhet et al. [233] found that alkaline hydrolysis of PHA films increased adsorption of fibronectin and albumin. This effect could not be solely attributed to the altered wettability by the surface hydrolysis (functionalized with oxygen-based polar groups) but has to be seen in the context of the well-known complexity of protein interaction with solid substrates. Although hydrophobicity plays a major role in the attachment of proteins to biomaterials, other interactions like electrostatic charges (surface charges), dipole-dipole interactions, hydrogen bonding as well as sterical and entropical forces have also to be considered as providing important contributions [244].

6.2 Aim and Hypothesis

This investigation was based on the hypothesis that surface treatments (alkaline and rf-O₂ plasma) on the PHB and PHBV porous 3-D scaffolds would facilitate the adsorption of two bone marrow ECM proteins (collagen type I and fibronectin), enhance the cell affinity on the treated surface and cells expansion on the 3-D cell culture system.

6.3 Approach of Study

In the light of these recent findings and in order to provide a better understanding of the effective interactions arising from the physico-chemistry of PHA substrates towards cell affinity, the current study aimed to allow for a selection of the appropriate physico-chemical modifications to be used for the biological surface coating with two types of bone marrow main extracellular matrix (ECM) proteins (collagen type I and fibronectin) in the sense that the cell-biomaterial affinity could be increased sufficiently and in turn increasing cell growth. The surface of porous 3-D scaffolds of PHB and PHBV (4%, w/v) were modified by two different procedures: (i) alkaline hydrolysis (serial concentrations of aqueous NaOH solution) and (ii)
low radio frequency oxygen plasma (rf-O₂ plasma) treatment. The changes of its physico-chemical properties were confirmed by measurement of water contact angle, ζ-potential measurement, helium pycnometer, nitrogen absorption BET measurement and scanning electron microscopy (SEM). Other miscellaneous measurement was also conducted such as in vitro degradation in cell growth media. The cell-biomaterial affinity on PHB and PHBV scaffolds before and after the surface modification were also evaluated for a human lymphoid cell line (RL) (Table 6.1) by means of MTS assay and cell viability measurement.

Table 6.1: Chronic lymphocytic leukaemia (CLL) cell line properties. The table summarizes the main properties of the RL cell line.

<table>
<thead>
<tr>
<th>Cell properties</th>
<th>Cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell phenotype</td>
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<tr>
<td>Original disease of patient</td>
<td>non-Hodgkin's lymphoma</td>
</tr>
<tr>
<td>Patient data</td>
<td>52-year-old Caucasian male</td>
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<tr>
<td>Source of material</td>
<td>Ascites tissue</td>
</tr>
<tr>
<td>Year of establishment</td>
<td>1983</td>
</tr>
<tr>
<td>Culture Medium</td>
<td>90% RPMI 1640 + 10% FBS</td>
</tr>
<tr>
<td>Sub-cultivation routine</td>
<td>Maintain at 0.1 to 1 x 10⁶ cells/ml. Renew media every 2 to 3 days.</td>
</tr>
<tr>
<td>Doubling time</td>
<td>12 h</td>
</tr>
<tr>
<td>In situ morphology (cell size)</td>
<td>10 µm</td>
</tr>
</tbody>
</table>

6.4 Results

6.4.1 Effect of NaOH and Oxygen Plasma Treatment on Surface Hydrophilicity

Water contact angles of the untreated and treated solvent-cast thin films measured by sessile drop techniques are listed in Table 6.2. It was reported that the water contact angles of a typical hydrophobic surface were approximately 65° to 95° [245]. The average contact-angles of untreated PHB and PHBV solvent-cast thin films were 66.80 ± 0.2° and 79.24 ± 0.4° respectively which indicate high surface hydrophobicity. Hence, to identify which NaOH concentration suit best for the surface treatment, scaffolds and thin films were immersed in varies NaOH concentration (0.2M, 0.4M, 0.6M, 0.8M and 1.0M) and the selection/optimization was made based on the scaffold physical morphology and water contact angles (Figure 6.1). The observation showed that only NaOH concentrations of 0.4M and 0.6M were seemed to be the appropriate selection of all since the other NaOH concentrations (i.e., 0.2M, 0.8M and 1.0M) used in the treatment were resulted in either no changes in water contact angles (for solvent-cast thin films) or sustained from the structural integrity problem (several fragments detachment). However, only 0.6M NaOH PHB and PHBV treated polymeric scaffolds were selected for the cell-biomaterial affinity study because the water contact angle of their corresponding 0.4M
NaOH-treated solvent-cast thin films have changed to an extreme wetting surface (possibly less than 15°) and insignificant changes of contact angle as observed on PHB. As for the oxygen plasma treatment, both polymeric solvent-cast thin films were also changed to a complete wetting surface. Nevertheless, they were all considered for the cell-biomaterial affinity study to observe the cell behaviour on the completely wet surface.

**Figure 6.1**: Morphology of the polymeric porous 3-D scaffolds with an approximate size of 10 mm × 10 mm × 5 mm post alkaline surface treatment: (a, j) 0.8 mol/L NaOH; (b, k) 1.0 mol/L NaOH; (c, i) 0.6 mol/L NaOH; (d) Control - treated with deionized water; (e, g) 0.4 mol/L NaOH; (f, h) 0.2 mol/L NaOH.

**Table 6.2**: Water contact angle (θ_{H2O}) of PHB and PHBV 4% (w/v) solvent-cast thin films after (a) rf-O$_2$ plasma and (b) NaOH surface treatment.

<table>
<thead>
<tr>
<th>Polymeric porous 3-D scaffolds</th>
<th>Surface physico-chemistry [Contact angle, θ_{apparent} (°)]</th>
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<td></td>
<td>rf-O$_2$ plasma treatment</td>
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<tr>
<td></td>
<td>100 W, 10 min[a]</td>
</tr>
<tr>
<td>PHB (4%, w/v)</td>
<td>&lt; 25[b][c]</td>
</tr>
<tr>
<td>PHBV (4%, w/v)</td>
<td>&lt; 25[b][c]</td>
</tr>
</tbody>
</table>

**p < 0.01 as compared to 0.4 mol/L NaOH and untreated PHB (66.80 ± 0.2°) (n = 10).** [a] The optimized operational parameters based on the surface morphology observation and cellular response studied by Köse et al. [150, 151]. [b] The surface was completely wet by re-distilled water droplet (n = 10). The contact angle of a fully wetting surface was < 25° [103, 211]. [c] Thin films of PHB and PHBV were fabricated on the polypropylene (PP) sheet and then treated with both treatments.

6.4.2 Structural/Morphological Analysis: Scanning Electron Microscopy (SEM)

The surface properties of the polymer are very important because the surface is where the interaction between the cell and the material takes place. Prior to the NaOH and rf-oxygen plasma treatment, the surfaces of the scaffolds appeared to have cavities and pores that were possibly covered with a thin film formed during the solvent evaporation stage of the scaffold preparation. However, both polymeric scaffolds treated with NaOH seemed to develop a degree of sharpening of the morphological which has resulted in pronounced etching effects (Figure 6.2a to d). As for the oxygen plasma treatment, the surfaces also changed substantially upon
treatment probably because of the etching of the skin layer of the scaffolds have been smoothed thus exposing the pores created by the leaching of sodium chloride crystals (Figure 6.2e and f). Similarly, both polymeric scaffolds treated with both types of treatments presented distinguishable angular pores with some additional appearance of small voids as compared to before the treatment implying that they were left behind after solute leaching.

Figure 6.2: SEM of PHB and PHBV (4%, w/v) porous 3-D scaffolds subsequent to alkaline and rf-oxygen plasma treatment. (a) 0.4M NaOH PHB; (b) 0.4M NaOH PHBV; (c) 0.6M NaOH PHB; (d) 0.6M NaOH PHBV; (e) PHB rf-oxygen plasma; (f) PHBV rf-oxygen plasma. Treatment conditions for rf-oxygen plasma: 100 W, 10 min.
6.4.3 Weight Loss after Surface Treatment

The weight loss on different surface treatment conditions is shown in Table 6.3. When the PHB and PHBV scaffolds were placed in a 0.4M and 0.6M NaOH solution at 20 ± 1 °C, the weight loss was in the range of 2% to 6% (w/w) after 1 h of treatment. Only PHBV produced a smaller amount of weight loss as compared to PHB ($p < 0.05$) for the 0.6M NaOH treatment. As for the oxygen plasma treatment, the weight loss was considerably less than the above mentioned range ($p < 0.05$). In addition, both plasma- and NaOH-treated polymeric scaffolds have sustained no structural integrity problem (sturdiness of the scaffold) throughout the treatment period without any apparent detachable fraction of the scaffolds. Severe scaffold rupture was only observed for 0.8M and 1.0M NaOH solutions possibly due to the excessively attacked by hydroxide nucleophiles (from the strong alkaline) to the electrophilic C of the ester (carbonyl carbon: C=O), which leads to the cleavage of ester bonds and eventually produced structurally unstable material [246].

### Table 6.3: Weight loss of PHB and PHBV 4% (w/v) porous 3-D scaffolds after (a) rf-O$_2$ plasma and (b) NaOH surface treatment.

<table>
<thead>
<tr>
<th>Polymeric porous 3-D scaffolds</th>
<th>Weight loss (%) after surface treatments</th>
<th>rf-O$_2$ plasma treatment</th>
<th>NaOH treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100 W, 10 min[a]</td>
<td>0.4 mol L$^{-1}$</td>
</tr>
<tr>
<td>PHB (4%, w/v)</td>
<td></td>
<td>1.64 ± 0.15</td>
<td>3.58 ± 0.48*</td>
</tr>
<tr>
<td>PHBV (4%, w/v)</td>
<td></td>
<td>1.45 ± 0.25*</td>
<td>2.79 ± 0.75*</td>
</tr>
</tbody>
</table>

*$p < 0.05$ as compared with PHB or PHBV treated with rf-O$_2$ plasma ($n = 4$). $^\text{W}$ $p < 0.05$ as compared with 0.6M NaOH- treated PHB scaffolds ($n = 4$).

6.4.4 Material Characterization of Polymeric Porous 3-D Scaffolds: Physical Properties and Surface Area

Helium displacement pycnometry (AccuPyc 1330 V3.00, Micromeritics) was carried out to characterize the scaffolds in terms of their apparent density and porosity after surface treatment. The results showed that the porosity (the fraction of the surface that is void) of the untreated and treated PHB and PHBV were more or less the same (Table 6.4). It is quite interesting that NaOH and oxygen plasma treatment did not alter the porosity of the scaffold even though the SEM revealed lots of exposing pores created from the solute leaching process [151]. Therefore, both untreated and treated polymeric scaffolds were expected to allow better leukaemic cell growth inside the porous structure if the surface hydrophilicity is not taken into account. Apart from that, BET surface area of the untreated and treated PHB and PHBV scaffolds is also listed in Table 6.4. An obvious difference between the surface morphologies of the original and modified PHB and PHBV scaffolds was observed. It is important to note that the surface of the untreated PHB and PHBV scaffolds is relatively rough. However, after the
NaOH treatment, the surface turned rougher, which was a sign of NaOH etching or removal of the surface layer (Figure 6.2a to d). The observation was well in agreement with the BET surface area wherein it has shown a significant changed than the untreated and plasma-treated scaffolds \((p < 0.05)\). As for the oxygen plasma treatment, slight etching effects were observed on the plasma-treated samples leading to a limited degree of sharpening of the morphological features (Figure 6.2e and f) as compared to the NaOH-treated and untreated scaffolds \((p < 0.05)\) since the majority of the pore have been smoothed by the plasma radiation.

Table 6.4: Physical properties of PHB and PHBV (4%, w/v) porous 3-D scaffolds before and after surface treatment.

<table>
<thead>
<tr>
<th>Physical properties</th>
<th>Polymeric scaffolds (4%, w/v)</th>
<th>Before treatment</th>
<th>Alkaline treatment (0.6 M)</th>
<th>rf-O₂ plasma treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHB</td>
<td>PHBV</td>
<td>PHB</td>
<td>PHBV</td>
</tr>
<tr>
<td>BET surface area, (A_s), m²/g [a]</td>
<td>0.70 ± 0.02</td>
<td>0.82 ± 0.03</td>
<td>0.97 ± 0.01*</td>
<td>0.91 ± 0.01*</td>
</tr>
<tr>
<td>Geometrical bulk density, g/cm³</td>
<td>0.084 ± 0.15*</td>
<td>0.072 ± 0.28</td>
<td>0.077 ± 0.19*Ψ</td>
<td>0.070 ± 0.17*Ψ</td>
</tr>
<tr>
<td>Skeletal density, g/cm³[b][c]</td>
<td>0.47 ± 0.52*</td>
<td>0.92 ± 0.14</td>
<td>0.44 ± 0.52Ψ</td>
<td>0.89 ± 0.14ΨΨ</td>
</tr>
<tr>
<td>Porosity, %</td>
<td>82.12 ± 0.18*</td>
<td>92.17 ± 0.73</td>
<td>80.97 ± 0.21</td>
<td>91.03 ± 0.52</td>
</tr>
</tbody>
</table>

*\((p < 0.05)\) - Results were considered statistically significant \((n = 4)\) as compared to before the treatment. \(^Ψ(p < 0.05)\) - Results were considered statistically significant \((n = 4)\) as compared to rf-O₂ plasma treatment. [a] BET surface area (m²/g) = Total skeletal surface area (m²)/skeletal mass (g). [b] \(ρ_s\) is the skeletal density of the crushed scaffolds, which was determined from helium pycnometry. [c] The higher pore volume (the higher the amount of absorbate intruded), the lower the skeletal volume.

6.4.5 Surface Properties of Polymeric Porous 3-D Scaffolds: ζ-Potential

Figure 6.3a and b shows the measured ζ-potential as a function of pH \((ζ = f(pH))\) for plasma- and NaOH-treated PHB and PHBV scaffolds as obtained by streaming potential measurements. Both polymers showed a similar characteristic. Unaltered PHB and PHBV surfaces showed an isoelectric point of pH 3.8 and 3.1 respectively which indicate the slightly acidic character of the surface. In this case, a clear difference between the samples can be seen (Table 6.5). The iep shifts, and the decrease in the modulus of the ζ-plateau value as compared to the untreated [(PHB: Δ(iep) = 0 to 1.1; Δ(ζ-plateau) = -15 mV to -91 mV and PHBV: Δ(iep) = 0.1 to 0.4; Δ(ζ-plateau) = -16 mV to -91 mV)]. All scaffolds demonstrated a distinct downshift in iep and an
increased modulus of \( \zeta \)-plateau with the increase of the NaOH concentration except for the 0.4M NaOH-treated PHB which produced almost the same profile as the untreated PHB scaffold.

**Figure 6.3**: Measurement of \( \zeta \)-potential as a function of pH (\( \zeta = f(pH) \)) for plasma- and NaOH-treated PHB and PHBV scaffolds as obtained by streaming potential measurements. Shown are (a) PHB and (b) PHBV porous 3-D scaffolds before and after rf-oxygen plasma and NaOH treatment. Arrows highlight the shift of the iep after NaOH treatment.
This phenomenon can be attributed to the increased number of acidic dissociating surface functional groups. Theoretically, during the NaOH hydrolysis, the hydroxide anion (OH\(^{-}\)) hydrolyses the ester groups resulting in the breaking of the polymer chain and the formation of carboxylic acid and hydroxyl groups on the termini of the two new chains [247]. This will lead to a certain increment of negatively charged ether carbon group (C-O) and carboxyl group (-C(=O)OH) due to the increased number of hydroxyl group (-OH) and reduction of ester bond (O-C=O) respectively. Whilst for the rf-oxygen plasma treatment, there are also certain increment in ether carbon group (C-O) content and a C-C bond content decrease with possibly no ester linkages cleaved at the polymer surface. Apart from that, the \(\zeta\)-potential measurements correlated well with the water contact angle measurements (Table 6.2). A complete wetting (less than 25\(^{\circ}\)) [103] was observed on both polymeric plasma-treated and NaOH-treated PHBV solvent-cast thin films whilst the water drops spread further across the NaOH-treated PHB thin film surface, as compared to the untreated thin films (except for the 0.4M NaOH treated PHB scaffolds).

Table 6.5: \(\zeta\)-potential results: iep and \(\zeta_{plateau}\) values of the \(\zeta = f(pH)\) for PHB and PHBV (4%, w/v) porous 3-D scaffolds before and after surface treatment.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>iep</th>
<th>(\zeta_{plateau}) (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB, untreated</td>
<td>3.8</td>
<td>-29</td>
</tr>
<tr>
<td>PHB, untreated, EtOH (2 h)</td>
<td>3.7</td>
<td>-29</td>
</tr>
<tr>
<td>PHB, NaOH 0.4M</td>
<td>3.7</td>
<td>-31</td>
</tr>
<tr>
<td>PHB, NaOH 0.6M</td>
<td>2.7</td>
<td>-81</td>
</tr>
<tr>
<td>PHB, 100W 10 min</td>
<td>-</td>
<td>-120</td>
</tr>
<tr>
<td>PHBV, untreated</td>
<td>3.1</td>
<td>-37</td>
</tr>
<tr>
<td>PHBV, untreated, EtOH (2 h)</td>
<td>3.2</td>
<td>-36</td>
</tr>
<tr>
<td>PHBV, NaOH 0.4M</td>
<td>3.0</td>
<td>-53</td>
</tr>
<tr>
<td>PHBV, NaOH 0.6M</td>
<td>2.7</td>
<td>-93</td>
</tr>
<tr>
<td>PHBV, 100W 10 min</td>
<td>-</td>
<td>-128</td>
</tr>
</tbody>
</table>

6.4.6 Effect of Sterilization on the Surface Properties of Polymeric Porous 3-D Scaffolds

Some well established techniques to sterilize 3-D tissue engineering scaffolds are disinfection in 70%, (v/v) ethanol (EtOH), ethylene oxide (ETO), radio frequency glow discharged (RFGD) plasma sterilization method and more recently a combination of ultra-violet light (8 min) and ethanol [6]. All untreated and treated PHB and PHBV (4%, w/v) porous 3-D scaffolds were sterilized by using a combination of 8 min under UV light and immersion for 2 h in ethanol (70%, v/v). \(\zeta\)-potential measurements (Figure 6.3) show that sterilizing the polymer scaffolds as mentioned above has no impact on the surface charge. Taking the untreated PHB and PHBV scaffolds as an example, there were no significant changes in the iep (PHB: 3.7; PHBV: 3.0) and \(\zeta_{plateau}\) value (PHB: -29 mV; PHBV: -36 mV) pre- and post-sterilization. This is
shown in Table 5. Moreover, the wetting characteristics of both polymers thin film surface pre- and post-sterilization also revealed that there was good correlation with the $\zeta$-potential measurements where no significant changes in water contact angles (Table 6.6).

**Table 6.6:** Water contact angle ($\theta_{\text{H}_2\text{O}}$) of untreated PHB and PHBV (4%, w/v) solvent-cast thin films pre- and post-sterilization ($n = 4$).

<table>
<thead>
<tr>
<th>Surface physico-chemistry</th>
<th>Polymeric porous 3-D scaffolds</th>
<th>PHB (4%, w/v)</th>
<th>PHBV (4%, w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before sterilization process</strong></td>
<td>Contact angle, $\theta_{\text{apparent}}$ ($^\circ$)</td>
<td>66.80 $\pm$ 0.2</td>
<td>79.24 $\pm$ 0.4</td>
</tr>
<tr>
<td><strong>After sterilization process</strong></td>
<td>Contact angle, $\theta_{\text{apparent}}$ ($^\circ$)</td>
<td>65.43 $\pm$ 0.3</td>
<td>78.11 $\pm$ 0.5</td>
</tr>
</tbody>
</table>

**6.4.7 In Vitro Degradation Study of Polymeric Porous 3-D Scaffolds**

**6.4.7.1 pH Measurements**

As polymer degradation leads to acidic by-products, we investigated the potential effects on the pH surrounding the scaffolds. The pH measurements were performed in the absence of cells to eliminate potential changes caused by cell growth (Figure 6.4b). The pH of all the scaffold-soaked culture medium fluctuated relatively in the range of pH 7 to 8 until day 21, after which, the pH became increasingly acidic and reached to a final value of $5.60 \pm 0.10$ (PHBV) and $6.07 \pm 0.04$ (PHB) at day 56. The pH of the control (culture media without scaffold) remained constant in the range of 7.4 to 7.8 ($p > 0.05$) throughout the experiments. In comparison of that control, the pH of a media with PHB scaffold was significantly lower from day 21 to the end of the experiments (day 56) ($p < 0.05$) whilst PHBV scaffold, the pH of a medium started to become acidic from day 35 until day 56 ($p < 0.05$).

**6.4.7.2 Gravimetric Analysis**

Gravimetric measurement for both polymeric porous 3-D scaffolds revealed that there were significant changes to its weight over a period of 60 days. However, both PHB and PHBV scaffolds sustained their structural integrity and morphology over a period of 60 days although accumulation of medium particulates was observed within 47 days of incubation (Figure 6.4a). It was observed that the mass of PHB scaffold decreased gradually ($p < 0.05$) within 47 days of incubation by $17.35 \pm 3.75\%$ of its initial weight. Meanwhile, the mass of PHBV scaffold decreased rapidly within 14 days of incubation and then slowly degraded until day 47 with the total loss of $36.33 \pm 9.73\%$ from its initial weight ($p < 0.05$). Then, both polymers were seemed to stabilize from day 47 until day 66. Moreover, the extent of the weight losses seemed to be
well correlated with the pH analysis on the range of day 21 to day 47 which showed a prominent pH drop to indicate the occurrences of accumulated acidic degradation products (i.e., carboxylic acid groups) due to the autocatalytic hydrolysis effect. However, only PHBV scaffold became ruptured (pointed by the arrows) and shattered into several large fragments at day 66 to indicate the effect of accumulated acidic by-product inside the porous structure (Figure 6.5b).

**Figure 6.4:** Kinetics of the *in vitro* degradation process for PHB and PHBV (4%, w/v) porous 3-D scaffolds were measured via (a) mass and (b) pH. The polymeric porous 3-D scaffolds were submerged in cell growth media and incubated at 37 °C. Samples were periodically removed and dried under vacuum prior to the analysis (n = 4). Control was a cell growth media without porous 3-D scaffolds. (Ψ) p < 0.05 for the value compared to each of the polymers or control (pH analysis) and (*) p < 0.05 for the value change compared to the previous value.
Figure 6.5: Morphology of the polymeric porous 3-D scaffolds with an approximate size of 10 mm × 10 mm × 5 mm pre- and post-incubation in cell growth media. (a) PHBV at week 4 and (b) PHBV at week 9. (c) PHB at week 4 and (d) PHB at week 9. Ruptured PHBV scaffolds (as circled in the picture (b)) were observed at week 9 and some of the incubated scaffold were shattered into several big pieces (n = 4).

6.4.8 Cell culture on Porous 3-D scaffolds: Chronic Lymphocytic Leukaemia (CLL)

6.4.8.1 Seeding Efficiency of Chronic Lymphocytic Leukaemia Cell Line (RL)

The ability to support the attachment and promote the proliferation of cultured cells is a prerequisite of a functional scaffold [82]. To evaluate the cellular behaviour on the treated porous 3-D scaffolds of PHB and PHBV, chronic lymphocytic leukaemia cell line (RL) was initially seeded and cultured for 24 h. The seeding efficiency shows the percentage of cells that remained in the scaffold after 24 h of seeding. It was observed that the seeding efficiency of all untreated and treated PHB and PHBV were not statistically different in the range of 81.54 ± 15.07% to 95.34 ± 9.23% (p > 0.05). Moreover, it seems to indicate that RL prefers to attach and interact initially on all scaffold surfaces regardless of its surface hydrophilicity/hydrophobicity and morphological conditions.
6.4.8.2 Effect of Surface Treatments on Cell-Biomaterial Affinity

After the cell seeding efficiency had been determined, all the untreated and treated polymeric scaffolds were then subjected to a short-term cell proliferation of 14 days. The MTS assay was carried out to make further comparison on a different type of treated scaffolds with the untreated scaffolds via absorbance measurements ($\lambda = 490$ nm) as shown in Figure 6.6. Evidently, the proliferation of RL on day 7 and day 14 for all treated and untreated polymeric scaffolds increased tremendously in comparison with day 1 ($p < 0.01$). As on day 7, the treated scaffolds cellular growth (except NaOH-treated PHB) was significantly higher than the untreated PHBV scaffold only ($p < 0.05$). On day 14, the cellular growth of all treated polymeric scaffolds showed a significant increased of absorbance as compared to day 7 ($p < 0.05$). On top of that, the cellular growth of all treated scaffolds on day 14 was statistically higher than the untreated scaffolds ($p < 0.05$). Viability of the cells extracted from the scaffolds was assessed at days 0, 7 and 14 (Figure 6.7). Cells cultivated in and supported by all of the treated 3-D porous scaffolds had the best viability compared with those supported by the untreated scaffolds ($p < 0.05$). Overall, it seems that this abnormal lymphoblastic cell line (CLL) has a specific preference on choosing which surface properties and material characteristics (i.e., surface wettability and morphological conditions) to attach and growth effectively. Moreover, this phenomenon showed that the cells adapted efficiently to their new 3-D microenvironment (Figure 6.8a to f), and it is expected to support long-term proliferation (i.e., more than 2 weeks) in cytokine-free conditions. However, it may be subjected to the surface properties such as wettability, electronegativity and chemical composition that determines the availability, conformational and orientational of the absorbed ECM proteins to trigger further cell adhesion, spreading and growth [248].
**Figure 6.6:** Cellular growth of a CLL cell line (RL) on PHB and PHBV (4%, w/v) porous 3-D scaffolds without treatment (control) and with surface treatment (alkaline and rf-oxygen plasma). The MTS assay was used to measure the proliferation of CLL cell line (RL) on all scaffolds. (*) $p < 0.05$ for absorbance decreased/increased as compared to the previous day. Results shown were means (±sd) of 6 separate instances, each in quadruplicates wells ($n = 4$) obtained by subtraction from cell-free equivalents, to eliminate $A_{490}$ produced by the scaffold + media (negative control) alone.

**Figure 6.7:** Viability of the RL cells seeded onto the treated and untreated PHB and PHBV porous 3-D scaffolds during a period of 14 days. *$p < 0.05$, compared between groups. For all panels, results were means (±sd), $n = 2$ replicates per experiment, $N = 6$ separate experiments.
**Figure 6.8:** SEM of cell-loaded PHBV and PHB treated scaffolds. (a) RL cells cultured in 0.6M NaOH PHB scaffolds at day 14. (b) Magnified views (1100x) of (a). (c) RL cells cultured in 0.6M NaOH treated PHBV scaffolds at day 14. (d) Magnified views (550x) of (c). (e) RL cells cultured in plasma-treated PHBV scaffolds at day 14. (f) Magnified views (950x) of (e). The cells (arrows) were observed to have penetrated the pores of the inner section of the scaffolds with large aggregates seen.

### 6.5 Discussion

Most polymeric materials demonstrated poor cell adhesion properties due to their low surface energy, chemical inertness and smooth surface. For this reason, it is very essential for the polymeric scaffolds used in the biomedical application to be surface modified by some additional treatments to raise surface bioactivity, thus to enhance hydrophilic properties as well.
as the adhesive properties. It has been approved that surface treatment is an effective method to improve the cell compatibility of polymers by improving the surface hydrophilic property [249]. In fact, surface properties such as the availability of certain functional groups, domain structure, electrical charge, hydrophilicity/hydrophobicity, interfacial adaptability and surface roughness are considered to determine the outcome of cells interacting with the materials which in turn, can induce several cascade reactions and activation phenomena [250]. Therefore, for different type of materials (e.g., PLLA, PHB, PHBV, etc.) it is thought that their different cell affinity may be due to their different surface properties.

The results in our work show that surface treatment at a specific NaOH concentration might be considered to be an effective method to improve surface hydrophilicity of the polymeric scaffolds at the appropriate level (not too hydrophilic and hydrophobic except for PHBV, which produced a highly hydrophilic surface). The discrepancy between NaOH-treated PHB and PHBV could possibly due to the surface roughness and large availability of the polar-containing functional group of PHBV solvent-cast thin films (due to the less crystalline region for easy solvent penetration) which ultimately induced the spreading coefficient of the water droplet on the surface during the water contact angle measurement. However, the improved surface properties based on the optimized operational parameters used by Kose et al. [150, 151] have rendered both of the polymeric scaffolds surface to excessively hydrophilic (Table 6.2). For that reason, it would be suggested that a proper optimization process needs to be re-examined by coupling with the water contact angle measurement since the authors were fundamentally correlated different plasma operational parameters with only the surface morphology and cell-biomaterial responses. Meanwhile, the improvement in the hydrophilic property of the polymeric material is normally attributed to changes in surface chemical and physical property. It was reported that the main mechanism that leads to the wettability improvement of polymeric scaffolds by using rf-O$_2$ plasma and alkali treatment is surface functionalization by the insertion of polar groups as well as topography changed (surface roughness) [251-254]. This phenomenon has been shown by Li et al. [254] where the hydrophilic property of the surface-hydrolyzed PHBHHx matrices was improved due to the increase of specific surface areas and surface roughening. In this work also, the microstructure in Figure 6.2a to d clearly shows that the NaOH treatment led to a rougher surface (Table 6.4) with many pores on the surface due to the etching function of NaOH which provides mechanical interlocking and in turn contributes to the improvement of hydrophilic properties [232]. On the other hand, the weight lost during the surface modifications should also be taken into consideration. Fortunately, both treated polymeric scaffolds sustained for minor losses of less than 5% (w/w) and though retaining their structural integrity (sturdiness of the scaffold) throughout the whole treatment period. However, further experimental work could be done by optimizing several processing parameters such as concentration of NaOH (i.e., from the range of 0.4M to 0.6M), time of treatment, temperature...
and power of plasma radiation so that the weight loss could be controlled and minimized [246].
The physico-chemical characteristic of the modified PHB and PHBV surfaces showed distinct
differences (subjected to type of treatment applied). The hydrophobic and slightly acidic surface
of the original PHB and PHBV has been altered significantly into a hydrophilic material due to
the insertion of oxygen-containing polar group from the NaOH and rf-O$_2$ plasma treatments.
The later wetting condition measured by means of water contact angle has verified the change to
a certain degree with the largest effect caused by the rf-O$_2$ plasma (Table 6.2). Additionally, the
abundant formation of negatively charged ether carbon (C-O) is evident to the electrokinetic
measurements as shown in Table 6.5 and Figure 6.3 to confirm the changes of the surface
properties after the treatments as compared to the untreated scaffolds.

Biological polymers (natural polymers) degrade rapidly in the biological medium when
appropriate hydrolytic enzymes are available [151]. However, as for the synthetic organic
polymers which are mostly produced via chemical synthesis, they disappear slowly from the
environment [207]. Irrespective of polymer categories, the rate and mode of degradation of the
polymers usually influence their service life, mechanical properties and the response of the
biological system towards them [151, 255]. Many previous studies have used gravimetric and
pH analysis to determine the degradability of polymers in the form of thin film and porous
scaffold. However, most of the pH change caused by the degradation of the biomaterial was so
insignificant that it might not be used as a degradation indicator. For that reason, density and
weight changes, on the other hand, are significant enough to serve as an appropriate indicator of
degradation. Results in our work indicate that the pH and weight changes were well correlated
as time progresses (Figure 6.4). The sample weights changed dramatically within 14 days in the
cell culture media (especially for PHBV). At this phase, it believed that the samples absorb the
solvent (a water-based media), start to break by swelling and at the same time eroded the
scaffold surface. Later, both polymeric scaffolds degraded slowly from day 28 up to day 47 due
to the autocatalytic degradation inside the bulk scaffolds [218]. On top of that, this drastic
weight changed in the first 14 days was possibly due to the chain scission created a polyester
chain shortening as it absorbed large amount of solvent [255]. These data indicate that the
microenvironment of the loaded cells inside the scaffold will become less restrictive as time
progresses (at least after the initial 14 days) and therefore, increases in cell numbers within the
scaffold structure will not be excessively hindered by the confined space in the scaffold [151,
255].

PHB and PHBV polymers have a significant role to play in tissue engineering and in the
development of living tissue products for therapeutic applications [2, 66, 256]. In this study, a
cell line of chronic lymphocytic leukaemia (RL) was grown on the untreated and treated PHB
and PHBV scaffolds and their metabolic activity for day 1, 7 and day 14 was measured by
means of MTS assay. Comparisons were made between the untreated and treated scaffolds to
identify which physico-chemical modifications suit best for biological surface coating apart from improving cell-biomaterial affinity. On the 7th and 14th day of incubation, the cellular growth increased immensely for all untreated and treated polymeric scaffolds as compared to day 0 \((p < 0.05)\) (Figure 6.6). On balance, the cellular growth of all treated scaffolds showed tremendous improvement as compared to all untreated scaffolds. All of these data has shown that the adhesion and growth of the chronic lymphocytic leukaemia cell line (RL) seemed to have specific preferences to a different polymeric surface physico-chemical characteristic in which largely affected the adsorption of protein monolayer on the treated polymeric surface. However, the NaOH treatment (0.6M) might be the appropriate choice of surface treatment and later for the biological surface coating due to their appropriate wetting changed (the surface is not too hydrophilic or hydrophobic except for the PHBV) as compared to the plasma treatment even though the weight loss during the treatment revealed slightly higher than the plasma treatment. In addition, many studies have demonstrated that cell adhere, spread and grow more easily on moderately hydrophilic substrates than on hydrophobic or very hydrophilic ones [257-260]. For that reason, choosing the appropriate wettability could increase the adsorption of particular extracellular matrix proteins (to provide cell attachment recognition sites) from the supplemented serum-containing culture media and during the biological surface coating which in turn, favoring the cellular attachment, spreading, migration and growth [121]. Besides that, the treated polymeric scaffolds will have the advantages of adsorbing extra amount of the ECM proteins (e.g., collagen type I and fibronectin) during the biological surface coating which will be useful in increasing cell adhesion, spreading and growth on the polymer surface for a long culture period (i.e., up to 6 weeks). As for the untreated polymeric scaffolds, they might have a problem in retaining high cellular growth and cell spreading across the porous structure for a long culture period (more than 14 days) although the result showed good cell attachment and growth up to 14 days. The main problem could be due to the lack of these adsorbed ECM proteins that provide cell attachment recognition site and mechanical strength [6].

6.6 Conclusion

In the present study, controlled surface hydrolysis and surface rendering by NaOH and rf-O₂ plasma respectively are proposed to functionalize and modify the surface of PHB and PHBV scaffolds. The results show that the surface hydrophilicity of PHB and PHBV were improved noticeably after NaOH treatment. However, the rf-oxygen plasma treatment rendered the surface of PHB and PHBV scaffolds by becoming too hydrophilic (fully wetting). Moreover, it seems that both alkaline and rf-oxygen plasma treatments have altered the morphology of the scaffold by making more voids available for occupation by the CLL cell line (RL) in which otherwise covered by a thin film formed during the solvent evaporation stage of scaffold
preparation. The analysis of the structural and material characteristics of the treated PHB and PHBV showed that the surface morphology and porosity were noticeably similar in this regard except for the BET surface area. Thus, both the plasma and NaOH treatments have proven their worth to modify the surface properties of the scaffold without having any structural and sturdiness problems. Therefore, the concentration of 0.6M NaOH was chosen due to the appropriate amount of the hydrophilicity they embraced apart from having a stable and well interconnected scaffold structure. It is undoubtedly that the treated polymeric scaffolds will have the advantage of attracting the ECM proteins from the supplemented-serum growth media or during the biological surface coating which in turn, will provide a good cell adhesion during the initial stage and expansion. pH change caused by the heterogeneous degradation of the biomaterial was quite well correlated with the weight changed to indicate the occurrences of degradation. Weight changes, on the other hand, also significantly served as an appropriate indicator of degradation. The decrease of the weight upon degradation without the loss of structural integrity is a good sign for the well-being of the loaded cells. In this study, CLL cell line (RL) was proliferated immensely on all treated polymeric scaffolds as compared to the untreated after 14 days of incubation. This result indicates a great potential in developing an in vitro 3-D biomimicry of the human haematopoietic microenvironment model for the study of leukaemia.
CHAPTER 7 Development of Biomimetic Polymeric Porous Scaffolds of an *In Vitro* Three-Dimensional (3-D) Human Leukaemia Model

7.1 Introduction

The development of biomaterials for tissue engineering applications has recently focussed on the design of biomimetic materials that are capable of eliciting specific responses mediated by biomolecular recognition signals which can be manipulated by altering the design parameters of the material [81]. In many cases, the lack of these biomolecular recognition signals on the material surfaces coupled with their inevitable hydrophobicity has resulted in the inability of the material (fabricated as 3-D porous scaffolds) to mimic the 3-D microenvironment bone marrow (BM) niches for regulating the self-renewal haematopoietic stem cells (HSCs) functions. The biomolecular recognition signals of the niches consist of stroma cells, such as macrophages, adipocytes and fibroblasts in the BM and control cellular behaviour either in the function or dysfunction of normal or malignant bone marrow [261]. Therefore, in order to mimic the BM in both its 3-D structure and its provision of appropriate bio-signals for cell attachment, the surface and bulk modification of the biodegradable polymer via chemical or physical methods with bioactive molecules such as a native long chain of ECM proteins offers an ideal solution to overcome the limitations of the scaffolds.

However, to the extent of our knowledge, there are no studies concerning the coating of the native long chain of ECM proteins onto the surface of PHB and PHBV porous 3-D scaffolds. Only one relevant study conducted by Dong *et al.* [262] can be highlighted in this investigation wherein an amphiphilic PHA granule binding protein (PhaP-RGD) was used to bind to the hydrophobic surfaces of PHBV matrices without the use of surface treatment to improve cell growth. However, one study actually established and modelled the 3-D culture system of leukaemia cell lines (AML: HL-60, K-562 and Kasumi-6) by using variety of synthetic polymers [6]. For that reason, we intend to follow the previous mentioned protocols to prove that other leukaemia entities (such as CLL: RL and JeKo-1) can be modelled in a similar manner. Moreover, the coating of the scaffolds with ECM proteins would actually create an adhesive interface and a strong mechanical contact between the scaffold material and the cells whose organization and production modulates and enhances cell adhesion through transmembrane integrin receptors. Meanwhile, the incorporation of the ECM proteins to the biodegradable polymeric scaffolds is that not only would they provide the cell-binding sequence for cell adhesion, but also provide secondary interactions with other ECM proteins and interactions with growth factors. This would stabilize the cell binding thus enhancing cell adhesion which results in better cell growth and maturation [263]. Before the establishment of an *in vitro* 3-D biomimicry of the human haematopoietic microenvironment of the AML studied...
by Blanco et al. [6] there were only traditional two-dimensional (2-D) cultures and in vivo animal models of AML available to explain the molecular determinants of leukaemogenesis, the cellular and microenvironmental elements that enhance leukaemia growth and protect the leukaemic stem cells from chemotherapy [264, 265]. However, the methods were too difficult to investigate due to these three major limitations. This difficulty arises from; i) 2-D cultures cannot support long-term primary leukaemic cell growth without exogenous growth factors or stromal cell support, ii) 2-D culture conditions cannot structurally provide the native haematopoietic microenvironment (HM) for human leukaemic cells, especially the 3-D niches which protect the leukaemic stem cell and iii) animal models, although superior to 2-D cultures, cannot completely replicate the human microenvironment and require extensive animal facilities and expertise for use [266, 267].

7.2 Aim and Hypothesis

It was hypothesised that by using a 3-D culture system that resembled the highly porous structure of the cancellous bone (containing soft abnormal bone marrow tissue); a good representation of the original leukaemic bone marrow microenvironment will be reproduced. Leukaemic cell culture could be maintained with its original phenotypic features, independent of cytokine addition and the natural leukaemic cells expansion could be studied in its natural state in vitro.

7.3 Approach of Study

In order to recreate an ideal 3-D BM microenvironment for the investigation of leukaemia in vitro, polymeric scaffolds need to be fabricated and optimized with desirable physical properties (e.g., high porosity, adequate pore size and distribution, a high surface area-to-volume ratio through the presence of a network of channels and interconnected pores) and wettability which will facilitate enhanced penetration by cells and media deep into the scaffolds and the formation of cellular associations [157, 268]. Furthermore, polymeric scaffolds need to be biocompatible and should offer bio-recognition signals that support cellular growth and tissue formation through, possibly, immobilization with ECM proteins. In this investigation, 2 different CLL cell lines, namely RL and JeKo-1, were used as a substitute for human primary leukaemia, a heterogeneous disorder, in order to account for differences in the growth requirements of the different leukaemia subtypes as well as assist in the development of the in vitro 3-D leukaemia model (Table 7.1). The leukaemic cell lines were tested on the polyhydroxyalkanoates (PHA), a class of biodegradable polyesters, in particular PHB and PHBV. Prior to the coating of the proteins, both polymeric scaffolds were subjected to the NaOH treatment (0.6M) to accelerate
the adsorption of ECM proteins by providing large amounts of polar groups through polar interaction and hydrogen bonding. Bio-recognition signals were provided to the selected scaffolds by coating with either the highest or the lowest concentration of fibronectin and/or collagen based on the study carried out by Blanco et al. [6] This is to provide an adhesive interface (fibronectin) or strong mechanical contact (collagen) between the scaffold material and the CLL cells [269].

Table 7.1: Chronic lymphocytic leukaemia cell lines (JeKo-1 and RL) main properties used in the optimization process.

<table>
<thead>
<tr>
<th>Cell properties</th>
<th>JeKo-1 [270, 271]</th>
<th>RL [272]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell phenotype</td>
<td>Lymphoblast</td>
<td>B lymphoblast</td>
</tr>
<tr>
<td>Original disease of patient</td>
<td>Mantle cell lymphoma</td>
<td>Non-Hodgkin's lymphoma</td>
</tr>
<tr>
<td>Patient data</td>
<td>78-year-old Caucasian female</td>
<td>52-year-old Caucasian male</td>
</tr>
<tr>
<td>Source of material</td>
<td>Peripheral blood</td>
<td>Ascites tissue</td>
</tr>
<tr>
<td>Year of establishment</td>
<td>N/A</td>
<td>1983</td>
</tr>
<tr>
<td>Culture medium</td>
<td>80% RPMI 1640 + 20% FBS</td>
<td>90% RPMI 1640 + 10% FBS</td>
</tr>
<tr>
<td>Sub-cultivation routine</td>
<td>Maintain at &lt; 2.5 x 10⁶ cells/ml. Renew media every 2 to 3 days</td>
<td>Maintain at 0.1 to 1 x 10⁶ cells/ml. Renew media every 2 to 3 days</td>
</tr>
<tr>
<td>Doubling time</td>
<td>55 h</td>
<td>12 h</td>
</tr>
<tr>
<td>In situ morphology</td>
<td>7 µm</td>
<td>10 µm</td>
</tr>
</tbody>
</table>

7.4 Results

7.4.1 Unmodified Polymeric Scaffolds Evaluation

PHB and PHBV polymeric scaffolds were evaluated for their ability to support the growth and maintenance of two cell lines, RL and JeKo-1 over a period of 6 weeks. Unmodified PHB and PHBV displayed a seeding efficiency for RL and JeKo-1 higher than 50% \( (p < 0.05) \) 24 h (RL: 91.33 ± 1.17% (PHB), 72.86 ± 11.06% (PHBV); JeKo-1: 59 ± 3.61% (PHBV)) following inoculation except PHB cultured with JeKo-1 (55.74 ± 18.78%) (Figure 7.1). Both polymers were able to support long-term (over a period of 6 weeks) cellular growth (Figures 7.2 and 7.3). CLL-cell-line-specific cell growth kinetics was consistently observed in both the PHB and PHBV scaffolds. Both scaffolds with RL cells showed a statistically significant higher absorbance (i.e., metabolic activity of cells) starting from week 3 up to week 6 of culture \( (p < 0.05) \), compared with that of JeKo-1; this absorbance stabilized after week 4. In contrast, the JeKo-1 cell line exhibited slower, two-stage growth kinetics, peaking at different culture phases which were at week 1 (PHB), week 3 (PHBV) and week 6 (PHB and PHBV). This growth kinetics in 3-D was consistent with what is known of the RL and JeKo-1 cell lines in standard 2-D cultures (Table 7.1: RL had a faster growing time (doubling time), established as a culture faster than the JeKo-1 cell line).
Figure 7.1: Cell seeding efficiency on ECM-coated scaffolds. The PHB and PHBV scaffolds were coated with collagen type I (125 µg/ml or 62.5 µg/ml), fibronectin (50 µg/ml or 25 µg/ml), and collagen and fibronectin (62.5 µg/ml and 25 µg/ml, respectively). The CLL cell lines used were (a) RL and (b) JeKo-1. (*)Significant differences with \( p < 0.05 \) between the samples were highlighted by lines. \( \Psi \)The tested scaffolds were displayed a seeding efficiency of over 50\% (\( p < 0.05 \)). The cultures were performed in parallel on 2 separate occasions, each in quadruplicates.
7.4.2 Treated Polymeric Scaffolds Evaluation (0.6M NaOH)

As shown in Figure 7.1, majority of the treated PHB and PHBV displayed a seeding efficiency higher than 50% ($p < 0.05$) 24 h (RL: $91.30 \pm 3.06\%$ (PHB), $73 \pm 7\%$ (PHBV); JeKo-1: $61.17 \pm 6.10\%$ (PHB), $81.33 \pm 6.11\%$ (PHBV)) following inoculation. Moreover, PHBV-treated scaffold exhibited higher seeding efficiency than the unmodified and 25 $\mu$g/ml fibronectin-coated PHBV scaffold cultured with JeKo-1 ($p < 0.05$). As shown in Figure 7.2 and Figure 7.3, both polymers were able to support long-term (over a period of 6 weeks) cellular growth. Both scaffolds with RL cells showed a statistically significant higher absorbance starting from week 2 to week 3 up to week 6 of culture ($p < 0.05$), compared with that of the other cell line (JeKo-1). Only PHB exhibited stable absorbance 1 week earlier than the unmodified foams (after week 3). As for the JeKo-1 cell line culture, the cell also exhibited a slower increase in metabolic activity with two-stage growth kinetics, peaking at different culture phases which were at week 1 and week 6 (PHB). However, PHBV stabilized after 1 week of culture peaking at week 2 and remained stable until week 6. As to compare cellular growth trends between the treated and untreated (unmodified) scaffolds, both polymers seemed to establish a similar growth trend except for the treated PHB RL culture and treated PHBV JeKo-1 which was stabilized earlier after week 3 and week 1 respectively.

7.4.3 Polymeric Scaffolds Coating with ECM Proteins

In order to mimic the bone marrow microenvironment and enhance the cell culture, PHB and PHBV scaffolds were coated with two of the main bone marrow ECM proteins, collagen type I and fibronectin, evaluated at either high (125 $\mu$g/ml for collagen type I and 50 $\mu$g/ml for fibronectin) or low concentrations (62.5 $\mu$g/ml for collagen type I and 25 $\mu$g/ml for fibronectin). The effect of the protein coating of PHB and PHBV on the seeding efficiency and cell growth of the CLL lines was compared with that on uncoated scaffolds. The seeding efficiency shows the percentage of cells that remain in the scaffold after 24 h of seeding. For the RL cell line, seeding efficiency for majority of the protein-coated scaffolds were consistently over 50% ($p < 0.05$) 24 h following inoculation except 125 $\mu$g/ml and 62.5 $\mu$g/ml collagen-coated PHBV scaffolds ($71.17 \pm 15.91\%$ (125 $\mu$g/ml), $75.17 \pm 13.38\%$ (62.5 $\mu$g/ml)). As for the JeKo-1 cell line, seeding efficiency for some of the protein-coated PHBV scaffolds was over 50% ($p < 0.05$) 24 h ($75.10 \pm 2.05\%$ (Fibronectin 50 $\mu$g/ml), $69.33 \pm 8.08\%$ (Collagen 125 $\mu$g/ml), $90.33 \pm 6.11\%$ (Collagen 62.5 $\mu$g/ml)). However, none of the protein-coated PHB scaffolds achieved over than 50% seeding efficiency.

Cellular proliferation of the 2 CLL cell lines cultivated on ECM-coated PHB and PHBV was measured using the MTS assay over a period of 6 weeks. Although quite different growth
patterns were evident at each time point, a common finding in all of the cultures was that cell growth on the ECM-coated scaffold was at least as good as and at most time points better than that on the control uncoated scaffold. In the cell-line specific analysis, RL growth was enhanced at the early stages of the culture (the first 2 weeks) when supported by both of the polymers coated with collagen (irrespective of the concentration); peaking at week 2. This is shown in Figure 7.2 (Figure 7.2a when seeded onto PHB and Figure 7.2b when seeded onto PHBV). By week 4, the culture was best supported by PHB-coated collagen (62.5 µg/ml) as compared with fibronectin and other collagen concentration ($p < 0.05$). On the other hand, the same cell line displayed similar behaviour when seeded in PHBV, with enhanced growth in the collagen coated scaffold (irrespective of the concentration) at the early stages of culture (by week 2) and later improvement in proliferation (after week 5) with collagen 125 µg/ml coating. Meanwhile, the differences between both polymers only occurred at week 5 with all PHB protein-coated scaffolds exhibiting a slightly higher growth than PHBV ($p < 0.05$). Overall, an enhanced RL growth is observed when coating the scaffolds with ECM proteins ($p < 0.05$); with an initial higher proliferation onto the PHB and PHBV collagen-coated scaffolds (irrespective of the collagen concentration) which changed to the specific collagen concentration in the last stages of the culture.

Growth trend lines were added to the collagen-coated scaffolds with 62.5 µg/ml (the best protein concentration to produce homogenous results) and to the untreated scaffolds to show that coating results in a two step process. First, when the cells were seeded they needed to adapt to the new environment and remained without differentiation for a few days to adapt. Second, once adapted, they start expanding until they reach a peak proliferation point. For the protein-coated scaffolds this period (peak proliferation point) took 2 to 3 weeks and when seeded onto the uncoated scaffold it took 4 to 5 weeks.

As for JeKo-1, the cells grew slower than the RL cell line on both collagen- and fibronectin-coated scaffolds as indicated by the lower absorbance readings obtained with similar trend as observed in 2-D cultures. However, both polymers exhibited the same growth trend with the 62.5 µg/ml collagen-coated scaffolds (peaking at week 2) although the absorbance reading was considered lower than the RL cellular proliferation ($p < 0.05$) (Figure 7.3: (a) PHB and (b) PHBV). With cultures on PHB, the initial cellular growth of 125 µg/ml collagen-coated scaffold was higher than 62.5 µg/ml until both polymers were peaking at week 2 ($p < 0.05$). Whilst on PHBV, 62.5µg/ml exhibited higher initial cellular growth than the 125µg/ml collagen-coated scaffold with the same peaking time point (week 2) ($p < 0.05$).
Figure 7.2: Cellular proliferation of RL cells on ECM protein-coated PHB and PHBV scaffolds. RL cells cultured in (a) PHB and (b) PHBV scaffolds coated with collagen type I and/or fibronectin were assessed by standard MTS assay. $^* p < 0.05$ for the value change compared to the previous value. Week 0.14 represented an initial cellular growth of cell seeded scaffolds after 24 h incubation. A comparison of the growth trends on the 62.5 µg/ml collagen-coated scaffolds (dotted line), the treated 0.6M NaOH (A) and the uncoated controls (B) (straight line) revealed a significant difference ($p < 0.05$). Cultures were performed in parallel on 2 separate occasions, each in quadruplicates.

Meanwhile, PHBV 62.5 µg/ml collagen-coated scaffolds exhibited higher cellular growth than PHB at week 2 ($p < 0.05$). Whilst on PHB, the 125 µg/ml collagen-coated and 50 µg/ml fibronectin-coated scaffolds exhibited higher cellular growth than PHBV at week 2 and week 3 respectively ($p < 0.05$). In contrast to the behaviour observed with the collagen coated scaffolds, fibronectin-coated conditions displayed a similar growth trend of RL cellular proliferation on
the untreated and treated scaffolds which was peaking at week 4 to week 5. For the untreated (control) and treated scaffolds, this culture adaptation and proliferation period took 4 to 5 weeks again for both scaffolds after their first peaking at week 1 to week 3. After the cells adapted to their new environment, the proliferation stabilized; in the case of the collagen coated scaffold the cultures stabilized at week 2 and in the untreated (control) and treated scaffolds the culture did not stabilized until week 5.

Figure 7.3: Cellular proliferation of JeKo-1 cells on ECM protein-coated PHB and PHBV scaffolds. JeKo-1 cells cultured in (a) PHB and (b) PHBV scaffolds coated with collagen type I and/or fibronectin was assessed. *p < 0.05 for the value change compared to the previous value. Week 0.14 represented an initial cellular growth of cell seeded scaffolds after 24 h incubation. A comparison of the growth trends on the 62.5 µg/ml collagen-coated scaffolds (dotted line), the treated 0.6M NaOH (B) and the uncoated controls (A) (straight line) revealed a significant difference (p < 0.05). The cultures were performed in parallel on 2 separate occasions, each in quadruplicates.
7.4.4 Examination of the 3-D *In Vitro* Microenvironment

The 3-D microenvironment provided by the PHB and PHBV scaffolds was characterized using SEM at 2 and 4 weeks after seeding. The RL cell line grew in large clusters (Figure 7.5a and b), similar to that observed in 2-D (Figure 7.4a and b). In contrast, JeKo-1 cells aggregated within the pores without clustering (Figure 7.5c and d). Over a period of 4 weeks, the cells had migrated into the central pores of the scaffold establishing and defining “niches-like” areas of growth. Cellular density increased with culture time and by week 4 the central pores of the scaffolds were populated by cells that had adhered to the walls of the pores, regardless of the cell line tested. Consistent with previous data, all scaffolds coated with ECM proteins supported higher cell growth compared with that of the untreated and NaOH-treated scaffolds. The analysis of cell-loaded scaffold structure throughout the culture period (irrespective of cell and polymer type) indicated that large voids and degraded fragments were generated as time progressed (especially at week 2 and week 4). The observation was well in agreement with the *in vitro* degradation kinetics of the scaffolds in cell growth media study (data not shown). The cell-free scaffolds at week 2 and week 4 (negative control: picture not shown) exhibited similar 3-D microenvironment as the cell-loaded scaffolds. Therefore, with the less restrictive space in the porous structure as time progresses, this would allow an increase of cell number which was consistent with the previous cell growth kinetics data.

**Figure 7.4:** RL cell morphology under light microscope in 2-D culture system. Panel (a) shows the 2-D culture of RL cells at day 1 of culture (100 µm scale). Panel (b) shows the 2-D culture of RL cells at day 3 of culture (50 µm scale).
Figure 7.5: SEM of PHB and PHBV scaffolds coated with ECM proteins. (a) RL cells cultured in the PHB scaffold coated with 62.5 µg/ml collagen at week 2. (b) RL cells cultured in the PHBV scaffold coated with 62.5 µg/ml collagen at week 2. (c) JeKo-1 cells cultured in the PHB scaffold coated with 50 µg/ml fibronectin at week 4. (d) JeKo-1 cells cultured in the PHBV scaffold coated with 50 µg/ml fibronectin at week 4. The cells (indicated by arrows) have penetrated deep inside the central section of the scaffolds.

7.4.5 Effect of Collagen Solution Residual on Cell Growth Media

Protein-coated scaffolds (collagen type 1) were subjected to 3 h of incubations at 37 °C and 5% CO₂ in cell growth media to observe any changes to the pH and media colour due to the excess collagen solution inside the scaffolds. No media changes were conducted until the end of the incubation. The cell growth media were prepared according to the recommendation of the cell provider and the initial pH (cell growth media without a scaffold) was measured with the value of 7.16 at 20 ± 1 °C (room temperature). It was observed that as the time of the incubation progresses, there were insignificant differences of pH between all polymers preconditioned with collagen type 1 in comparison with the control (p > 0.05). Moreover, there was also no changed to the colour of the media as compared to the samples that have not preconditioned with collagen solution. These results indicate that the amount of any excess collagen left inside the porous 3-D scaffolds have been removed sufficiently with PBS under vacuum condition (2 mbar) prior to the cell seeding. This is important to consider that if the cell growth media
became acidic; this could result in an initial loss of cells post seeding and later during the proliferation phase. Meanwhile, the results were in support of the work done during the cell seeding where most of the protein-coated polymers (collagen type 1) result was over 50% of cell seeding efficiency irrespective of cell types (Figure 7.1).

7.4.6 Long Term In Vitro Degradation Study of Polymeric Porous 3-D Scaffolds

Degradation behaviour of porous scaffolds plays an important role in the engineering process of a new tissue. The rate of degradation may affect many cellular processes including cell growth, tissue regeneration and host response [31, 151]. Visual assessment of both polymeric porous 3-D scaffolds in PBS for 214 days of incubation revealed that their structural integrity and morphology were intact although accumulation of medium particulates was observed in the initial incubation period of day 7 up to day 48. Accumulation of particulates from day 50 was too small as it remained plateau until day 214 (Figure 7.6). The accumulation of medium particulates for both polymers was consistent with the mass analysis results as it changed in a similar manner. It was observed that mass of PHB porous 3-D scaffolds was gradually decreased ($p < 0.01$) from day 7 to day 48 by 13 ± 4.6% of their initial weight (Figure 7.6). Meanwhile, the mass of PHBV porous 3-D scaffolds decreased rapidly at day 7 ($p < 0.01$) and gradually reduced from day 7 to day 37 by 14 ± 3.7% of its initial weight. The weight loss started to stabilize from day 50 until day 312 with no structural damage and rupture. Unlike PHB, PHBV started to give off large chunks of its structure from day 214 and eventually ruptured into several big parts at day 312 (weight loss: 28 ± 3.7% of their initial weight). The extent of weight loss in this investigation (gravimetric analysis) seemed to be very significant due to the surface erosion. However, unlike the pH analysis on the same period, there was no prominent pH drop to indicate any accumulation of acidic degradation products due to the autocatalytic hydrolysis effect. However, when the scaffolds were incubated in the cell growth media, the pH of the medium became significantly acidic at week 4 until week 6 which eventually cause the structure of the scaffolds to rupture at week 6 (data not shown). This was probably due to the autocatalytic degradation process which induced the breakage of the ester linkages to form acidic by-products.

In summary, it appears that the samples might have absorbed the solvent (water) and strain by swelling for 50 days and by then chain scission had created chains short enough to leave the matrix. This caused the changes in the weight and structural integrity. Then, the samples became stabilized with no weight change until day 312 (except PHBV). These all indicate that the microenvironment of the loaded cells becomes less restrictive in time (at least after the initial 2 months) and therefore increases in the cell numbers within the porous scaffold as it will not be hindered by the confined space in the porous structure. However, if the cell growth media
simulated fluid scaffolds were taken into account, the scaffolds would not sustain its structural integrity up to 2 months. For that reason, these optimization studies were only conducted in a confined time frame (6 weeks) where the structural of the cell-loaded scaffolds were still intact for cellular proliferation.

Figure 7.6: Kinetics of *in vitro* degradation process for PHB and PHBV (4%, w/v) porous 3-D scaffolds were measured via gravimetric analysis. The polymeric porous 3-D scaffolds were submerged in phosphate buffered saline (PBS) and incubated at 37 °C. Samples were periodically removed and dried under vacuum prior to the analysis. "Significant differences with \( p < 0.01 \) between the samples were highlighted by lines (\( n = 4 \))." Samples were ruptured into several large pieces.

7.5 Discussion

The interaction of the cells with the material where they are seeded is a result of specific cell surface adhesion receptors which are integrins and ECM proteins that have a cell-binding domain. However, the synthetic polymeric scaffolds tested in many studies lack the natural recognition sites of the cells to the material and are very hydrophobic, making it difficult for the cells to interact with the material. It is well known that hydrophobic surfaces favour the adsorption of proteins from aqueous solutions thermodynamically but it may induce strongly irreversible adsorption and denature the protein’s native conformation and bioactivity (a natural conformation of a protein is a prerequisite for its bioactivity) [120]. Even though the mechanical properties and biocompatibility criteria needed for some of the tissue engineering area has been met previously by the naturally synthesized polymeric scaffolds selected: PHB and PHBV [21, 151, 273] it proved that it was not enough. In order to overcome the hydrophobicity problem, a surface treatment was employed by using an optimized concentration of NaOH (0.6M). Since it has now been well accepted in the biomaterial community that both very
hydrophilic and hydrophobic surface are not good for cell attachment and proliferation, it is better to produce a moderate wettability so that proper amounts of proteins such as the fibronectin and collagen used in this study could be adsorbed and at the same time preserve their natural conformation, resulting in a positive cell response [120, 259, 274].

In addition, as scaffolds designed to mimic the bone marrow should resemble the in vivo architecture, morphology and function in order to optimize cellular proliferation in vitro, it was thought to mimic its composition as well in the sense to provide bio-recognitions signalling site for later interaction. On top of that, scaffold physico-chemical properties such as hydrophobicity and/or hydrophilicity, surface area, porosity and pore size should also considered as critical as the above mentioned specifications to influence cell adhesion, spreading, proliferation and differentiation [157, 166]. Fundamentally, bone marrow is formed of three different constituents: living cells; non-living organic matter such as collagen and non-living inorganic crystals (hydroxycarbonate apatite, HCA). The crystal structure is deposited within an organic matrix formed mainly (95%) of collagen type I [73]. Since ECM-coating of scaffolds is an important parameter to consider in the tissue engineering of 3-D cellular cultures [275-277], it was decided to investigate this further and to coat the scaffolds with two very common ECM proteins: collagen type I and fibronectin. Given that cellular growth can be affected by the method used to coat scaffolds with proteins, it was very important to achieve homogeneous coating and suitable results. Most methods have been based on the immersion of polymer films and matrices [275] which may result in non-uniform distribution and poor penetration of the protein into the scaffold. In this investigation, the simple and non-destructive method of vacuum-immersion protein coating was employed. This method was selected due to fact that the previous study carried out by Blanco et al. [6] using centrifugation process has resulted in structural damage. This method was probably the best method so far for these types of polymers which have issues (e.g., structural integrity) in dealing with the centrifugal force. Previous studies (pore interconnectivity observation through color staining using Alizarin Red S) have shown that this method might have resulted in a good penetration and homogeneous protein coating of the scaffold even in the central sections (Appendix D).

Although the CLL cell lines have already been adapted to 2-D cytokine-independent static cultures, to the extent of our knowledge, their 3-D culture has not been demonstrated and no appropriate culture models of leukaemic cells exist except the studies done by Blanco et al. [6] using AML cell lines. Conventionally, haematopoietic cell cultures have relied on the 2-D growth configuration provided by T-flasks or well-plates which do not recapitulate the haematopoietic inductive microenvironment (HIM) required for supporting the development of multiple cell types at different maturation stages as well as the maintenance of the haematopoietic stem cells [6]. In contrast, ample evidence exists that 3-D culture systems provide a growth environment that promote cell-cell and cell-matrix contact, recreating the
haematopoietic niches, as well as being able to support high cell density. Their attributes make the 3-D culture systems advantageous [261]. Therefore, direct comparison between 2-D and 3-D cultures is not appropriate in terms of their growth kinetics and requirements.

Meanwhile, selecting appropriate parameters for the PHB and PHBV scaffolds that support the growth of all two CLL lines would provide a suitable growth environment for the culture of human primary leukaemic cells. The CLL cells displayed cell line-specific growth kinetics. The growth kinetics of RL is three times higher than that of JeKo-1 and established rapidly in the 3-D culture systems generated by PHB and PHBV. Even though, PHB and PHBV have different properties in terms of porosity (PHB: 81.97% and PHBV: 92.17%) and specific pore volume (PHB: 9.30 cm$^3$/g and PHBV: 10.51 cm$^3$/g) no big difference was observed between the two scaffolds for the fast-growing RL and the slow-growing JeKo-1 cells suggesting that the scaffolds did not present any mass transport limitations for 3-D leukaemia cultures. Since the above mentioned growth kinetics cannot guarantee the best selection of the polymers for studying primary leukaemia cell, other prominent characteristics between PHB and PHBV were summarized in Appendix H. In this table, it summarized the advantages of using PHBV as compared with the PHB from the start of its fabrication process, material characteristics, physico-chemical modifications, in vitro degradation and lastly cellular growth response on the other types of leukaemic cell line, e.g., AML: HL-60 (Chapter 5). All the essential factors that are summarized in Appendix H came from the optimized parameters that have been studied beforehand (Appendix G). In this respect, the PHBV scaffolds were considered advantageous for the use in developing an in vitro model of the primary leukaemia.

Extensive studies have been performed to render materials biomimetic. The surface modification of biomaterials with bioactive molecules is a simple way to make biomimetic materials. The early work has used the long chains of ECM proteins such as fibronectin (FN), vitronectin (VN) and laminin (LN) for surface modifications [81]. The use of peptides, e.g. RGD, YIGSR, etc. instead of ECM proteins as coating material to optimise scaffolds was additionally considered in this investigation. However, the use of complete ECM proteins was considered to be better for porous 3-D scaffolds because not only they could provide the cell-binding sequence for cell adhesion, but also provide secondary interactions with other ECM proteins and growth factors. Small peptides have lower binding site to cells and selective activity for distinct integrin subtypes and are easily cleaved by enzymes [121]. As a result, 3-D systems were developed: scaffolds made of 2 different naturally synthesized polymers: PHB and PHBV and treated with 0.6M NaOH (based on the optimized surface treatment), to culture CLL cell lines and the potential of coating with different concentrations of collagen type I and fibronectin to improve cellular proliferation was tested. Overall, there was no big difference between the PHB and PHBV protein-coated scaffolds with the fast growing RL and the slow-growing JeKo-1 cells. However, there was a big difference in the kinetics of cellular growth.
between the untreated, treated and treated with protein-coated scaffolds. Specifically, it was observed that both polymers coated with 62.5 µg/ml of collagen type I has shown a homogeneous result with similar cellular growth kinetics peaking at week 2 that was validated with the two cell lines. It sustained the best growth of leukaemic cells compared with that of untreated, treated, fibronectin and a higher concentration of collagen (125 µg/ml). This difference can be attributed to the improvement of the treated porous scaffolds architecture and wettability which have proven to produce more voids and increase the chances of sufficient protein adsorption. These extra spaces coupled with an increase in surface hydrophilicity would literally allow more sites of the treated scaffold to be coated with protein monolayer due to the increased perfusion of the media containing proteins deep into the scaffold. With more protein monolayer covered on the pore surface, the cell-cell and cell-matrix contact are becoming better which has been demonstrated to improve cell expansion. This was in agreement with our results which showed that coating with collagen, primarily or fibronectin was beneficial in a CLL cell type-specific manner. Moreover, collagen can accelerate cell adhesion when compared with other ECM proteins [278]. This due to the fact that a large amount of polar groups on the surface (such as -OH, -COO-) could provide many sites to obtain the collagen by polar interaction and hydrogen bonding. This may have explained the superiority of the collagen-coated scaffolds with all cell lines.

To conclude this investigation, after considering all factors (Appendix G and Appendix H), the combination of the treated PHBV with collagen 62.5 µg/ml was best for the 3-D cultivation of the CLL cell lines studied in this work and later for the human primary leukaemia cell. Although the fibronectin-coated scaffolds on the slow-growing JeKo-1 cells produced the highest absorbance between week 5 to week 6, selecting a long period of culture (more than 4 weeks) for the cell culture stabilization would not definitely compromise the structural damage of the PHB and PHBV scaffold due to the acidic degradation observed in the in vitro degradation in cell growth media (Chapter 6, Figure 6.5b). For these protein-coated scaffolds, culture stabilization was accelerated and the cultures became steady by week 1 to week 2 depending on the cell line whilst in the case of uncoated scaffolds, it occurred after week 4 and some of the condition could not be stabilized even at the end of the investigation. For this reason, coating the scaffold with collagen and fibronectin accelerated the growth kinetics of the cells perhaps through enhanced adherence of the cells to the scaffold.

7.6 Conclusions

We have shown that a highly porous scaffold (PHB and PHBV), similar to that defined by the highly porous structure of the spongy cancellous bone (containing soft bone marrow tissue), combined with bone marrow-specific ECM proteins (collagen type I and fibronectin) can
provide a 3-D structure for sustainable \textit{in vitro} cultivation of CLL cells lines for at least 4 weeks and provides a platform for the study of human primary leukaemia cell \textit{in vitro}. The increased hydrophilicity and availability of functional groups after physico-chemical modification would allow increased perfusion of protein molecules, media and cells deep into the scaffolds and thus increasing cell attachment, proliferation and migration. On balance, both treated polymeric porous scaffolds coated with collagen type I at a concentration of 62.5 µg/ml sustained the growth of all CLL cell lines tested \textit{in vitro} well, although each cell line preferentially grew with different cellular growth rates at different phases of the cultures. However, after considering all prominent factors as shown in Appendix H, the combination of the treated PHBV-collagen 62.5 µg/ml is best for the 3-D cultivation of the studied CLL cell lines and later the primary leukaemia cell. These microenvironmental differences in this novel experimental system \textit{in vitro} may provide specific understanding in that both cell-matrix and cell-cell interactions, the later investigation of the pathogenesis of the disease and exploration of novel treatments for leukaemia.
CHAPTER 8 Generation of an Abnormal 3-D \textit{In Vitro} Bone Marrow Biomimicry

8.1 Introduction

Leukaemia is a form of cancer that begins in the blood-forming cells of bone marrow known as hematopoietic stem cells (HSCs). This form of cancer usually occurs when there is an excess of abnormal white blood cells (known as leukocytes) in the blood and lymphoid tissues. For that reason, a study on this abnormal bone marrow which produced the leukaemic stem cells is needed to elucidate the molecular determinants of leukaemogenesis and to identify the cellular and microenvironmental elements that enhance leukaemia growth [6]. To do so, we need to look into the building block of the bone marrow that produces HSCs. In general, in order to sustain blood cell formation, a unique microenvironment of haematopoietic stem cells (HSCs) is essentially required [279]. The availability of stroma cells such as macrophages, adipocytes and fibroblasts which formed the bone marrow niches make this 3-D microenvironment an essential location for hematopoiesis [6, 261]. In addition, the stroma cells elaborate extracellular matrix proteins including fibronectin, collagen, vitronectin and tenascin which create specialized compartments with localized chemokines and cytokines resulting in the regulation of proliferation, differentiation and self-renewal of HSCs [280]. The organization/regulation of the bone marrow niches is critical for the function or dysfunction of normal or malignant bone marrow [281]. Acute myeloid leukaemia is one such malignant bone marrow condition [282]. Other leukaemia cells such as chronic lymphocytic leukaemia (CLL) could also come from lymphoid tissues of the lymphocytes lineages.

Acute myeloid leukaemia (AML) is characterized by a block in differentiation and an unregulated proliferation of myeloid progenitor cells [283]. AML are a group of heterogeneous haematological malignancies with a similar morphology both in children and mostly in adults [284] and affecting nearly 5 in every 100,000 men each year [285]. In view of the occurrence of leukaemia’s as a disease, the need to find the way on how treat it remains a big challenge. The traditional two-dimensional (2-D) cultures and \textit{in vivo} animal (murine) models of AML have helped to characterize the leukaemic progenitors, defining molecules that regulate leukaemic proliferation and differentiation and screening for drugs for efficacy in treating it [264, 285]. However, there are still some difficulties which arise that limit the current techniques used in the study of AML. First, the long-term primary leukaemic cell growth in 2-D cultures cannot be done successfully without the support of exogenous growth factors or stromal cell. Second, 2-D culture conditions cannot structurally provide the native haematopoietic microenvironment (HM) for human AML cells and can only survive for a few days in culture media with foetal calf serum. Third, the animal (murine) models, although superior to 2-D cultures, cannot completely replicate the human microenvironment due to the physiological differences between
mice and human and also require extensive animal facilities and expertise for use [286, 287]. To overcome these challenges, an in vitro 3-D biomimicry of the human haematopoietic microenvironment is developed for the study of AML [288].

The culturing process of HSCs has been traditionally done in a 2-D culture system developed by Dexter in tissue culture flasks/plates [289]. However, HSCs cannot be cultivated in vitro without the addition of the growth factors which usually need high concentrations of cytokines (in the range of ng/ml) [290]. The drawbacks of this 2-D culture system is that it is too expensive, it develops early maturation of the HSCs that could limit its self-renewal capability and do not correspond to those found naturally in the BM, in the range of pg/ml [291]. Hence, the culture of BM haematopoietic cells in 3-D has been investigated as an alternative since it provides a more physiologically accurate microenvironment. Since then, several 3-D cultures system have been developed using hematopoietic cells including the use of encapsulated or cultured on hydrogel matrices [292], tantalum coated biomaterial [290], porous biomatrix CellFoam™ [293], macroporous microspheres [294, 295] or synthetic polymers [292]. Although these scaffolds may resemble some elements of the BM architecture and facilitate cell-cell and cell-matrix interactions, these methods involve the addition of cytokine cocktails to the culture, resulting in unwanted differentiation [7]. Only one study has successfully developed a long-term 3-D culture system that resembled 3-D niches of bone marrow microenvironment fabricated from synthetic polymers (PU and PLGA) using cord blood MNCs without the use of exogenous growth factors [7]. These cultures provide a different microenvironment from that of the 2-D flask cultures and can be used as a model for in vitro haematopoiesis.

8.2 Aim and Hypothesis

As the BM microenvironment in vivo is distributed in a 3-D space and plays a critical role in hematopoiesis, maintaining stem cell potential and controlling proliferation and differentiation, it has some factors (i.e., growth factors) that in absence or presence may result in one outcome or another. The identification of these situations although still unclear it could help elucidate these questions. As a result, it was hypothesised that by recreating the 3-D bone marrow biomimicry, the human primary AML MNCs would, when seeded onto the scaffolds, secrete their own ECM proteins and the cells would start proliferating in the artificial scaffolds without the need of adding exogenous cytokines and truly recreating the natural in vivo environment. For this chapter a 3-D culture system that could expand human primary AML MNCs without the addition of exogenous cytokines by mimicking the BM microenvironment was developed.
8.3 Approach of Study

We have previously shown that 3-D scaffolds made of either poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) and poly(3-hydroxybutyric acid) (PHB) were able to sustain long-term cultures of leukaemic cell lines (Chapter 7). We have now extended our evaluation of these scaffolds to a long-term culture and expansion of unselected human primary acute myeloid leukaemia blood MNCs as summarized in Table 8.1. In order to enhance this BM biomimicry, we coated PHBV with an extracellular matrix (ECM) protein, with an ideal concentration of collagen type I (62.5 µg/ml) that can provide strong mechanical contact between the scaffold material and the cells (Chapter 7).

Table 8.1: Human primary acute myeloid leukaemia (AML) main properties.

<table>
<thead>
<tr>
<th>Cell Properties</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of material</td>
<td>Peripheral blood</td>
<td>Peripheral blood</td>
<td>Aspirated bone marrow</td>
</tr>
<tr>
<td>Type</td>
<td>Secondary</td>
<td>Secondary</td>
<td>Relapsed-Secondary</td>
</tr>
<tr>
<td>Volume of MNCs supernatant</td>
<td>~7 ml</td>
<td>~10 ml</td>
<td>~9 ml</td>
</tr>
<tr>
<td>Cell number/vial (10 ml blood)</td>
<td>16.86 × 10^6 cells</td>
<td>21.32 × 10^6 cells</td>
<td>1.29 × 10^6 cells</td>
</tr>
<tr>
<td>Culture medium</td>
<td>70% IMDM + 30% FBS + 1% PS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultivation routine</td>
<td>Renew media every 2 to 3 days</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

8.4 Results

A polymeric scaffold with different conditions: Unmodified (control) and protein-coated PHBV were tested and seeded with AML blood MNCs. The aim was to derive a cytokine-free 3-D culture system by recreating the 3-D porous structure of the BM. Additionally, when the cells are seeded onto the scaffolds, they would secrete their own ECM proteins and start proliferating in the artificial scaffolds without the need of adding exogenous cytokines and truly recreating the natural in vivo environment. Previous studies on PHAs particularly PHB and PHBV scaffolds seeded with CLL cell lines suggested that protein coating of the polymer could improve its performance. The same technique applied before was used here but human primary AML blood MNCs in vitro was used instead. PHBV was coated with collagen type I, using an ideal concentration of 62.5 µg/ml to add a mechanical strength between the cell and the material surface. The effect of protein coating of PHBV was investigated and compared with the uncoated scaffolds (control).

8.4.1 AML Blood MNCs Proliferation

Twenty-four hours after primary human AML MNCs of 2 different patients were seeded into the scaffolds, the seeding efficiency was in the range of 46% to 82% (Patient 1: 51.21 ± 5.43% (control PHBV), 62.50 ± 11.45% (protein-coated PHBV). Patient 2: 60.52 ± 10.56% (control
PHBV), 71.30 ± 10.48% (protein-coated PHBV)), with no significant difference between them, indicating that majority of the cells remained in situ within the scaffolds (Figure 8.1). Furthermore, the seeding efficiency of the examined scaffolds when seeded with AML blood MNCs was observed to have the same trend when seeded with AML (Chapter 5) and CLL cell lines (Chapter 7) as to indicate a nature-like behaviour of non-anchorage cell types which produced low cell attachment when seeded on biomaterials.

![Figure 8.1: Cell seeding efficiency of the unmodified and ECM-coated scaffolds seeded with AML blood MNCs. The cultures were performed in parallel on 2 separate occasions, each in duplicate.](image)

Cellular proliferation was evaluated over a period of 28 days without the addition of exogenous cytokines. All scaffolds supported the proliferation of primary human AML MNCs in long-term culture (Figure 8.2) and was best in the PHBV scaffolds coated with collagen, peaking at day 14 (Figure 8.2a and b; \( p < 0.05 \)). Cells cultivated on the unmodified PHBV (control) were seen to peak 1 week later (day 21) with lower levels but similar growth kinetics of patient 1 and 2 respectively. Viability of primary leukaemic cells extracted from the scaffolds was assessed at day 0, 7, 14, 21 and 28 (Figure 8.3a and b). Cells cultivated in and supported by the protein-coated 3-D PHBV scaffolds had the best viability (86.47 ± 2.8% (patient 1); 73.24 ± 1.7% (patient 2)) compared with that supported by the unmodified PHBV scaffolds (55.21 ± 2.2% (patient 1); 45.87 ± 1.6% (patient 2); \( p < 0.05 \)) at the end of the 28 day cytokine-free culture. In contrast, AML MNCs (patient 3) in 2-D culture did not survive beyond 5 days without the addition of cytokines. Together, the viability and proliferation data indicated that viable cells derived from primary human AML MNCs could be supported and expanded long-
term in cytokine-free conditions, best in collagen-coated PHBV scaffolds in vitro. We next characterized the cell-scaffold construct morphologically.

![Figure 8.2](image)

**Figure 8.2:** Primary human AML cell support and proliferation on 3-D scaffolds. MNCs from blood samples of 2 different patients with AML (labelled as patient 1 (a) and patient 2 (b)) were cultured on scaffolds for a period of 28 days in the absence of cytokines. Cellular proliferation was assessed by standard MTS assay. From day 7, cellular proliferation was best in PHBV scaffolds coated with collagen type I, highest at day 14 prior to termination of all cultures. Results are plotted after normalization with control scaffolds (scaffolds without cells at the same time point of culture). *p < 0.05 compared within culture day. For all panels, results are means ± sd, n = 2 replicates per experiment, N = 2 separate experiments.
Figure 8.3: Viability of primary human AML MNCs cultured in unmodified and protein-coated PHBV scaffolds during a period of 28 days in the absence of cytokines. Three different patients were utilized: (a) Patient 1 (3-D culture) and 3 (2-D culture) and (b) Patient 2 (3-D culture). The cells were 100% viable when seeded and decreased over time to day 28. In contrast, standard 2-D 24-well plate cultures of AML MNCs (patient 3: sample aspirated from the bone marrow) were non-viable by day 5. *p < 0.05, comparison between groups. For all panels, results are means ± sd, n = 2 replicates per experiment, N = 2 separate experiments (MTS assay) and N = 3 separate experiments (viability).
8.4.2 3-D Microenvironment

Characterization of the 3-D scaffolds and cell morphology was done using SEM for cell distribution and surface topography after 0, 14 and 28 days of cell culture in the scaffold. Scaffolds were sectioned in the outer and central regions of the rectangular cube. The SEM micrographs (Figure 8.4) demonstrate how over a period of 28 days on both unmodified and protein-coated PHBV scaffolds; the cells migrated into the pores establishing themselves in “niche-like” areas. Cellular density in the pores increased with time and by day 28, the walls of the scaffolds were covered by cells. When SEM of the cell-seeded unmodified (control) and protein-coated PHBV scaffolds were compared, the cells adhered better when seeded onto protein-coated PHBV scaffolds. The cells adhered closely to each other indicating that there was a good cell-cell and cell-matrix interaction on the protein-coated PHBV scaffolds apart from the formation of fibres-like structure which could possibly be the ECM proteins secreted by the cells.
Day/PHA | PHBV Collagen | PHBV Control
--- | --- | ---
Day 0 | ![Image](image1.png) | ![Image](image2.png)
Day 14 | ![Image](image3.png) | ![Image](image4.png)
Day 28 | ![Image](image5.png) | ![Image](image6.png)

**Figure 8.4:** Scaffold topography and morphology of AML MNCs by SEM over 28 days of cytokine-free culture. At day 0, MNCs are seen nestling into the pore surfaces. The enucleated red blood cells (indicated by arrows) contaminating the MNCs fraction is visible in the two scaffolds tested, together with the rest of the MNCs seeded. By day 14, MNCs have expanded in number and start to cover the walls of the pores within all scaffolds. Growth of a small colony of cells within the middle section of the scaffold is observed in the protein-coated PHBV condition from day 14 to day 28.

**8.5 Discussion**

We have developed an *in vitro* 3-D culture system that expands functional leukaemia cells without the addition of exogenous cytokines. By studying 2-D and 3-D growths and comparing the results, it was discovered that the 3-D structure facilitated the *in vitro* expansion of AML
blood MNCs without any external support. For the last 10 years, researchers have been able to expand a long-term leukaemia MNCs in vitro without the addition of exogenous cytokines [285]. Prior to this success, there has been a lack of a consistent cell culture system for growing leukaemic cells since the use of traditional two-dimensional (2-D) cultures which cannot support long-term primary leukaemic cell growth without exogenous growth factors or stromal cell support and structurally provide the native haematopoietic microenvironment (HM) for human leukaemic cell [6]. Hence, a long-term leukaemia 3-D in vitro cell culture system by mimicking the 3-D abnormal or malignant bone marrow (BM) niches is needed for characterizing the leukaemic progenitors, defining molecules that regulate leukaemic proliferation and differentiation and screening potential chemotherapeutic agents.

Highly porous scaffolds were fabricated from PHBV and provided an ideal system for three-dimensional human AML blood MNCs culture; these 3-D systems composed of an ECM protein and a surface treated 3-D scaffold. In previous studies (Chapter 6), the role of two proteins: collagen and fibronectin using different concentrations was studied and it was concluded that the combination of PHBV coated with 62.5 µg/ml was best to support the 3-D growth of the CLL cells studied. As the AML and CLL cell lines have been cultured successfully by using variety of synthetic polymers (e.g., PU, PLA, PLLA, PLGA, etc.), it was hypothesized that other classes of polymeric materials (e.g., PHB and PHBV) could also be used to make a porous 3-D scaffold and ultimately expand these leukaemic cells for a long-term growth (up to 4 weeks). The use of this material for making 3-D bone marrow biomimicry scaffold to culture human AML blood MNCs in vitro was a novel approach to observe a long-term proliferation without the addition of expensive growth factors. Until now, these polymeric materials (PHB and PHBV) have not been used for the study of leukaemia and most of the 3-D structure made from these polymers was in thin films with the thickness of less than 1 mm (if no inorganic salts and porous preform porogen involved). The polymer chosen in this study was selected due to the high-quality results obtained in the previous studies for biomedical applications on top of being biocompatible and FDA approved [9, 23]. In this study, protein-coated PHBV showed a higher number of metabolically active cells than the unmodified PHBV (control) when seeded with AML blood MNCs. These results can be compared to the ones obtained previously when using AML and CLL cell lines where protein-coated PHBV overall had also showed better results. This could be explained by the superiority of the ECM protein (e.g., collagen type I) which affects cellular adhesion, viability, ingrowth and distribution on the protein-coated PHBV scaffolds [7].

Also hydrophobicity had an effect in the differences in metabolically active cells (MTS) between the unmodified and surface-treated plus protein-coated PHBV scaffolds. This is due to the fact that hydrophilic surface favourably influenced the cells during the initial stage of contact and later for cells spreading, proliferation and differentiation [120, 259, 274, 278].
cells adhere and grow better on hydrophilic scaffolds than on hydrophobic surfaces; thus it is expected that the surface-treated plus protein-coated PHBV scaffold seeded with AML blood MNCs showed higher number of metabolically active cells than the unmodified PHBV. Furthermore, it can be explained by a large amount of polar groups on the surface (such as -OH, -COO-) which could provide many sites for collagen adsorption by polar interaction and hydrogen bonding [278]. Hence, the sufficient amount of the adsorbed protein on the surface has in fact provided a proper microenvironment for AML blood MNCs culture. Although there were no differences between the seeding efficiency of the collagen coated and unmodified PHBV scaffolds when using PHBV coated with collagen type I; the collagen seemed to accelerate cell growth faster than the unmodified scaffolds and also of any other ECM proteins.

Cellular expansion was measured using the MTS assay where the results proved cellular expansion in the form of increased absorbance. It can be appreciated that the first few days in culture the cells expand quickly on the protein-coated scaffolds and they commenced a rapid expansion peaking at day 14. Whilst the unmodified scaffolds seems to expand quite slowly for the first 7 days until it peaked at day 21. The above mentioned phenomenon was possibly due to the time required for the microenvironment to be established (factors that affected the establishment: surface hydrophilicity/hydrophobicity and ECM surface coating) and the production of self-sustaining growth factors. Although cytokines were not directly measured in this study, in the absence of exogenous cytokines added to the cultures, AML blood MNCs would not have been viable or expanded if cytokines were not produced by cells within the microenvironment in situ, including potentially stromal cells [7]. On the other hand, the corroboration of the MTS assay absorbance values with the DNA quantification which related to cell number was not executed due to the difficulty of extracting the cells from the scaffold as reported from the previous study [7]. It was observed that the longer the culture lasted the more difficult it became to extract the seeded cells. The usage of certain agents only (e.g., proteinase-K or collagenase) might not be enough to release the adhering cells on the protein-coated material surface. A combination of physical means such as shaking, agitation and centrifugation coupled with enzymatic agents for protein digestion would probably help to harvest more cells. The MTS assay for the cell extracted scaffolds (manually extraction) and centrifuged at 1000 rpm for 5 min showed that there was a changed of the growth media into dark-purplish colour after 3 h of incubation (data not shown) to indicate the availability of the remaining cells inside the scaffolds. For that reason only the MTS assay results were used to confirm the hypothesis that AML blood MNCs were expanded in vitro without the addition of cytokines. Viability variance between the unmodified and protein-coated PHBV could be explained by the surface hydrophilicity/hydrophobicity and ECM surface coating although both type of the scaffolds have the same physical properties (Chapter 6: Table 6.4).
The expansion in number is linked with the production of stromal cells and growth factors. Using the scanning electron micrographs taken, it can be supposed that there was a stroma formation (Figure 8.4: PHBV with collagen at day 14 and day 28) and growth factor secretion as the cells would not be able to expand in numbers if there were not. On top of that, the SEM pictures showed an increasing number of cells in the pores that populate the scaffold as time progresses. However, due to the structural integrity problem of the PHBV scaffolds, the Haematoxylin & Eosin (H & E) immunostaining was unable to carry out for comparing the data with the scanning electron micrographs. This is to prove that there was collagen secretion apart from the one present in the coated scaffolds. A flexible and sponge-like PHBV scaffold is essentially required so that during all the rigorous steps for the preparation of the H & E immunostaining, the scaffolds are not destroyed and ruptured into pieces.

Overall, there are several important aspects that need to be taken into serious contemplation. First, the requirement of the scaffolds to be flexible, sponge-like and robust for further analysis is essential so that the microenvironmental structure is not destroyed during the handling process. Second, the method improvement of the extraction/detachment of the cells from the scaffolds in order to obtain the highest amount of cells. Apart from the suggestion of the improvement of the fabrication process and cell extraction procedures, other aspects of expanding the studies using this 3-D culture system to the next levels are still ongoing such as the possibility of a serum-free culture, determining the microenvironmental niches supported by the scaffolds and responsiveness of cells within the niches to external signals.

8.6 Conclusions

In summary, we have achieved successful long-term culture and expansion of AML blood MNCs without the addition of exogenous cytokines using a 3-D scaffold fabricated with PHBV and coated with collagen type I. This 3-D bone marrow biomimicry can be used for diagnosis or prognosis (predicting the likely outcome of an illness) of the abnormal hematopoiesis in a relevant in vitro system and has the potential to be applied to drug discovery platforms and expansion protocols for cellular therapies and transplantation.
CHAPTER 9 Overall Conclusions and Future Work

9.1 Overall Conclusions

The main goal of this study was to develop an in vitro 3-D long-term culture of the human primary AML blood mononuclear cells without the addition of exogenous cytokines. In order to do so, microbial biosynthetic polymers were fabricated, characterized, optimized and selected. Overall conclusions as a result of the efforts toward this goal are summarized in the following sections.

9.1.1 Summary of Contributions

9.1.1.1 Fabrication of Porous 3-D Scaffolds

To produce a porous 3-D scaffold with an improved thickness greater than 1 mm (in relative to the conventionally made PHA matrices) from this type of microbial biosynthetic polymer (e.g., PHB and PHBV) was a big challenged. Most of the scaffolds fabricated from these polymers were not in the porous 3-D structure. The applicability and reproducibility of the breakthrough technique to fabricate the porous 3-D scaffold was demonstrated on PHB and PHBV, manufactured using the solvent-casting particulate-leaching (SCPL) process. Several factors have been identified in the successful of fabricating the porous 3-D scaffolds: (1) Identification of an ideal polymer concentration. (2) Controlling the rapid phase separation of the polymeric solution due to the rapid solvent evaporation. This has been demonstrated by keeping the polymer solution plus porogen cast inside a small orifice of lyophilization flask and put it inside a fume cupboard with constant velocity air suction. This is to avoid any development of thin films on top of the scaffold (etching effect). (3) The usage of cold deionized water (DIW) during the salt leaching process avoids fast surface erosion. Fast salt leaching processes will facilitate internal structural damage. As the whole structure of the dried cast was randomly compacted, a few parts of it might not be compacted properly during the drying process. These loose sections might contain large big chunks of agglomerated salt particles that were packed together tightly. Contacts with hot water for instance, will cause the water to rapidly diffuse through the loose sections due to the expansion of the pores wall (the polymer becomes rubber-like condition) and rip off a large chunk of the compacted cast which will eventually leave a big hole on the scaffolds. Although the process was considered time consuming (it could take up to 2 days), the final structural formation of the porous 3-D scaffolds was indeed promising for tissue engineering applications. The porous 3-D scaffolds were characterized using conventional characterization techniques prior to the 3-D cell culture work.
and have demonstrated high porosity, acceptable range of pore diameters and relatively good interconnectivity (the results can be viewed in Appendix D).

9.1.1.2 Development of 3-D Biomimicry Culture System

It was an ideal prospect to mimic the highly porous structure of the spongy cancellous bone and its composition (e.g., soft marrow tissue containing HSCs and non-HSCs) in order to overcome the problem of culturing AML cells in vitro. It seemed that by using a three dimensional configuration that resembled the in vivo bone marrow, the same microenvironment was developed. Hence, the 3-D model culture system has been a successful approach. The leukaemic cells (e.g., CLL and AML cell lines) can be expanded for long periods of time (up to 6 weeks). They were all investigated in their natural environment without the addition of external growth factors. To validate the three dimensional model, the human primary AML blood mononuclear cells was cultivated on the optimized porous 3-D scaffolds. The in vitro expansion of human AML blood mononuclear cells represented a possibility to be applied to drug discovery platforms and expansion protocols for cellular therapies. The hypothesis was that by providing a 3-D environment in the form of a scaffold, it will facilitate the expansion of the cells in its native state (the same that it was previously assumed for the CLL and AML cell lines). The hypothesis was proven and the cells expanded without the addition of cytokines although further studies about the differentiation pattern of the cells are still required and need further investigation.

Initially, the research was commenced by choosing two types of polymeric scaffolds (e.g. the PHB and PHBV) which were produced in our laboratory using the solvent-casting particulate-leaching (SCPL) process. Both of the polymeric scaffolds were initially tested with the AML cell line to observe the cells response on the material without adjusting its native surface chemistry and physical properties. As the cells seemed to be irresponsive, the surface modifications were employed. The surface of the scaffolds was treated with NaOH and rf-O2 plasma and the selection of the most suitable surface-treated scaffolds for the CLL cell line expansion was carried out. Afterwards, the polymeric scaffolds were coated with two very important and common ECM proteins: collagen type I and fibronectin. The cell seeding of the protein coated scaffolds showed an enhanced affinity in comparison with the uncoated and untreated scaffolds. Protein was incorporated using the vacuum-immersion method to produce homogenous surface protein coating and it resulted in better cell spreading and proliferation. Although the seeding efficiency of certain cell lines was higher when using the uncoated scaffolds, overall and after the long term studies, the protein-coated scaffolds did improve cellular expansion by peaking as early as week 2 to week 3 as compared to the uncoated and untreated scaffolds which peaked at week 4 to week 5.
Overall, it can be concluded that the scaffold that supported leukaemic cell growth in vitro at its best was the scaffold made from PHBV and coated with 62.5 µg/ml of collagen type I. Nevertheless even though collagen coating aided cellular expansion further investigations are required about different and more efficient methods of coating. Protein absorption should also be quantified and the design of a perfusion system where the collagen could be circulated efficiently through the sample should be examined as well.

9.1.2 Future Work

9.1.2.1 Fabrication of Porous Scaffolds with Optimal Pore Size and Interconnectivity

Pore diameters, interconnectivity and porosity are important design criteria for the fabrication of polymeric 3-D porous scaffolds. They are important parameters to consider during the fabrication technique as different cells can attach and spread on a broad range of pore sizes. In addition, good interconnectivity would facilitate the delivery of nutrient, oxygen and metabolites which are beneficial to the proliferation of cells. It was reported that pore size should be close to 20 µm for the ingrowth of hepatocytes, 20 to 150 µm for skin regeneration and in the range of 200 to 400 µm for bone regeneration [296]. Various microcellular and porous scaffolds morphologies is achievable by adjusting the parameters during the solvent-casting particulate-leaching (SCPL) process. The shape and the size of the porogen play a vital effect on the porosity. Meanwhile, the usage of pressure (compression) during the scaffolds moulding affect the pore interconnectivity. It was reported that the sphere shape with face-to-face contact coupled with the compression pressure can in fact be used to control of the pore size and interconnectivity of the scaffold (the higher the pressure, the bigger the pore interconnectivity) [202]. Considering such design parameters, the pore diameter and interconnectivity of the scaffold could be adjusted to satisfy the various cell growths for tissue engineering prior to the surface treatment and to verify whether pore size and interconnectivity really matters.

9.1.2.2 Adsorption of Collagen Type I to Polymer Surfaces

To allow better uptake of collagen during exposure of the polymeric scaffolds to the collagen solution, a perfusion system is recommended so that the homogeneous closed system where the collagen solution could be circulated throughout the sample can be created. The present method involved with many steps just to make sure that the porous structure are not blocked by the protein. An alternative approach to expose the polymeric scaffolds to collagen solution would be the incorporation of collagen in the polymer solution prior to the SCPL process.
9.1.2.3 The Amount of Protein Adsorbed

There have been many studies focused on protein coating of polymeric surfaces to improve cellular affinity and collagen type I has been the molecule mostly used in these cases. The problem of measuring protein adsorption still remains uncertain. It is believed that there are several interactions needed in order for the protein to be adsorbed onto the solid surface and that the polymeric scaffold surface chemistry affects the adsorption of the proteins; furthermore the amount of protein adsorbed by the scaffold correlates with the number of surface attached cells. A recent technique using Atomic Force Microscopy (AFM) has been employed based on the analysis of AFM height histogram obtained from height images to quantify the additional thickness added to the disks height (bare surface) during protein adsorption [297, 298].
REFERENCES


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233. Rouxhet, L., Duhoux, F., Borecky, O., Legras, R., Schneider, Y.-J., *Adsorption of albumin, collagen, and fibronectin on the surface of poly(hydroxybutyrate-hydroxyvalerate) (PHB/HV) and of poly(-caprolactone) (PCL) films modified by an


NaCl Conductivity Standard Curve

Figure A.1: Relationship between conductivity of sodium chloride (Sigma-Aldrich®) solution with respect to its different concentration (mg/ml). Conductivity of deionized water was 2.73 µS/cm. It was a linear correlation between conductivity (mS/cm) and the concentration of sodium chloride (mg/ml); \( Y = 2.8475x + 8.5027 \); \( R^2 = 0.9999 \) (\( n = 3 \))
Morphology of Dried Porous 3-D Scaffolds after Salt-Leaching Process

Figure B.1: Morphology of the polymeric porous 3-D scaffolds in a cylindrical shape prepared using the solvent-casting particulate-leaching (SCPL) technique. They were later cut in cuboids with an approximate size of 5 mm × 10 mm × 10 mm: (a) Aerial view of the PHB 4% (w/v) porous 3-D scaffold; (b) Side view of the PHB 4% (w/v) porous 3-D scaffold with the thickness of 5.25 ± 0.36 mm; (c) Aerial view of the PHBV 4% (w/v) porous 3-D scaffold; (d) Side view of the PHBV 4% (w/v) porous 3-D scaffold with the thickness of 4.40 ± 0.52 mm. *d*: thickness.
Wettibility of Polymeric Porous 3-D Scaffolds

Figure C.1 compares the PHB and PHBV (4%, w/v) porous 3-D scaffolds for their absorbability (wettibility) in cell culture medium (IMDM). When the PHBV was put into the cell growth media, the scaffold was submerged within 105 min. However, PHB was submerged with faster absorbability rate than PHBV (within 60 min) owing to the less hydrophobic character than PHBV. The fast wetting and sinking of a scaffold into cell culture medium are highly desirable for tissue engineering applications because the cells can be directly seeded and cultured in scaffolds without any prewetting treatments (e.g., ethanol) which could induce structural deformation [162].

Figure C.1: Photographs showing the submerging of (a) PHBV (4%, w/v) porous 3-D scaffold and (b) PHB (4%, w/v) porous 3-D scaffold into the cell culture growth media (IMDM) as a function of time.
APPENDIX D

Pore Interconnectivity Observation Through Colour Staining

1) **Chemical for staining:** Alirazin Red S (Sigma-Aldrich™) was dissolved in deionized water (DIW) with the concentration of 0.4% (w/v).

2) **Instrument for optical microscopic imaging:** Dino-Lite AM-311s USB Microscope 10x - 200x + LED.

3) **Software:** Image Analyzer 1.33 MeeSoft™ (grayscale mapping exposure).

4) **Method:** A simple experiment was performed to observe the pores interconnectivity in which the polymeric porous 3-D scaffolds were soaked in the color staining solution of Alirazin Red S (0.4% w/v) by means of the normal soaking (without pressure) and forced entry (under vacuum pressure of 2 mbar) for 60 min to 105 min and 5 min respectively. After the soaking, the sample was dried, sectioned and pictured prior to the image analysis by mean of inverted colour mapping exposure. Pores with red colour apparently had been accessible, either directly or via neighboring pores.

5) **Results:**

(a) Visible observation

<table>
<thead>
<tr>
<th>Methods</th>
<th>Visible observation of porous 3-D scaffolds[a]</th>
<th>PHB (4%, w/v)</th>
<th>PHBV (4%, w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal soaking</td>
<td>Mild colour stained with one big unstained spot</td>
<td>Mild colour stained with scattered unstained spots</td>
<td></td>
</tr>
<tr>
<td>Forced entry (2 mbar)</td>
<td>Colour stained almost homogeneously</td>
<td>Colour stained almost homogeneously</td>
<td></td>
</tr>
</tbody>
</table>

[a] Visible observation on the vertical cross-section of the porous 3-D scaffolds.
Figure D.1: Photographs showing the vertical cross section of polymeric porous 3-D scaffolds stained by using the Alizarin Red S (Sigma-Aldrich™). Two different methods were conducted which are by means of the normal soaking (without pressure) and forced entry (under vacuum pressure of 2 mbar for 5 min).
**Table D.2:**

<table>
<thead>
<tr>
<th>Methods/parameter</th>
<th>PHB (4%, w/v) porous 3-D scaffolds [b]</th>
<th>PHBV (4%, w/v) porous 3-D scaffolds [b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal soaking (soaking period: ~60 min to 105 min)</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Interconnectivity (%) [a]</td>
<td>70.12 ± 3.54</td>
<td>74.28 ± 1.52*</td>
</tr>
<tr>
<td>Control (unstained 3-D scaffolds)</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>Forced entry (vacuum pressure of 2 mbar, 5 min)</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Interconnectivity (%) [a]</td>
<td>78.00 ± 0.41</td>
<td>85.82 ± 0.56*</td>
</tr>
</tbody>
</table>

[a] % Interconnectivity was calculated based on the TOTAL AREAS UNDER THE CURVE of grayscale mapping exposure histogram. [b] Optical microscopic images were taken by using Dino-Lite AM-311s USB Microscope 10x to 200x. *p < 0.05 as compared with PHB (n = 4).

% Interconnectivity = \( \frac{\sum \text{Areas under the curve (black coloured area)}}{\sum \text{Areas under the curve (black coloured area)} + \text{Areas under the curve (white coloured area)}} \) \times 100%.

**Figure D.2:** Microscopic images (10x and 20x magnification) demonstrating the vertical cross section of polymeric porous 3-D scaffolds stained by using the Alirazin Red S (Sigma-Aldrich™).
APPENDIX E

Example Calculation of Surface Roughness Ratio (r) Based on the Wenzel’s Theory

Table E.1: Surface roughness ratio (r) of polymeric porous 3-D scaffolds.

<table>
<thead>
<tr>
<th>Physical properties</th>
<th>Polymeric porous 3-D scaffolds</th>
<th>PHB (4%, w/v)</th>
<th>PHBV (4%, w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface roughness ratio, r</td>
<td>1.41 ± 0.02</td>
<td>1.62 ± 0.03*</td>
<td></td>
</tr>
<tr>
<td>BET surface area, A_s, m²/g</td>
<td>0.70 ± 0.02</td>
<td>0.82 ± 0.03*</td>
<td></td>
</tr>
</tbody>
</table>

*(p < 0.05) - Results were considered statistically significant (n = 4) as compared with PHB.

Assumptions:
1. The weight of porous 3-D scaffolds in a cylindrical shape measured is the same amount of polymer used in the SCPL process = 2.4 g = 4% (w/v) PHB and PHBV.
2. The efficacy of SCPL = 100%.
3. There is no loss of polymer materials during the fabrication and leaching process.

PHB
1. Diameter of porous 3-D scaffold measured by Vernier caliper = 8.22 cm.
2. So, radius = 4.11 cm.
3. Thickness = 0.55 cm.
4. The geometric surface area of 3-D scaffold (cylindrical shape) = \(2\pi r^2 + 2\pi rh\)
   \(\text{Surface Area} = \text{Areas of top and bottom} + \text{Area of the side}\)
   \(\text{Surface Area} = 2[\text{Area of top}] + (\text{perimeter of top})h\)
5. BET surface area (result is given by the instrument) = Specific surface area (m²/g).
6. PHB BET surface area = 0.7029 m²/g × 2.4 g (weight of porous 3-D scaffold) = 1.69 m²
7. Roughness factor (r) = True surface area of porous 3-D scaffolds/Geometric surface area of porous 3-D scaffolds = 1.69 m²/1.20 m² = 1.41

PHBV
1. Diameter of porous 3-D scaffold measured by Vernier caliper = 8.40 cm.
2. So, radius = 4.20 cm.
3. Thickness = 0.44 cm.
4. The geometric surface area of porous 3-D scaffold (cylindrical shape) = \(2\pi r^2 + 2\pi rh\)
   \(\text{Surface Area} = \text{Areas of top and bottom} + \text{Area of the side}\)
   \(\text{Surface Area} = 2[\text{Area of top}] + (\text{perimeter of top})h\)
5. PHBV BET surface area = 0.8192 m²/g × 2.4 g (weight of porous 3-D scaffold) = 1.97 m²
6. Roughness factor (r) = True surface area of porous 3-D scaffolds/Geometric surface area of porous 3-D scaffolds = 1.97 m²/1.22 m² = 1.62
APPENDIX F

1. Surface Thermodynamics (Surface Free Energy on the Thin Film)

Method: The Fowkes equation is chosen as the equation of state (EOS). In this case, the adhesion energy at SL interfaces/work of adhesion at SL interfaces/total energy change of separating liquid & solid adhesion (E_{adh}) is a sum of two partial contributions, each of which is expressed as the geometric mean polar of two adhesion parameters, \( \gamma_p \) and \( \gamma_s \), the first of which characterizes the "adhesive power" of the liquid and the second is the "adhesive power" of the solid. The surface energy of the solvent-cast thin film was determined by the contact angles of two types of reference liquids (H\(_2\)O and CH\(_2\)I\(_2\)). The material surface free energy (\( \gamma_s \)) was calculated based on the equations as shown below:

\[
E_{adh} = W_{SL} = \gamma_L(1 + \cos \theta) = 2[(\gamma_L^d \gamma_s^d)^{1/2} + (\gamma_L^p \gamma_s^p)^{1/2}] \rightarrow \text{(Dupree, Young and Fowkes equation)}
\]

\[
\gamma_s = \gamma_s^d + \gamma_s^p \rightarrow \text{(Surface free energy)}
\]

<table>
<thead>
<tr>
<th>Test liquid</th>
<th>( \gamma_L ) (mN/m)</th>
<th>( \gamma_s^d ) (mN/m)</th>
<th>( \gamma_s^p ) (mN/m)</th>
<th>( \gamma_L ) (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (H(_2)O)</td>
<td>72.8</td>
<td>21.8</td>
<td>51.0</td>
<td></td>
</tr>
<tr>
<td>Di-iodomethane (CH(_2)I(_2))</td>
<td>50.8</td>
<td>50.8</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Results:

CH\(_2\)I\(_2\) = (50.8)\((1+\cos42.80) = 2[(50.8 \times \gamma_L^d)^{1/2} + (0 \times \gamma_s^p)^{1/2}], (50.8)(1.73) = (14.24)\gamma_L^d = 87.88/14.24 = (\gamma_L^d)^{1/2}, \gamma_s^d = (6.17)^2 = 38.08 \text{ mN/m}

H\(_2\)O = (72.8)\((1+\cos67.80) = 2[(21.8 \times \gamma_L^d)^{1/2} + (51.0 \times \gamma_s^p)^{1/2}] = (72.8)(1.38) = (21.8)(38.08)^{1/2} + (251.0 \times \gamma_s^p)^{1/2}, 100 = 57.62 + [(14.28)(\gamma_s^p)^{1/2}], 100 - 57.62 = [(14.28)(\gamma_s^p)^{1/2}], 42.38/14.28 = (\gamma_s^p)^{1/2}, \gamma_s^p = (8.81)^2 = 8.81 \text{ mN/m}

\[
\therefore \text{SFE of PHB (} \gamma_s = \gamma_s^d + \gamma_s^p \text{)} = 8.81 + 38.08 = 46.89 \text{ mN/m}
\]

| Sample | \( \theta_{H2O} \) (deg.) | \( \theta_{CH2I2} \) (deg.) | Dispersive comp. (\( \gamma_s^d \)) | Polar comp. (\( \gamma_s^p \)) | SFE (\( \gamma_s = \gamma_s^d + \gamma_s^p \)) |
|--------|-----------------|-----------------|-----------------|-----------------|----------------|---|
| PHB    | 67.80 \pm 1.2   | 42.80 \pm 0.5   | 38.08 \pm 0.2 mN/m | 8.81 \pm 1.3 mN/m | 46.89 \pm 1.0 mN/m |

SFE at the SL interface based on the Young equation = \( \gamma_{SL} = 46.89 - (72.8)\cos(67.80) = 19.38 \text{ mN/m} \)

Dupree equation (work of adhesive at interfacial) - \( E_{adh} = W_{SL} = \gamma_L + \gamma_s - \gamma_{LS} = 72.8 + 46.89 - 19.39 = 100.30 \text{ mN/m} \)

OR \( E_{adh} = W_{SL} = \gamma_p(1 + \cos \theta) = 72.8(1 + \cos67.80) = 100.30 \text{ mN/m}, ([+] represent a non-spontaneous process is occurred (the liquid water beads up. No spreading occurred). An external energy (e.g., surface treatment) is needed to overcome the strong water intermolecular cohesive forces so that the water can spread on the polymer surface).

Work of cohesion of water [\( W_{LL} = \Delta G = 2\gamma_L \)] if they are separated into 2 sections of liquid which form 2 new surface area = 2(72.8 mN/m) = 145.8 mN/m.

Spreading coefficient (water over a thin film) = \( \gamma_{SL} = W_{SL} - W_{LL} = 100.30 - 145.8 = -45.50 \text{ mN/m} \) ([-] spreading coefficient means water will not spread over a thin film surface).

| Sample | \( \theta_{H2O} \) (deg.) | Interfacial (\( \gamma_{SL} \)) | Work of adhesive (\( W_{SL} \)) | (\( \gamma_{SL} \)) | (\( W_{SL} - W_{LL} \)) |
|--------|----------------|-----------------|----------------|-----------------|----------------|---|
| PHB    | 67.80 \pm 1.2 | 19.38 \pm 0.6 mN/m | +100.30 \pm 1.1 mN/m | -45.50 \pm 1.3 mN/m |

If \( W_{SL} > W_{LL} \rightarrow \) The work of adhesive at SL interface (e.g., hydrogen bonding [polar component], dipole-dipole [dispersive component] and Van der Waal [dispersive component]) is strong enough to overcome the work of cohesive of water to promote spreading on the solid surface.

If \( W_{SL} < W_{LL} \rightarrow \) No wetting occurred.
Discussion: According to Blanco et al. [6], POLYMERIC MATERIAL which is hydrophobic and POSSESES HIGH IN INTERFACIAL FREE ENERGY IN AQUEOUS SOLUTION tends to unfavourably influence cells and tissues during the initial stages of cell-biomaterial. However, not all polymer materials have high interfacial free energy (γSL). As for PHB/PHBV, the surface free energy at solid surface is much higher than at the interface (γs > γSL). That is why we observed that the AML and CLL cell lines had a fairly good attachment on both polymeric surfaces (Chapter 5 and 6). The hydrophilic surface condition has more than 100 mN/m or at least more than the water surface tension 72.8 mN/m (γs > γSL - SFE@solid is always higher than SFE@SL). As for the hydrophobic surface (<100 mN/m), the condition could be γs > γSL or γs < γSL. If the case of γs > γSL (as observed on PHB & PHBV), the surface is still considered to have the ability to be wet by the liquid (e.g., water) because the SFE@solid is higher than SFE@SL. In fact, this will affect the adsorption of ECM proteins whereby the proteins will rearrange its molecular structure to adsorb onto the hydrophilic surface. The protein structure will again rearrange to allow the exposed RGD peptides (biosignaling templates) for cell attachment via ligand-receptor interactions. BUT if γs < γSL (as observed on the other highly hydrophobic synthetic polymers (e.g., PS, PE, PMMA, PLA, etc.)), the problem will start to occur during the initial contact of polymeric surface with the serum-supplemented aqueous media where only small surface area will be spread by the aqueous cell media. This will give only a small area and amount of proteins to adsorb onto the surface and eventually make the cell to unfavourably attach to the surface due to the low availability of biosignaling compounds.

2. Cassie-Baxter Contact Angle Corrections for the Heterogeneous Wetting on the Porous 3-D Scaffolds

<table>
<thead>
<tr>
<th>Surface physico-chemistry</th>
<th>Polymeric porous 3-D scaffolds</th>
<th>Control (Thin films)</th>
<th>[a][b][c]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHB (4%, w/v)</td>
<td>PHBV (4%, w/v)</td>
<td>PHB (4%, w/v)</td>
</tr>
<tr>
<td>Surface roughness ratio, r</td>
<td>1.41 ± 0.02</td>
<td>1.62 ± 0.03</td>
<td>N/A</td>
</tr>
<tr>
<td>Fraction of fluid contact area, f</td>
<td>0.52 ± 0.04</td>
<td>0.34 ± 0.02</td>
<td>N/A</td>
</tr>
<tr>
<td>Contact angle, θapparent (deg.)</td>
<td>65.79 ± 1.3</td>
<td>67.55 ± 1.1</td>
<td>66.80 ± 0.2</td>
</tr>
<tr>
<td>True contact angle, θtrue (deg.)</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

[a] Fabricated by using the solvent casting. [b] Equilibrium contact angle of solvent-cast thin films on polypropylene sheet (n = 10). [c] Contact angle of polypropylene (PP) sheet without PHB and PHBV coating = 92.43 ± 0.3° [d] Cassie-Baxter regime for the heterogeneous wetting: cosθtrue = [τf.cosθapparent] + f - 1 (roughness ratio of the wet area, τf was assumed to be as an average roughness ratio, r of polymeric porous 3-D scaffolds). [e] Initial true contact angle of the redistilled water droplet resting on the porous 3-D scaffolds (n = 10). [f] Fraction of fluid area in contact with the material surface, f = 1 - fair. A planar water droplet area is assumed to be in a circle shape with a diameter of ~400 µm (DSA software). All pores inside the droplet targeted area were assumed to be in a circle shape where the total area of pores which contained air can be calculated (fair is calculated by selecting random pore areas from the SEM (n = 3)).

Calculations:

PHB: cosθtrue = τf.cosθapparent + f - 1 = cos93.95 = [(1.41)(0.52)(cosθ)] + 0.52 - 1 -0.07 = [0.733cosθ] - 0.48, -0.07 + 0.48 = [0.733cosθ], cosθ = 0.41, θ = cos⁻¹[0.41] = 65.79°

PHBV: cosθtrue = τf.cosθapparent + f - 1 = cos117.20 = [(1.62)(0.34)(cosθ)] + 0.34 - 1 -0.45 = [0.55cosθ] - 0.66, -0.45 + 0.66 = [0.55cosθ], cosθ = 0.38, θ = cos⁻¹[0.38] = 67.55°

Discussion: A heterogeneous wetting condition of Cassie-Baxter regime was selected in describing the wetting condition of the porous material and predicting the corrected apparent contact angle. Both polymeric porous 3-D scaffolds were observed to amplify the theoretical/corrected apparent water contact angle (θapparent) ranging from approximately 28° to 49° (θtrue - θapparent). These theoretical/corrected apparent water contact angles (θtrue) were considered close enough to the solvent-cast thin films (p > 0.05: applicable only for PHB) to indicate that the Cassie-Baxter regime was quite relevant in explaining the highly observed surface hydrophobicity. In fact, both polymeric porous 3-D scaffolds and the corresponding thin films were considered as hydrophobic materials as they have a high degree of water contact angle of more than 65°. Thus, the highly observed surface hydrophobicity of the polymeric porous 3-D scaffolds in comparison with that of solvent-cast thin films (in accordance with the Cassie-Baxter regime) was probably due to the surface heterogeneity (e.g., availability of the surface roughness which could lead to hysteresis), air trapped inside the pore grooves (enhances the hydrophobicity because the drop is then partially sitting on air) and contaminants of salt on the rough surfaces (which could lead to a decrease of solid-gas (γs) surface energy).
APPENDIX G

Optimized Parameters Obtained From the Optimization Processes

Table G.1: Optimized parameters obtained from the optimization processes to enhance cell-biomaterial adhesion and interactions.

<table>
<thead>
<tr>
<th>(1) Structural optimization</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polymer concentration</strong></td>
<td>4% (w/v) - Homogeneous polymer solution. Fixed parameters: Temperature, pressure and volume. Independent variables: Time, concentration and type of polymers. Dependent variable: Homogeneity of the solution (visual assessment)</td>
</tr>
<tr>
<td><strong>Thickness of the 3-D scaffolds</strong></td>
<td>4 mm to 5 mm (recommended dimension of the porous 3-D scaffolds to be put inside the 24-well cell culture plate: ~10 mm × ~10 mm × ~5 mm). Fixed parameters: Salt weight fraction, % w/w, temperature, pressure and volume. Independent variables: Polymer concentrations and type of polymers. Dependent variable: Thickness</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(2) Physico-chemical optimization via surface treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mode of treatment</strong></td>
<td>Wet chemical method: 0.6M NaOH. Fixed parameters: Temperature, pressure, volume, scaffolds dimension and time. Independent variables: Type of polymers and NaOH concentrations. Dependent variables: Structural integrity (visual assessment), wetting (contact angles) and weight loss</td>
</tr>
<tr>
<td><strong>Post optimization outcome</strong></td>
<td>1) Good structural integrity</td>
</tr>
<tr>
<td><strong>Structural morphology</strong></td>
<td>2) More void developed with good interconnected open pores</td>
</tr>
<tr>
<td><strong>Post optimization outcome</strong></td>
<td>3) Weight loss less than 5% (w/w)</td>
</tr>
<tr>
<td><strong>Surface chemistry</strong></td>
<td>A significant decrease of hydrophobic properties. Fully wetting condition (contact angle less than 25°) occurred for all polymers. Both polymers exhibited well in cellular attachment and proliferation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(3) Cell-biomaterial adhesion and interaction optimization via biological surface coating</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ECM protein concentration</strong></td>
<td>Fixed parameters: Polymer concentration, thickness, type of surface treatment, scaffolds dimension and cell seeding density. Independent variables: Protein concentrations, type of polymers, culture time and type of cells. Dependent variable: Absorbance (490 nm). <strong>REMARKS:</strong> On balance, both PHBV and PHB coated with collagen type I at a concentration of 62.5 µg/ml have the ability to sustain the growth of all CLL cell lines tested <em>in vitro</em> well, although each cell line preferentially grew with different concentrations of collagen and fibronectin at different phases of the cultures</td>
</tr>
</tbody>
</table>
APPENDIX H

Comprehensive Comparison of Both Polymers and Criterion of Choosing PHBV-Collagen as a Biomaterial

**Table H.1:** Comprehensive comparison of both polymers and criterion of choosing PHBV-collagen as a biomaterial to study the biology and treatment of primary leukaemia in an *in vitro* biomimicry.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>PHB 4% (w/v)</th>
<th>PHBV 4% (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fabrication process (Drying of polymer cast)</td>
<td>Rapid phase separation produced detachable thin films</td>
<td>No detachable thin films occurred</td>
</tr>
<tr>
<td>Fabrication process (Salt-leaching process)</td>
<td>Easily to detach whilst handling the wet scaffold</td>
<td>Stable structure whilst handling</td>
</tr>
<tr>
<td>Porosity</td>
<td>82%</td>
<td>93% (extra void spaces)</td>
</tr>
<tr>
<td>Weight loss after surface treatment (0.6M NaOH)</td>
<td>5.28% (w/w): Easily to rupture whilst handling the wet scaffold</td>
<td>3.26% (w/w): Rigid wet scaffold structure</td>
</tr>
<tr>
<td>In <em>vitro</em> degradation in cell growth media</td>
<td>Both polymeric scaffolds have the tendency to produce acidic degradation between week 4 to week 5 onwards</td>
<td></td>
</tr>
<tr>
<td>Cellular growth response from other leukaemic cell types (AML: HL-60) (Refer to Chapter 5, Section 5.4.11)</td>
<td>Slow cellular growth for the untreated scaffolds until week 2</td>
<td>Better cellular growth until week 2 for the untreated scaffolds. This is due to the highly porous nature of PHBV 3-D scaffolds which have provided extra surfaces onto which the cells can growth adequately</td>
</tr>
</tbody>
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