Fabrication and characterisation of 3D porous bioactive glass-ceramic/polymer composite scaffolds for tissue engineering.

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Declaration

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

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Abstract

Designing tissue engineering scaffolds with the required mechanical properties and favourable microstructure to promote cell attachment, growth and new tissue formation is one of the key challenges in the tissue engineering field. An important class of scaffolds for bone tissue engineering is based on bioceramics and bioactive glasses. The primary disadvantage of these materials is their low fracture resistance under load and their high brittleness. These drawbacks are exacerbated by the fact that optimal scaffolds must be highly porous (>90% porosity). As a main focus of this thesis, a novel approach was investigated to enhance the structural integrity, fracture strength and toughness of partially sintered 45S5 Bioglass® based glass-ceramic scaffolds by polymer infiltration and to develop an understanding of the interaction of these two phases in the final composite structure. Commercially available synthetic poly(D,L-Lactic acid) (PDLLA) was incorporated as a coating onto the partially sintered Bioglass® based scaffolds by dipping technique. Two natural polymers synthesised from bacteria, which exhibit different properties to those of PDLLA, were also investigated: i.e. poly(3-hydroxybutryate) (P(3HB)) and poly(3-hydroxyoctanoate) (P(3HO)). The work of fracture of partially sintered 45S5 Bioglass® scaffolds was significantly improved by forming interpenetrating polymer-bioceramic microstructures which mimic the composite structure of bone. It was demonstrated that coating with polymers such as PDLLA, P(3HB) and P(3HO) does not impede the bioactivity of the scaffolds but the extent of bioactivity, given by the kinetic of HA formation, was seen to depend on polymer type and on scaffold sintering conditions. Polymer coated 45S5 Bioglass® pellets sintered at the same condition as the scaffolds and immersed in SBF were investigated to better evaluate the bioactivity mechanism and interfacial properties of the materials. It was demonstrated that polymer coated 45S5 Bioglass® based glass-ceramic scaffolds can have higher bioactivity and improved fracture toughness when the basic scaffold structure is sintered at relative lower sintering temperatures leaving residual open porosity which can be efficiently infiltrated by the polymer.

A bilayered scaffold structure was also designed and fabricated to develop for the first time a porous bioactive glass-ceramic scaffold coated with PDLLA nanofibers. Electrospinning was used to deposit a PDLLA fibrous layer on top of the bioactive
glass scaffold. These scaffolds were developed for osteochondral tissue engineering applications. SBF studies showed that the extent of mineralisation of the PDLLA fibres depended on the fibrous mesh thickness. PDLLA fibres deposited for 2 hours did not mineralise when immersed for 7, 14 and 28 days in SBF making the structure suitable for osteochondral defect applications. Initial in vitro cell response studies showed that the bilayered scaffolds were non toxic and chondrocyte cells were able to proliferate on the PDLLA fibre layers, demonstrating the potential of the novel scaffolds for osteochondral tissue engineering.
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\[ \text{Ra} = y_1 + y_2 + \ldots + y_n/n \ldots \]  \hspace{1cm} (4.4) \hspace{1cm} 83

\[ k = \mu t Q/ A \Delta p \]  \hspace{1cm} (4.5) \hspace{1cm} 83

\[ \Delta p\text{Total} = \Delta p + \Delta p\text{sec} \]  \hspace{1cm} (4.6) \hspace{1cm} 84

\[ \Delta p\text{sec} = 2Q^2 p/\pi [1/d_1^2 - 1/d_2^2] \]  \hspace{1cm} (4.7) \hspace{1cm} 84

\[ \begin{align*}
\text{p-nitrophenylphosphatase} & \quad + H_2O \\
\text{ALP} & \quad \rightarrow \\
p-nitrophenol & \quad + HPO_3^- + H^+
\end{align*} \]  \hspace{1cm} (5.1) \hspace{1cm} 100
Chapter One

1. Introduction

Highly porous scaffolds with 3-dimensional (3D) open pore structure are required for most bone tissue engineering applications. Current synthetic scaffolds are usually made from bioceramics or polymers, but a better combination of mechanical and biological properties may be achieved with a composite or hybrid material. Bioceramic-polymer composites will mimic better the extracellular matrix (ECM) of the host bone tissue, considering the complex composite structure of bone. Likewise, for osteochondral tissue engineering, one should consider the need of simultaneous regeneration of both cartilage and subchondral bone when designing novel osteochondral constructs. In this case, again composite materials may represent a better alternative to monolithic ceramics or single phase polymers. In the present research project, based on previous results obtained by Chen and Boccaccini [1], the addition of a well-known biodegradable polymer, i.e. poly(DL-lactide) (PDLLA), to a 3D 45S5 Bioglass® based scaffold has been investigated. The scaffolds were fabricated by the foam replica technique [1] and polymer coating and infiltration were obtained by a simple immersion procedure developed in this study. The characterisation of scaffolds includes microstructural analysis, bioactivity in simulated body fluid (SBF) and mechanical property determination. The use of poly(3-hydroxybutyrate) (P(3HB)) as a highly biocompatible, natural biodegradable polymer in combination with 3D 45S5 Bioglass® based scaffolds is also of great interest to fabricate composite bioactive scaffolds which were also developed in this project. A different type of natural biodegradable polymer, in this case, poly(3-hydroxyoctanoate) (P(3HO)), an elastomeric polymer, has also been investigated. P(3HB) and P(3HO) biodegradable polymers were synthesised in collaboration with researchers at University of Westminster (UK). Scaffolds coated and infiltrated with PDLLA, P(3HB) and P(3HO) were compared, in terms of mechanical properties, degradation behaviour and bioactivity. The composite scaffolds produced serve as robust 3D systems to study relevant properties, such as the mechanical properties (compressive strength and work of fracture) and bioactivity, as well as to establish the mechanism of reinforcement imparted by the polymer by characterisation of the
fracture behaviour of the polymer/Bioglass® interface. The development of an optimised PDLLA nanofiber coating on the 45S5 Bioglass®-based scaffolds, utilising electrospinning, was also investigated. In addition, the development of a new bilayered construct based on PDLLA fibrous layer, obtained by electrospinning, on the surface of a PDLLA coated Bioglass® scaffolds was investigated. This novel construct represents a suitable matrix for osteochondral tissue engineering applications, and a preliminary cell culture study utilising chondrocyte cells (ADTC5) was carried out.

This thesis is organised as follows. Chapter 2 provides a comprehensive literature review, aiming at covering the state-of the art of the research areas of relevance for this project in order to rationalise the materials, techniques and strategies adopted in the project. Chapter 3 presents the aims and objectives of the project and the experimental strategies followed to realise these aims and objectives. Chapter 4 describes the experimental procedure and analytical techniques used, while Chapter 5 focuses on the fabrication and characterisation of the PDLLA, P(3HB) and P(3HO) coated 3D 45S5 Bioglass®-based scaffolds, describing the results obtained which are comprehensively discussed. Chapter 6 focuses on the development of PDLLA nano fibre coatings on 45S5 Bioglass® sintered pellets and 45S5 Bioglass® 3D scaffolds, based on electrospinning technique. In Chapter 6 the complete characterisation of the layered scaffolds is presented including a preliminary cell culture study on the novel constructs. Chapter 7 concludes the thesis summarising the results and drawing conclusions as well as presenting suggestions for further work.
Chapter Two

2. Literature Review

2.1 Tissue engineering: definition and principles

Tissue engineering (TE), a scientific field which started in 1987 in USA [2], has made important contributions to the progress of medical research. In this innovative biomedical approach, the fundamental principle that the body is able to heal itself is conveniently applied [3]. The organs of the body are known to have an ability to repair or recover when they are diseased or injured. Regenerative medicine is a more recent term that came into use during the 1990s, and to some it is synonymous with stem cell technology or cell therapy [4]. Thus, these two areas; tissue engineering and regenerative medicine, are very much complementary and many researchers use the two terms interchangeably.

Fundamentally, biological tissues consist of the cells, the extracellular matrix and the signalling systems. A common tissue engineering strategy involves the use of scaffolds, made from biomaterials, which serve primarily to support and delivery of cells. Scaffolds can be enriched with signalling molecules which can be bound to them or infused into them [5]. Then the designed scaffold, which is cultured with sufficient cells in vitro, is implanted and surgeons manipulate the local environment in order for the scaffold to integrate in the host body. Under these ideal conditions, the body is capable of healing itself.

In summary, tissue engineering requires a designed, engineered scaffold that is populated with cells and signalling molecules to induce the regeneration ability of the host body, aiming at regenerating functional tissues. Tissue engineering is thus an alternative to conventional organ transplantation and tissue reconstruction.

Tissue engineering has been also defined as the application of principles and methods of engineering and life sciences to obtain a fundamental understanding of the structure-function relationship in normal and pathological mammalian tissue and the
development of biological substitutes to restore, maintain or improve tissue function [6]. Similarly, Galletti, Hellman and Nerem [7] in 1995 defined TE as the basic science and development of biological substitutes for implantation into the body or the fostering of tissue remodelling for the purpose of replacing, repeating, regenerating, reconstructing, or enhancing biological function.

Tissue engineering principles have been applied to a large number of tissues, including bone, cartilage, skin, liver, etc., and the field continues to grow with the contribution of cellular biology, materials science and medicine, as core disciplines.

### 2.2 Rational for bone tissue engineering

Bone tissue engineering is being developed as an alternative therapeutic treatment to tackle the shortcomings of conventional clinical treatment of bone fracture and disease, i.e. transplantation and implantation [8]. For example, one of the shortcomings in the application of bone graft is the size of the defect and the viability of the host body. In an autograft procedure, there could be significant donor site morbidity and therefore the need for multiple surgeries. In allografting, however, there are problems with donor tissue scarcity and the risk of tissue rejection due to diseased or infectious donor tissue. In addition, the survivability of an orthopaedic prostheses for a reasonable period of 15 years is 75% to 85% and requirements for longer than 30 years durable prostheses is increasing due to the enlargement of the aging population [9;10].

Therefore, tissue engineering is expected to have a significant impact in the improvement of human health and the life of patients in the future. It can be anticipated that the tissue engineering approach has the potential to solve the transplantation crisis caused by donor shortage, immune rejection, pathogen transfer and multiple surgeries [11].
2.3 The bone tissue engineering approach

Tissue engineering of hard tissue has the purpose of filling of a wound site where bone has been lost through trauma or disease with a scaffold, as mentioned above, to regenerate bone tissue and to restore the mechanical function of bone. Biological substitutes such as cells and biomolecules are used, in addition to the biomaterials scaffolds, to maximise the regeneration capacity and to allow for greater success in developing therapeutic strategies for replacement, repair, maintenance and enhancement of bone tissue function [2].

As indicated above, natural tissues consist of three components: cells, extracellular matrix (ECM), and signalling systems (e.g. growth factors) [12]. Thus, the bone tissue engineering approach is concerned with the design of suitable 3D scaffolds made from engineering materials which will act as an extracellular matrix (ECM) and will be the substrate for bone cells to attach to and proliferate in vitro to facilitate tissue regeneration in vivo. The use of signalling molecules has a potential to markedly increase scaffold effectiveness [5]. Fig. 2.1 illustrates the tissue engineering triad.

Living cells can either migrate into the scaffolds after implantation (acellular approach) or can be introduced into the scaffolds in cell culture before implantation (cellular approach). In the acellular approach, guided regeneration of tissues can be achieved by using scaffolds as templates for ingrowth of host cells and bone tissue in vivo. In the cellular approach, however, cells are cultured within a scaffold in vitro before being implanted as part of an engineered device. The success of the cellular approach for tissue engineering of bone is critically dependent on the development of the scaffold which must resemble the ECM of the host tissue.

Cells can be isolated as fully differentiated cells of the tissue intended to be engineered, or they can be manipulated to produce the desired function when isolated from other tissues or a stem cell source. In both approaches, the engineered scaffold should induce the specific cell type attachment and proliferation either in vitro or in vivo being thus one of the most important components in the regeneration of new functional bone tissue.
2.4 Challenges in tissue engineering and ideal scaffolds for bone tissue engineering

As mentioned above, tissue engineering requires a multi disciplinary approach, including cell and molecular biology, biochemistry, materials science and engineering. Materials scientists and engineers encounter a variety of challenges when designing an ideal scaffold. Tissue engineering advancement depends on the progress of the science and technology in these areas. The main challenges can be grouped into three categories, namely understanding cells and their mass transfer requirements, the fabrication of materials to provide scaffolds and templates and the interaction between materials and cells.

One major requirement for successful tissue regeneration is acquiring a sufficient amount of relevant cells. Stem cell biology involving embryonic stem cells shows promise for tissue engineering [13]. As first step, tissue is harvested as allograph, autograph or xenograph to yield the required cells. The cells are then transferred to the scaffolds where further remodelling can occur. Large masses of cells for tissue engineering need to be kept alive, not only in vitro but also in vivo. To achieve this, systems including in vitro flow bioreactors and in vivo strategies to maintain healthy, functional cell populations are being considered [14].
The challenges for material scientists are linked to developing optimal chemical and physical configuration of new biomaterials for scaffolds and to understand the interaction of scaffolds with cells to produce engineered organs. Scaffolds are usually made of biodegradable materials. They can be naturally occurring materials, synthetic materials and hybrid materials, which need to be compatible with living systems and with cells in vitro and in vivo. The interface between cells and scaffolds must be clearly understood so that biomaterial surface chemistry and topography can be optimised. Design of scaffold/cell interfaces is one of the major challenges in the field, and should be considered at molecular, nanostructural and microstructural level.

The design of biomaterials for tissue engineering scaffolds can also incorporate biological signalling to induce tissue growth and regeneration, for example the release of growth and differentiation factors, design of specific receptors and anchorage sites as well as 3D site specificity using computer assisted design and manufacturing techniques are being considered.

As mentioned above, the scaffold should ideally mimic the ECM of the tissue that needs to be regenerated. The criteria for an ideal scaffold for bone TE are summarised in Table 2.1.

**Table 2.1 Criteria for an ideal scaffold for bone tissue engineering [15-17].**

1. The scaffold materials should be biocompatible and non toxic to the body.
2. The scaffolds should act as a 3D template for the in-vivo and in-vitro bone growth, i.e. the structure of the scaffolds should be similar to the structure of trabecular bone.
3. Interconnected pores of porosity > 90% with macropores of size between 300 and 500 microns are required for cell penetration, tissue ingrowth, vascularisation and nutrient delivery.
4. The scaffolds material should promote osteoconduction and osteoinduction with the host bone.
5. Scaffold surfaces should exhibit texture and topography that can promote cell adhesion and adsorption of biological metabolites.
6. The scaffolds should influence the genes in bone generating cells to enable efficient cell differentiation and proliferation.
7. The mechanical properties of the scaffolds should match those of the host tissue and scaffolds should bond to host tissue (bioactivity).
8. Ideally, the scaffold degradation kinetics should be matched to the rate of new tissue regeneration.
9. The manufacturing technique should enable the production of scaffolds of irregular or complex shapes depending on the site of application.

2.5 Osteochondral tissue engineering

Articular cartilage injuries occur frequently as a result of trauma, tumour or osteoarthritis [18]. Articular cartilage is normally made of hyaline cartilage which is composed of a complex organization of type II collagen and other minor collagens in combination with hyaluronic acid and cartilage-specific proteoglycan termed aggrecan. Osteochondral defect is a term used to indicate joint damage of the articular cartilage and the underlying bone (subcondral bone) which requires a unique repair response to that of chondral defects [19].

However, the repair response typically leads to formation of fibrocartilage in the defect void [20] which will often be observed after long-term follow-up using conventional surgical procedures such as abrasion arthroplasty, microfracture and subcondral bone drilling [21-23]. Several studies have shown that tissue engineering strategies have potential for regeneration of cartilage [24-27].

For osteochondral tissue engineering combinations of both bone and cartilage tissue engineering principles can potentially be met by using engineered osteochondral (bone-cartilage) composite scaffolds of predefined size and shape generated in vitro using autologous cells. The bone region of the engineered osteochondral composite may further help anchor the graft within the defects, since a bone-to-bone interface integrates better and faster than cartilage-to-cartilage interfaces [28].

Many strategies dealing with tissue engineering scaffolds for osteochondral repair employ the design of bilayered scaffolds that could regenerate both cartilage and
subchondral bone involving different combinations of materials, morphologies and properties in both parts of the scaffolds. Common approaches involve: 1) seeding autologous chondrocytes at the top of the 3D scaffolds to create a cell-scaffold construct for in vivo implantation [29-32], 2) two different cartilage and bone scaffolds that have been joined (or assembled) together either before or during surgical implantation [33;34] and 3) an integrated bilayered structure that allows for a complete transition between the bone and the cartilage layers without requiring a joining mechanism [35-37]. For these strategies, also several bioreactors have been described, as reviewed in refs [38;39] and proposed by several authors [40]. Moreover, several strategies with single-layer materials can be followed, as recently reviewed by Mano and Reis [18]. Nevertheless, it is widely accepted that a bilayered structure would be more challenging to produce but more suitable for regenerating an osteochondral defect. Such bilayered scaffold should be able to incorporate/induce different types of cells in a favourable environment requiring different chemical surroundings and mechanical requirements, leading to the growth of the two different tissues with different biological requirements. Essentially, this means that the bilayered scaffolds can be designed to better mimic the native ECM for each tissue type independently, rather than trying to fabricate a construct that attempts to compensate for the functional requirements of both cartilage and bone in a single structure.

A characteristic interfacial region present in bilayered scaffolds would provide segregation of the consequential up growth of osseous tissues into the cartilage region as well as allowing for a straightforward cell seeding process for in vitro cell culture. Unlike traditional scaffolds, these bilayered structures incorporate various inclusions and coatings to form unique composite layered morphologies.

2.6 Materials for bone tissue engineering scaffolds

In developing successful scaffolds for bone tissue engineering, the goal is to produce a bone-matrix-like material. Natural bone matrix is a composite composed of biological ceramic (a natural apatite) and biological polymer. The inorganic part of the bone; Ca_{10}(PO_4)(OH)_2 (carbonated hydroxyapatite), provides the relatively high
compressive strength of bone. The biological polymer; collagen fibres, are tough and flexible, and thus tolerate stretching, twisting, and bending. It is hence not surprising that ceramics, polymers and their composites have been chosen for developing scaffolds for bone tissue engineering applications which are reviewed in this section.

2.6.1 Bioactive glasses

As early as in 1969, Hench and colleagues discovered that certain silicate glass compositions had excellent biocompatibility as well as the ability of bone bonding [41]. A common characteristic of bioactive glasses and glass-ceramics is the formation of a biological hydroxyl carbonate apatite (HCA) layer, in relevant biological conditions, that bonds to bone [41].

Rapid formation of a HCA layer on the surface of bioactive glasses occurs in five stages. A high level of bioactivity means that these stages occur fast, e.g. in a matter of hours. The surface reactions (stages 1-5) on a bioactive glass in a relevant aqueous solution, e.g. simulated body fluid, are summarized below [41]:

Stage (1). Rapid exchange of Na\(^+\) or K\(^+\) with H\(^+\) or H\(_3\)O\(^+\) from solution:
\[
\text{Si-O-Na}^+ + \text{H}^+ + \text{OH}^- \rightarrow \text{Si-OH} + \text{Na}^+_{(solution)} + \text{OH}^- 
\]

Stage (2). Loss of soluble silica in the form of Si(OH)\(_4\) to the solution resulting from breakage of Si-O-Si bonds and formation of Si-OH (Silanols) at the glass solution interface:
\[
\text{Si-O-Si} + \text{H}_2\text{O} \rightarrow \text{Si-OH} + \text{OH-Si}. 
\]

Stage (3). Condensation and repolymerisation of a SiO\(_2\) rich layer on the surface that is depleted in alkalis and alkaline earth cations:
\[
2(\text{Si-OH}) + 2(\text{OH-Si}) \rightarrow -\text{Si-O-Si-O-Si-O-} 
\]

Stage (4). Migration of Ca\(^{2+}\) and PO\(_4\)^{3-} groups to the surface through the SiO\(_2\) rich layer forming a CaO-P\(_2\)O\(_5\)-rich film on top of the SiO\(_2\)-rich layer, followed by growth of an amorphous CaO-P\(_2\)O\(_5\)-rich film by incorporation of soluble calcium and phosphate from the solution.
Stage (5). Crystallisation of the amorphous CaO-P\(_2\)O\(_5\) film by incorporation of OH, CO\(_3\)^{2-}, (or F anions) from the solution to form a mixed HCA layer (or hydroxyl fluoroapatite, HCFA layer).

Figure 2.2 shows schematically that calcium ions dissolved from bioactive glasses and glass-ceramics increase the ion activity in the surrounding body fluid, and the hydrated silica on the surface of glasses and glass-ceramics provides favourable sites for apatite growth.

According to Hench [9], bioactive materials are classified into two classes: Class A and Class B materials. Class A bioactive materials are both osteoconductive and osteoproducive [9], as consequence of rapid reactions involving critical concentration of soluble Si, Ca, P and Na ions that give rise to both an intracellular and an extracellular response on the material surfaces and the physiological environment. Class B materials are osteoconductive, i.e. they induce bone migration along an interface, due to slower surface reactions, minimal ionic release and only extracellular responses occur at the interface [9]. Class A bioactive materials elicit bone and soft tissue bonding while class B bioactive materials only show bone bonding. Silicate bioactive glasses, the materials of interest in this investigation, are Class A bioactive materials, while hydroxyapatite is a Class B bioactive material [9].
As mentioned above, bioactive glass was discovered more than 30 years ago [43] and provided for the first time an alternative material showing strong interfacial bonding of an implant with host bone tissue. A typical melt-derived bioactive glass composition known as 45S5 Bioglass® contains SiO₂(45wt%), Na₂O(24.5wt%), CaO(24.4wt%) and P₂O₅(6wt%). The composition was selected to provide a large amount of CaO with P₂O₅ in a Na₂O-SiO₂ matrix. The composition is very close to a ternary eutectic, and it is easy to melt. Commercial Bioglass® based products are being marketed under the names Perioglass® and Novabone®. Based on the success of Bioglass®, “third-generation” bioactive materials, composites, hybrid materials and macroporous foams are being designed for bone tissue engineering applications. It has been shown that bioactive glass activates genes that stimulate regeneration of bone by the direct affect of ion dissolution products of bioactive glasses. [44]

The 45S5 Bioglass® composition is able to develop a hydroxyapatite (HA) surface layer in contact with simulated body fluid in vitro. These HA crystals can bond to layers of collagen fibrils produced at the bone/Bioglass® interface by osteoblast cells in vivo. The chemical bonding of the HA layers to collagen creates strongly bonded interfaces [45]. A quantitative evaluation of the interfacial shear strength in rat and monkey models has shown that the strength of the interfacial bond between Bioglass® and cortical bone was equal to or greater than the strength of the host bone [46].

Bioactive glass bonding to tissues takes place by a series of ion-exchange and film-forming reactions as described in Figure 2.2. The bioactive glass bonding to soft tissue is composition dependent and only glass compositions with rapid reaction rates form a soft tissue bond [47]. Confirmation of Bioglass® bone bonding was demonstrated in 1976 when Bioglass® coated alumina implants were tested as load bearing prostheses in sheep [48]. The results showed bone bonding [49]. Small additions of K₂O and MgO to the basic Bioglass® composition has led to the bioactive glass called “Ceravital®”. This glass was implanted in animal models by Gross et al. [50] who found that the material bonded to bone with a mechanically strong interface.

The first five reaction stages that occur on bioactive glass surfaces in contact with physiological fluid lead to a rapid release of soluble ionic species and formation of a high surface area hydrated silica and a polycrystalline hydroxyl carbonate apatite
(HCA) bilayer on the glass surface (as discussed above). The reaction layers can enhance adsorption and desorption of growth factors and will influence the length of time required for macrophages to prepare the implant site for tissue repair and attachment, synchronised proliferation and differentiation of osteoblasts. Mineralization of the matrix follows soon thereafter and mature osteocytes, encased in a collagen-HCA matrix, are the final product after 12-16 days in vitro and in vivo.

Thus, the primary advantage that makes bioactive glasses promising scaffold materials is their rapid rate of surface reactions which lead to fast tissue bonding. However, it has been reported that crystallisation of bioactive glasses decreases the level of bioactivity [51] and it can even turn a bioactive glass into an inert material [52]. Recently results obtained at Imperial College London (UK) demonstrated that if sintering conditions during scaffold fabrication are well controlled, bioactive glass-ceramic scaffolds still show high degree of bioactivity [53]. These aspects of the development of partially crystallised Bioglass® based scaffolds will be investigated in detail in the present project.

The primary disadvantage of bioactive glass is its mechanical weakness and low fracture toughness. Its low tensile strength (in the range of 40-60MPa for dense components) makes it unsuitable for load-bearing applications. Thus, combining the mechanical properties of polymers with bioactive glass to produce optimised bioactive composites for bone repair is being highly investigated [54] and this topic will constitute a significant part of the present investigation.

### 2.6.2 Calcium phosphate ceramics (CPC)

Jarcho et al. in the USA [55], DeGroot et al. [56], and Denissen [57] in Europe and Akao et al. in Japan [58] have shown that ceramics made of calcium phosphate can be successfully used for replacing and augmenting bone tissue. The most widely used calcium phosphate based bioceramics are hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) [46]. Hydroxyapatite has the chemical formula Ca₁₀(PO₄)₆(OH)₂, a Ca/P ratio of 1.67 and it possesses a hexagonal structure. It is the most stable phase of various calcium phosphates. It is stable in body fluids and in dry or moist air up to 1200°C and does not decompose. HA has been shown to be bioactive [55].
The two main ways to prepare HA are wet chemical methods and solid-state reactions. Wet chemical methods involve the acid-base titration or co-precipitation from aqueous solutions that contain calcium nitrate and di-ammonium hydrogen phosphate; direct precipitation reaction between orthophosphoric acid solution and calcium hydroxide dispersed in water. In solid-state reactions, the mixed calcium compound is compressed and sintered above 950°C. There are also other options for preparation of HA powders such as sol-gel based methods and electro-crystallisation, spray pyrolysis, freeze-drying, microwave irradiation, mechanochemical methods as well as emulsion processing [59].

The rate of addition of reactants, pH and sintering temperature influence the stoichiometry (Ca/P ratio) and thus the nature of HA. Dried HA powder is amorphous while sintering at 900°C produces crystalline HA. Both Ca²⁺ and PO₄³⁻ ions, as well as the OH⁻ group in HA, can be replaced by other ions, several of them present in physiological surroundings. For biomedical purposes, carbonated apatite and fluorapatite are the materials of interest because of their similarity with bony apatite and decreased solubility in aqueous solution, respectively [60].

β-tricalcium phosphate (β-TCP) is represented by the chemical formula Ca₃(PO₄)₂, the Ca/P ratio being 1.5. β-TCP shows an X-ray diffraction (XRD) pattern of pure hexagonal crystal structure, and it is highly soluble in body fluid. Many studies indicate that the dissolution of HA in the human body after implantation is too low to achieve the optimal results in tissue engineering applications. On the other hand, the dissolution rate of β-TCP is too fast for bone bonding. Thus studies have focused on development of biodegradable scaffolds based on biphasic calcium phosphate composed of HA and β-TCP [61].

2.6.3 Apatite-wollastonite (A/W) glass-ceramics

In A/W glass-ceramics, the parent glass is the pseudo ternary system 3CaO.P₂O₅-CaO.SiO₂-MgO.CaO.2SiO₂. When the glass in bulk form is heated up to 1050°C at a rate of 5°C/min, fine grains of hydroxyapatite and fibrous β-wollastonite precipitate before complete densification and microcracking occur. β-wollastonite (CaO.SiO₂)
consisting of a silicate chain structure acts as the reinforcing phase [62]. To avoid crack formation, a small amount of CaF$_2$ is added to the parent glass, forming the composition MgO(4.6wt%), CaO(44.7wt%), SiO$_2$(34.0wt%), P$_2$O$_5$(6.2wt%) and CaF$_2$(0.5wt%) which is subjected to the same heat treatment. As a result, the glass powder can be fully densified at about 830°C, and oxyfluoroapatite (Ca$_{10}$(PO$_4$)$_6$(F$_2$O)) and wollastonite precipitate successively at 870°C and 900°C, respectively, to give a crack- and pore-free, dense and homogenous glass-ceramic [63].

A/W glass-ceramics can be easily machined into various shapes such as artificial vertebrae, intervertebral spacers, spinous process spacers and iliac spacers. The bending strength of A/W glass ceramics (215MPa) is twice that of dense sintered HA (112 MPa), which is due to the reinforcement effect of β-wollastonite as well as apatite. It has a relatively high fracture toughness of 2MPa.m$^{1/2}$ and high fracture surface energy of 15.9 Jm$^{-2}$.

The A/W glass-ceramic is capable of binding to living bone within a few weeks after implantation, and the implant does not deteriorate in vivo [64]. However, and surprisingly, there are no studies reported in the literature on the development of highly porous scaffolds made of A/W glass-ceramics for application in bone tissue engineering.

### 2.6.4 Ceravital® bioactive glass-ceramic

Ceravital®, is the trade name describing a number of different compositions of glasses and glass-ceramics. Their basic network components include SiO$_2$, Ca(PO$_2$)$_2$, CaO, Na$_2$O, MgO and K$_2$O with ceramic additions being Al$_2$O$_3$, Ta$_2$O$_5$, TiO$_2$, B$_2$O$_3$, Al(PO$_3$)$_3$, SrO, LA$_2$O$_3$, or Gd$_2$O$_3$. The only field in which glass-ceramic “Ceravital®” implants are clinically applied is in the replacement of the ossicular chain in the middle ear where the loads are minimal and the mechanical properties of the materials are sufficient [65].

However, in-vitro experiments showed that the solubility condition of these materials could be adjusted from high to low solubility, by addition of metal oxides to the melt.
This degradability property is attractive for tissue engineering, however no studies about the fabrication of highly porous scaffolds based on Ceravital® have been reported in the open literature.

2.6.5 Bioverit® bioactive glass-ceramic

Bioverit® is a mica-apatite glass-ceramic with chemical composition in the SiO$_2$-Al$_2$O$_3$-MgO-Na$_2$O-K$_2$O-F-CaO-P$_2$O$_5$ base glass system with special fluorophlogopite mica crystal (Na/KMg$_3$(AlSi$_3$O$_{10}$)F$_2$). The formation of these crystals is possible by heating the base glass composition at temperatures between 610°C and 1050°C. Mica crystals give the materials good machinability, and apatite crystals ensure the bioactivity of the implants.

Bioverit® has been used as implants especially as spacers in orthopaedic surgery, middle ear implants and dental tooth fillers. The in vitro reaction of the Bioverit® surface in Ringer’s solution and tris-buffer-solution indicates ion-exchange between the glass-ceramic and simulated body fluid. In vivo experiments with Bioverit® have shown good bioactive behaviour of the implants. The proven performance of the material in vivo is an attractive attribute for bone tissue engineering, however the author is not aware of any research work on the production of scaffolds from this material.

2.6.6 Natural biopolymers

Special interest has been paid to naturally occurring polymer materials for tissue engineering scaffolds due to their high biocompatibility and potential similarity to extracellular matrix (ECM) components. Among them collagen [66] and chitosan [67] are mostly investigated for bone tissue engineering. These polymers should not cause any inflammatory response when in contact with human tissue.

Other biopolymers of high biocompatibility are those developed from microbial sources. For example, polyhydroxyalkanoates (PHA) are a group of biodegradable and biocompatible polyesters produced by micro-organisms under unbalanced growth.
So far, only few PHA, including poly 3-hydroxybutyrate P(3HB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate, P(HBV), poly 4-hydroxybutyrate, P(4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate, P(HBHHX), and poly 3-hydroxyoctanoate P(HO) are available in sufficient quantity for applications in laboratory research [69]. Figure 2.3 shows the general chemical structure of PHAs. Poly 3-hydroxybutyrate, P(3HB), and poly 3-hydroxyhexanoate, P(HHX), structures can be obtained by replacing the R group with methyl or propyl group, respectively.

\[
\begin{array}{c}
\text{H} \\
\text{C} \\
\text{OR} \\
\end{array}
\begin{array}{c}
\text{C} \\
\text{O} \\
\end{array}
\begin{array}{c}
\underbrace{\text{CH}_{2m}} \\
\text{O} \\
\end{array}
\begin{array}{c}
\underbrace{\text{C}_{n}} \\
\end{array}
\]

Figure 2.3 General molecular structure of polyhydroxyalkanoates. m=1,2,3, yet m=1 is most common, n can range from 100 to several thousands. R is variable. When m=1, R=CH\(_3\), the monomer structure is 3-hydroxybutyrate, while m=1 and R=C\(_3\)H\(_7\), it is a 3-hydroxyhexanoate monomer.

P(3HB) is the most common member of the PHA family. It was discovered by Lemoige in 1926 [70]. The P(3HB) crystal is a relatively stiff, rigid material and has tensile strengths comparable to polypropylene. Doyle et al. [71] demonstrated that materials based on P(3HB) produce a consistent favourable bone tissue adaptation response with no evidence of an undesirable chronic inflammatory response after implantation periods of up to 12 months. Bone is rapidly formed close to the materials and 80% of the implant surface was seen to be in direct apposition to new bone. When porous composites of P(HBV) and sol-gel-bioactive glass (SGBG) are exposed to SBF for 12 hours (for bone tissue engineering scaffolds), results indicated that hydroxyl carbonate apatite (HCA) had formed on the surface of P(HVB)/SGBG scaffolds [72]. PHA have also been combined with HA [73] and bioactive glasses [74] in novel composites for tissue engineering scaffolds.

### 2.6.7 Synthetic polymers

The number of synthetic polymers that hold promise for bone tissue engineering is limited. Polymers, in particular the biodegradable ones, have a Young’s modulus much lower than bone and cannot be used directly in load bearing applications. Table 2.2 shows a summary of common synthetic polymers used in tissue engineering and
their relevant properties. There is also concern over the acidic degradation products of some polymers where hydrolysis occurs at the ester group giving an acidic by-product (e.g. PLA degrading to lactic acid). The synthetic polymers that are widely used for tissue engineering scaffolds include polyglycolic acid (PGA) and poly-(L)-lactic acid (PLLA), or their copolymer (PLGA) [75]. Poly(D,L)-Lactic acid (PDLLA) has been extensively investigated as well, for example as a biomedical coating of orthopaedic materials because of its excellent features with respect to implant performance [76]. PDLLA also shows excellent biocompatibility in vivo and a good osteoconductive potential [77]. In cartilage tissue engineering, PGA [78], PDLLA [79], and PGA-PLLA copolymers [80] have been studied for their efficacy as chondrocyte-delivering scaffolds in vitro and in vivo. Several investigators have also found that some non-biodegradable polymer substances, such as polytetrafluroethylene [81], polymethylmethacrylates [77], and composites of hydroxyapatite-Dacron® also facilitate restoration of articular surface.

Table 2.2 Physical properties of synthetic, biocompatible and biodegradable polymers investigated as scaffold materials [82].

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Melting Point, Tm (^\circ\text{C})</th>
<th>Glass Transition Tg,(^\circ\text{C})</th>
<th>Degradation time Month</th>
<th>Modulus ((\text{GPa}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDLLA</td>
<td>amorphous</td>
<td>55-60</td>
<td>12-16</td>
<td>1.9-2.4 (film)</td>
</tr>
<tr>
<td>PLLA</td>
<td>173-178</td>
<td>60-65</td>
<td>&gt; 24</td>
<td>1.2-3.0 (film)</td>
</tr>
<tr>
<td>PGA</td>
<td>225-230</td>
<td>35-40</td>
<td>6-12</td>
<td>7-14 (fibre)</td>
</tr>
<tr>
<td>PLGA</td>
<td>amorphous</td>
<td>45-55</td>
<td>adjustable</td>
<td>1.4-2.8</td>
</tr>
</tbody>
</table>

2.6.8 Synthetic composite materials

The development of composite materials for tissue engineering combining biopolymers and bioactive ceramic phases serves the purpose of enhancing the mechanical behaviour of the polymer, while maintaining its excellent biocompatibility and imparting bioactivity. Thus a better combination of mechanical and biological properties is usually achieved with a composite or hybrid structure.
Calcium phosphate ceramics (i.e. hydroxyapatite and tricalcium phosphate) and bioactive glasses (silica glasses containing calcium and phosphorus), are the most popular bioactive materials. They have demonstrated good biological properties and clinical successes. However, calcium phosphates and bioactive glasses are brittle; hence they cannot be used in load-bearing applications. In addition, their handling by the surgeon is difficult.

As mentioned above, many polymers have been proposed for applications in tissue engineering, either natural polymers (e.g. collagen, alginate, glycosaminoglycan, starch, chitin, poly(hydroxybutyrate) and chitosan) or synthetic (e.g. poly(lactic acid), poly(glycolic acid) and co-polymers of poly(lactic acid) and poly(glycolic acid). However, polymers usually exhibit low modulus and creep resistance compared to bone (whose Young’s modulus ranges between 0.5 and 20 GPa depending on bone type) [83]. This is a major drawback of biopolymers which limits their clinical applications as tissue engineering scaffolds, particularly in load bearing applications.

Using composites comprising biopolymers and an inorganic phase takes advantage of both polymer and ceramic properties, ideally to achieve materials with the required stiffness (close to the stiffness of bone), fracture strength and high toughness. Such composites can be based either on a polymer or a ceramic matrix and should be highly porous to enable cell penetration, tissue ingrowth, vascularisation and nutrient delivery, as outlined in Table 2.1 [1].

### 2.7 Technologies for bioactive glass processing

The most widely used methods for the fabrication of dense bioactive glasses are: 1) melt casting and 2) firing of compacted powder. The latter route involves the production of the desired body from a powder compact by viscous flow sintering.
2.7.1 Melt casting

The simplest form of melt casting involves melting a batch of powdered raw materials, followed by cooling and forming to produce a solid finished body (Fig. 2.4). For glasses that crystallise very easily, the solidification of the melt is accompanied by rapid nucleation and growth of crystals (i.e. grains). Uncontrolled grain growth generally leads to critical problems due to uncontrolled microstructure and low mechanical properties (e.g. low fracture strength). The melt casting method is limited to the fabrication of glasses and, by a controlled nucleation and crystal growth heat treatment, glass-ceramics.

One important variation of the melt casting method is the glass-ceramic process. This process involves heating a glass in controlled manner in a two-step process, firstly to nucleate and then to grow the crystals throughout the glass (Fig. 2.4). Glass-ceramics are by definition ≥ 50% crystalline by volume, and most are >90% crystalline. Since the process depends initially on the formation of a parent glass, it is limited to chemical compositions that can form glasses and in which crystallisation can be controlled by suitable heat treatments.
2.7.2 Firing of compacted powders (sintering)

In principle, the sintering of compacted powders can be used for the production of both glasses and polycrystalline ceramics. It is by far the most widely used method for the production of polycrystalline ceramics. The processing steps are shown in Fig. 2.5.

Figure 2.5 Basic flow chart for the production of polycrystalline ceramics by sintering of consolidated powders.

In its simplest form, this method involves the consolidation of a powder to form a porous, shaped powder compact (green body), which is then sintered at high temperature to produce a dense product.
2.7.3 Sol-Gel processing

Sol-gel processing is a chemically based method for producing ceramics, glasses, glass-ceramics and composites at much lower temperatures than the traditional processing methods. The process involves fine particles (1-100nm in diameter) being dispersed in either liquid or gases called colloids. When a colloid is sufficiently fluid and stable for a long period of time, the colloid is called a sol. The rigid solid formed by the evaporation of a solvent from a sol is called gel. By manipulating the sol-gel transformation behaviour, it is possible to form a variety of shapes quickly. The gel network can also be formed from hydrolysis and condensation of liquid metal-alkoxide precursors. An example of a metal-alkoxide precursor used to provide –Si-O-Si- network of bioactive gel-glasses is Si(OR)$_4$, where R is CH$_3$, C$_2$H$_5$, or C$_3$H$_7$. Other metal ions can also be used in addition to Si, such as, Ca, P, Ti, etc. Figure 2.6 shows the generic processing steps for sol-gel process to obtain ceramics and glasses.
A gel is dried when the physically adsorbed water is completely eliminated from the pores and this requires heating at controlled rate to temperatures of 120-180°C. Chemical stabilization of the dried gel is necessary to control the environmental stability of the material. Thermal treatment in the range of 500-900°C desorbs surface silanol (Si-OH) and eliminates a 3D network of silica rings from the gel. The surface chemistry is important to control the rate of HCA formation on the gel-glasses and their bioactivity. Stabilization also increases the density, strength and hardness of the gel and converts the network into a glass with network properties similar to melt-derived glasses. Densification of alkoxide-derived gel-glasses is completed in the range of 900-1150°C depending upon composition. Hydroxyls and adsorbed water must be removed from the gels prior to closure of pores or inhomogeneous microstructure will result. An important advantage of the sol-gel process is the ability to control the surface chemistry of the material by these thermal treatments.

2.8 Methods to produce macroporous ceramic scaffolds

An ideal scaffold is the one that mimics the extracellular matrix (ECM) of the tissue that is to be replaced so that it can act as a template in three dimensions onto which cells attach, multiply, migrate and function. In this section, several methods proposed in the literature for the fabrication of macroporous ceramic scaffolds are described.

2.8.1 Replica technique

![Figure 2.7 Schematic diagram of the replica technique for fabrication of porous ceramics [84].](image)

The earliest production of macroporous ceramics by the foam replica method dates back to the early 1960s, when Schwartzwalder and Somer [85] started using polymeric sponges as templates to prepare ceramic cellular structures of various pore
sizes, porosities and chemical compositions. In the polymer replica approach, synthetic e.g. polymer foams, typically polyurethane, (PU) [53] and natural (e.g. coral, wood [86]) templates of desired macrostructures can be used to fabricate macroporous ceramics. Figure 2.7 shows the schematic diagram of the replica technique for the fabrication of macroporous ceramics. The template is initially soaked into a ceramic suspension until the struts are homogeneously coated with the ceramic material. At this stage, the coatings should be viscous enough to avoid dripping by thixotropic effects. Thickening additives such as clays, colloidal silica, carboxymethyl cellulose and polyethylene oxide in combination with conventional dispersants can be used [86-89]. Moreover, binders and plasticizers are added to the initial suspension in order to prevent cracking of the struts during the subsequent heat-treatment process. The ceramic-coated polymeric template is subsequently dried and the polymer template is burnt out through careful heating between 300°C and 800°C and finally densified by sintering in an appropriate atmosphere at temperatures between 1,000 and 1,500°C, depending on the material.

Highly porous ceramics can be produced reaching open and interconnected porosity levels in the range 40%-95% with pores sizes between 200 micron and 3 mm. One possible disadvantage of the method is the tendency to produce a hole in the centre of each strut resulting from the removal of the polymer skeleton on heating. The presence of this hole can negatively affect the mechanical properties of the foams [53]. However, the approach of filling the hole with a polymer, as discussed below, leads to improved mechanical behaviour exploiting the interaction between the polymer and ceramic phases.

Alumina [90-92], titania [93], zirconia [94-96] and Bioglass® [53;97] foams are examples of scaffolds produced by the replica method using polymer sponges as the synthetic templates. A great variety of hydroxyapatite and calcium phosphate scaffolds have been also produced using both synthetic polymers templates as well as coral as natural templates [98-103]. The polymeric sponge method has also been proposed to manufacture macroporous calcium phosphate glass scaffolds of composition CaO-CaF2-P2O5-MgO-ZnO [104] and glass-reinforced HA foams [105]. Several approaches are being investigated to improve the mechanical properties of
foams produced by the replica techniques, which may lead to HA cellular structures of improved fracture strength [100].

2.8.2 Sacrificial template method

This method leads to porous materials having a negative replica of the original sacrificial template, as opposed to the (positive) replica obtained with the method described above. The technique involves the preparation of a biphasic composite comprising a homogenously dispersed sacrificial phase in a continuous matrix of ceramic or glass particles [106]. The sacrificial phase is then extracted from the partially consolidated matrix to generate pores within the microstructure. The removal of the sacrificial phase does not usually lead to flaws in the struts as is the case in positive replica methods. Therefore, the mechanical strength of the structures made by the sacrificial template method is usually higher than that of scaffolds fabricated by the replica method; however, porosity and pore interconnectivity are substantially lower [106;107]. Hydroxyapatite porous bodies produced from PMMA particles, PVB beads, wax and starch particles, as well as naphatane and sucrose [108-112] as sacrificial materials have been made by this method. Figure 2.8 shows the schematic diagram of the sacrificial template processes to produce porous ceramics.

Figure 2.8 Schematic diagram showing the sacrificial template method to produce porous ceramics. [84]
2.8.3 Direct foaming methods

![Figure 2.9](image)

Figure 2.9 Schematic diagram showing the direct foaming methods to produce a porous ceramic. [84]

In the direct foaming method, air is incorporated into a ceramic suspension which is then set in order to create a structure containing air bubbles [113;114]. In most cases, the consolidated foams are subsequently sintered at high temperatures to produce a high-strength porous ceramic. Stabilisation of air bubbles in the initial suspension is the most critical process. The stability of the air bubbles can be achieved by various surfactants and particle stabilisers. The foam structure prior to solidification is important because it influences the total porosity, pore size, wall thickness and microstructure of the final products. The porosity of foams produced from the direct foaming technique are hydroxyapatite and calcium phosphate scaffolds [115;116] obtained by gel-casting setting process as well as sol-gel derived bioactive glass scaffolds [113;117]. Figure 2.9 show the schematic process of direct forming methods to produce porous ceramics.

2.9 Bioceramic porous scaffolds coated with polymer layers

2.9.1 Calcium phosphate-based scaffolds

As mentioned above, calcium phosphates including HA, tricalcium phosphate (TCP) and calcium phosphate cements (CPC) play an important role in the development of scaffolds for bone tissue engineering. Miao et al. [118] have produced porous calcium phosphate ceramics with interconnected macroporous ( > 200µm) and microporosity (~ 5µm) as well as high porosities (~ 80%) by firing polyurethane (PU) foams coated with calcium phosphate cement at 1200°C. The open micropores of the struts were
infiltrated with poly(lactic-co-glycolic acid) (PLGA) to achieve an interpenetrating bioactive ceramic/biodegradable polymer composite structure. This work followed on from earlier study by the authors [119], where PLGA-coated porous CPC scaffolds were developed exhibiting compressive strength values of up to 4 MPa. In their most recent investigation Miao et al. [118] have also developed highly porous HA/TCP composite scaffolds (87% porosity) infiltrated with PLGA to form ceramic-polymer interpenetrating microstructures. In these composites the addition of PLGA led to a significant improvement on the compressive strength [120]. The mechanism based on crack bridging, previously investigated by Pezzotti et al. [121], was proposed to explain the strengthening and toughening achieved in the composites, evident by the presence of polymer ligaments that were stretched upon crack opening along the wake of the crack [120].

Li et al. [122] have produced macroporous HA ceramics with nanoporous struts. Subsequently, a commercially available biopolymer, Polyactive™, was incorporated into the struts by vacuum infiltration. As a result, the mechanical properties of the porous composites with interpenetrating organic/inorganic phases were found to improve significantly. Similar results were achieved in earlier investigations by Tencer et al. [123;124], who found that coating the internal surfaces of porous HA with biodegradable polymer (PDLLA) improved the compressive strength significantly but the coated material was shown to lack bioactivity.

Since bioactive silicate glasses exhibit higher bioactivity [125] or have faster rates of apatite formation compared to crystalline HA, bioactive glasses have been combined with HA scaffolds in bioactive composite coatings. Huang and Miao [126], for example, have used tetracalcium phosphate (Ca₄(PO₄)₂O; TTCP) and dicalcium phosphate anhydrous (CaHPO₄; DCPA) macroporous ceramics and PLGA/Bioglass® composites to coat HA scaffolds. The bioactive glass addition to the polymer coating increased the bioactivity of the scaffolds, as expected [125]. The replication technique was combined with H₂O₂ (hydrogen peroxide) pore forming method to produce the macroporous HA scaffolds which resulted in an increase in porosity but smaller pore sizes [126]. The HA scaffolds were first coated with 40wt% PLGA and further coated with bioactive glass/PLGA to increase the bioactivity as well as the compressive strength (5.8 MPa). Figure 2.10 A) shows a SEM micrograph of the microstructure of
Huang and Miao’s composites [126] showing that the PLGA phase fills the open micropores in the struts of the hydroxyapatite foam. It was observed that PLGA also filled the large defects (central hole) in the struts, as shown in Fig. 2.10 B).

Figure 2.10 SEM micrographs showing the microstructure of PLGA/HA composite scaffolds fabricated by Huang and Miao [126]: (A) PLGA phase (dark) filling open micropores in a HA strut (bright) and (B) PLGA phase filling the large defect in the centre of a strut.

Nakahira et al. [127] investigated hybrid hydroxyapatite/polymer composites by the infiltration of nylon into porous hydroxyapatite prepared from whisker-like powder at sintering temperatures between 800 and 1000°C. These HA/nylon composites exhibited a fracture toughness ($K_{IC}$) of 1.65MPam$^{1/2}$, and also showed good bioactivity according to results of SBF immersion tests.

A related study was published by Pezzotti et al. [128] who produced HA composites with relative porosity of 32% by cold-isostatic pressing followed by sintering. The HA structures containing percolated submicron porosity channels were infiltrated
with 6-nylon to produce composites with improved fracture properties. The results of this investigation also demonstrated the effect of different types of polymers with different mechanical properties on the overall fracture behaviour of the composites.

Miao et al. [129] have studied a composite consisting of three interpenetrating networks; tricalcium phosphate (TCP), HA and PLGA, which was produced in 3 steps. Firstly, the porous TCP network was produced by coating a PU foam with hydrolysable α-TCP slurry. Then, a HA network was derived from calcium phosphate cement (CPC) filled with the porous TCP network. Finally, the remaining open pore network in the HA/TCP composites was infiltrated with PLGA. These composites feature three phases with different degradation behaviour. It was postulated that bone would grow on the fastest degrading network (PLGA), while the remaining phases would remain intact thus maintaining their geometry and load bearing capability. The achieved compression strength of the PLGA coated material was remarkable at 30MPa; however the final porosity of the coated foams was not reported in the original study [129].

In other developments targeted to improve bone ingrowth and osseo integration, HA scaffolds have been coated with HA particles and polycaprolactone (PCL). The PCL matrix also acted as carrier for the antibiotic drug tetracycline hydrochloride which was entrapped within the coating scaffold layer [130;131]. HA scaffolds have also been coated with PLLA and compressive strength values of ~ 3MPa were achieved, however for a volume fraction of 70%, which is lower than the ideal value for bone tissue scaffolds [132]. With the PCL/HA composite coating, on the other hand, the mechanical properties such as compressive and elastic modulus were improved by several orders [130;131]. The release rate of the drug sustained for prolonged periods was found to be dependent on the degree of coating dissolution. In parallel study by the same group [133], HA porous scaffolds were coated with polymer (PCL)-HA hybrids for use as would healing and tissue regeneration substrates. The antibiotic vancomycin was incorporated in the PCL matrix at different concentrations and the drug release profile was determined. The encapsulated drug within the coated scaffolds was released in a highly sustained manner as compared to the rapid release of drugs directly adsorbed on the pure HA scaffolds [133]. These studies were the first to show the enhanced function of a scaffold achieved by applying a polymer.
coating. Not only are the mechanical properties improved but the scaffold also becomes a vehicle for targeted drug delivery. A complete review on the use of tissue scaffolds as drug delivery platforms has been published recently [134] showing that polymer coated calcium phosphate scaffolds are highly attractive for this application.

### 2.9.2 Bioactive glass and calcium silicate-based scaffolds

Chen et al. [1] have investigated the mechanical properties and bioactivity of Bioglass®-based scaffolds, before and after applying a PDLLA coating on the foam struts. They found that the bioactivity of scaffolds upon immersion in simulated body fluid (SBF) was maintained in the PDLLA-coated foams, while the transformation of the crystalline phase (Na$_2$Ca$_2$Si$_3$O$_9$) to amorphous calcium phosphate [53], which is a typical feature in Bioglass® derived glass-ceramic scaffolds, was delayed by the PDLLA coating. PDLLA was the first biodegradable polymer considered to coat Bioglass® scaffolds [1]; however, more recently, a new polymer based on polyhydroxyalkanoate has been investigated as an alternative coating material [135]. The polymer chosen, poly(3 hydroxybutyrate), P(3HB), is a natural thermoplastic polymer produced by many types of micro-organisms, which can be extracted as a stereoregular, optically active, isotactic polyester with purity and without any inclusion of catalyst residues [136]. In addition to its biocompatibility and biodegradability P(3HB) has been reported to have piezoelectric properties, which can stimulate bone growth and aid in healing of damaged or diseased tissue.

In the present project, also polymers of the PHA family, including P(3HB) and P(3HO) will be investigated, as discussed below. Bretcanu et al. [135] used for the first time bacteria-derived P(3HB) to infiltrate 45S5 Bioglass® scaffolds. These scaffolds are intended for the applications in cancellous bone substitution after trauma incidents. The pore morphology and macrostructure of the scaffolds before and after coating with P(3HB) as well as the coating homogeneity were investigated. It was found that polymer coating did not affect the interconnectivity of the pore structure; however, the coating was not fully homogenous as shown in Fig. 2.11. In terms of compressive strength of the coated and uncoated scaffolds, the polymer coated samples exhibited considerably increased compressive strength compared to uncoated
scaffolds. The formation of HA crystals on the surface of the scaffolds was investigated confirming the high bioactive character of the scaffolds. After two weeks of immersion in SBF a uniform layer of HA crystal was formed.

Figure 2.11 SEM image showing the microstructure of a Bioglass® scaffold coated by P(3HB) [135].

In separate developments, Wu et al. [137] have produced a highly porous interconnected (~ 99.9%) calcium silicate scaffold coated with PDLLA by the sponge replica technique. They reported compressive strength values of up to 1.45 MPa in air and 1.10MPa in PBS. The PDLLA modification was found to decrease the dissolution ratio of the calcium silicate scaffolds, while maintaining their apatite forming ability in SBF. In addition, the studies showed that PDLLA-modified scaffolds had a more uniform and continuous network of inner connectivity compared to non-modified scaffolds, in agreement with other investigations using Bioglass® [1] or HA [132], while also increasing the spreading and viability of human bone cells. The analysis of the literature on bioactive glass and glass-ceramic scaffolds has revealed the need for further optimisation work to improve the mechanical and biological performance of these novel scaffolds, this being one of the aspects to be covered in the present project.
2.9.3 Polymer-coated scaffolds based on alumina and titania

The concept of polymer coating and formation of interpenetrating polymer-ceramic microstructures has also been applied to scaffolds made from “bioinert” ceramics, such as alumina and titania. Peroglio et al. [83] have recently investigated alumina scaffolds coated with PCL. The coating was obtained by infiltrating the scaffolds with either a PCL solution or PCL nanodispersion. A typical fracture surface of a scaffold strut is shown in Fig. 2.12, which exhibits the presence of the polymer phase on the surface and penetrating into cracks in the alumina micro-structure. It was found that the elastic behaviour was primarily controlled by the ceramic scaffold, while the fracture energy mainly depends on the polymer phase. PCL additions of 10-20% to alumina scaffolds led to a 7- to 13-fold increase of the apparent fracture energy, in agreement with the results of Chen et al. for PDLLA-coated Bioglass® scaffolds [1]. The toughening mechanisms leading to this result was attributed to crack bridging by polymer fibrils [83]. The authors also showed that infiltrating PCL by nanodispersion did not result in a significant improvement of the mechanical behaviour of the scaffolds.

Figure 2.12 SEM image of the fracture surface of a PCL/alumina composite scaffold obtained by infiltrating alumina foams with a PCL solution [83]. The polymer phase is seen to coat the strut and to penetrate cracks in the alumina microstructure.

TiO$_2$ foam-like scaffolds with pore size ~ 300µm and >95% porosity were fabricated by the foam replication method by Novak et al. [138] in order to improve the structural integrity of the as-sintered foams, which exhibited low compression strength (< 0.045MPa), PDLLA or PDLLA/Bioglass® coatings were developed. The
PDLLA coating of a few microns in thickness was shown to improve the mechanical properties of the scaffolds: the compressive strength was increased by a factor of ~7 (0.3MPa). Moreover the composite coating containing Bioglass® particles was shown to impart the rutile TiO$_2$ scaffolds with the necessary bioactivity for the intended applications in bone tissue engineering. A dense hydroxyapatite layer was shown to form on the surface of the foams upon immersion in SBF for one week [138].

**2.10 Nanostructured 3D scaffolds with added functionality**

As mentioned above, scaffolds should act as an artificial extracellular matrix (ECM) and play a fundamental role in accommodating cell growth and proliferation. Since tissue-engineering scaffolds are intended as temporary artificial ECM to accommodate cells and to guide 3D tissue formation, materials that most closely resemble the critical features of the natural ECM are the most promising candidates. Structural protein fibres in a typical connective tissue, such as collagen and elastin fibres, have dimensions in the range from ten to several hundreds nanometers. Cells respond to the ECM through plasma membrane receptors that bond the matrix to the cell cytoskeleton. Because of a close connection between the cytoskeleton and the ECM through cell-surface receptors, cells sense and respond to the mechanical properties of their environment by converting mechanical signals into chemical signals. Therefore, the biophysical properties of the ECM influence various cells functions, including adhesion and migration. Structural fibrils and pores are often of a size compatible with cellular processes involved in migration, which may influence the mechanism by which cells migrate through the ECM [139]. As a result, cells can respond not only to micrometer-scale topography, but also to nanometre-scale topography [140].

Studies on 2D surfaces with various types of nanotopographies have been used to elucidate the mechanism by which cells respond to nano-scale features. Dalby *et al.* [141] investigated fibroblast responses to 2D nano-islands produced by polymer demixing. The results showed that the cells respond to the islands by broad gene up-regulation; with the topography affecting notably cell signalling, proliferation, cytoskeleton and production of ECM proteins. It has been suggested that topography alone can be used to elicit different responses from the same cell phenotype. With the
advance of nanotechnology, critical knowledge on the nano-scale organization of ECM components and how they interact with one another to organize a functional ECM is being generated. Atomic force microscopy (AFM) has become particularly important for the study of biological systems. Its major advantage is that it can produce high-resolution topographic images in aqueous and physiologically relevant conditions without the need to stain the specimen. More importantly, when operated in the force mode, AFM can reveal molecular information that is critical for the complete functioning of the native tissue [142].

In the present section several techniques developed for production of nanostructured 3D scaffolds will be briefly reviewed.

2.10.1 Nanostructured scaffolds by particle processing

Although advances have been made in the application of nanotechnology for other research fields including mechanical, electrical, catalytic and optical, relatively few advances have been made for biological applications (specifically, those involving bone regeneration), despite their promise to mimic the surface roughness that cells experience in vivo.

Studies by Webster et al. [143;144] have shown that bioactivity of biomolecules (protein such as fibronectin and vitronectin) is enhanced in terms of mediating osteoblast adhesion on nano-phase materials. Thapa et al. [144] have developed nano-structured PLGA and poly(ether urethane) (PU) formulations with surface feature dimensions ranging between 50 and 100 nm and tested their in vitro cytocompatibility properties with bladder smooth muscle cells.

Another recent study has shown that titania nano-size particles embedded in PLGA promoted osteoblast adhesion compared with conventional sized titania (> 100nm) in PLGA, since nano-particles of titania were more hydrophilic than those of conventional titania [145]. Similarly, Boccaccini et al. [146] have shown that bioactivity of PDLLA/TiO\textsubscript{2} composite film increased with increasing amount of TiO\textsubscript{2} nanoparticles incorporated in the films. Their studies also showed that TiO\textsubscript{2} nanoparticles alone have no significant effect on MG-63 cell viability.
As mentioned above, surface roughness is a crucial property influencing cell responses. Price and co workers [147] have demonstrated the importance of nanometer roughness based on the polymer casts of consolidated carbon nanofibre-based materials. Their study has indicated an increase in osteoblast adhesion on polymer casts of nanophase carbon fibres compared to conventional carbon fibres. Polymer casts of composites of polycarbonate urethane/carbon nanotubes also promoted osteoblast functions compared to conventional carbon tubes [148].

In conclusion, all the reviewed studies imply that cell response might be more sensitive to changes in surface roughness in the nanometer range (<100 nm) compared with conventional size (>100nm) and the sensitivity may vary with cell type.

2.10.2 Nanofibrous scaffolds by electrospinning

Electrospinning of fibres can be used to produce scaffold (ECM-like) structures similar to that of the fibrous proteins in native ECM albeit with different chemical compositions. The principle of electrospinning is to use an electric field to draw a polymer solution from an orifice to a collector, producing a submicron polymer fibre mesh with fibre diameter of several hundred nanometres. High voltages, usually 10-20 kV, are applied to generate a sufficient surface charge to overcome the surface tension in a pendant drop of the polymer fluid, resulting in a 2D membrane [149].

Due to the simplicity of this method, electrospinning has been widely used by a variety of research groups to prepare nanofibrillar matrices. Many studies have been carried out in the application of polymer nanofibers in the tissue engineering of bone [150], blood vessels [151], cartilage [79], cardiac tissue [152], peripheral nerve system [153], ligaments [154], liver [155], and skin [156]. In most of these studies, biodegradable polymer materials such as PCL, PLA, PGA and PLGA were used. In addition, naturally occurring macromolecules, such as collagen [157], silk protein [158], fibrinogen [159], elastin mimetic polypeptides [160], chitosan [161], dextran [162] and hyaluronic acid [163], have been fabricated into nanofibers by electrospinning. Nanofibers produced are expected to possess high axial strength
combined with extreme flexibility. The high surface area, high porosity and high spatial interconnectivity maximise the cell-nanofibres-scaffold interaction and promote tissue regeneration. As mentioned above, porosity plays a significant role in the transport of nutrients and cell migration during tissue regeneration.

In general, electrospun nanofibrous matrices are found to support cell adhesion and proliferation and cells tend to maintain their phenotype shape and guided growth according to the fiber orientation. Xu et al. [164] studied the potential of non-woven and aligned nanofiber matrices made from PLA-PCL and PLLA-PCL/Collagen as a scaffold for blood-vessel regeneration. A favourable interaction between the scaffold and human coronary artery smooth muscle cells (SMC’s) was demonstrated in the study. Attachment and migration of SMC’s along the axis of aligned nanofibers was significantly better than that on the polymer films. Challenges exist in utilising this technique to fabricate complex 3D scaffolds. Cells cultured on the fibrous membrane can penetrate into scaffolds only after the scaffolds have been degraded to a large extent. It is however difficult to produce nanofibers with diameters smaller than 50 nm using electro-spinning.

2.10.3 Nanofibrous scaffolds by self-assembly

Molecular self-assembly has recently emerged as a new approach to engineering artificial scaffolds that emulate the ECM both structurally and functionally. Unlike electrospinning, this technology not only incorporates specific biological components of the ECM, but also mimics the process of ECM assembly in a bottom-up manner [165]. Self assembly involves concerted action of weak and non-covalent interactions that result in hierarchical structures [166]. Stupp and co-workers [167] designed self-assembling molecules with a hydrophobic alkyl tail and hydrophilic oligopeptide head. The amphiphilicity of these molecules creates cylindrical nanofibres with well-defined diameters, which subsequently induce a liquid-to-gel transformation.

In one demonstration, the designed peptide amphiphile contains five key structural features which are conducive to in-situ directed self-assembly, reversible cross-linking, mineralization and cell adhesion. Upon acidification of the peptide solution
below pH4, cylindrical micelles form, in which the alkyl tails pack on the inside of the fibre and the peptide segments are displayed on the fibre surface [167]. Upon cross-linking of the cysteine residues on adjacent molecules, the stable nanofibers are able to direct the mineralization of hydroxyapatite. The resultant mineral is aligned with the direction of the self-assembled fibrils. These mineralised nanofibres resemble the lowest level of hierarchical organization of bone; their mechanical properties however are likely to be inferior to those of native bone. Probably is for this reason that no further study regarding the potential of this material as bone tissue engineering scaffold can be found in the specialised literature.

2.11 Bone and cartilage physiology.

2.11.1 Bone

There are two types of bone: a) compact (or cortical) bone and b) trabecular (or cancellous) bone. Cortical bone is found in the shaft (diaphyses) of long bone. It is consists of a number of irregularly spaced overlapping units termed Haversian systems (Fig 2.13 (A)) [168]. Each consists of a central Haversion canal-surrounded by concentric lamellae of bony tissue. Trabecular bone is found principally at the ends of long bones, and in vertebral bodies and flat bones. It is composed of a meshwork of trabeculae within which are intercommunicating spaces (Fig 2.13 (B)) [168]. The skeleton consists of approximately 80% cortical bone, largely in peripheral bones, and 20% trabecular bone, mainly in the axial skeleton. These amounts vary according to site and relate to the need for mechanical support. While trabecular bone accounts for the minority of total skeletal tissue, it is the site of greater bone turnover because its total surface area is greater than that of cortical bone.

Bones are generally richly supplied with blood, via periosteal vessels, vessels that enter close to the articular surfaces and nutrients passing obliquely through the cortex before dividing into longitudinally directed branches. Loss of the arterial supply to parts of a bone results in death of bone tissue, a process called avascular necrosis or osteonecrosis. In addition to its role as a support structure, the other primary function of bone is calcium homeostasis. More than 99.9% of the total body calcium resides in
the skeleton. The maintenance of normal serum calcium (1% of total body calcium) depends on the interplay of intestinal calcium absorption, renal excretion and skeletal mobilisation or uptake of calcium which is extremely important for maintenance of normal cellular functions.

The structural components of bone consist of extracellular matrix (largely mineralised), collagen and cells. The collagen fibres are of type I, comprise 90% of the total protein in bone and are oriented in a preferential direction giving lamellar bone its structure. Hydroxyapatite crystals are found within the collagen fibres and in the ground substance. The ground substance is primarily composed of glycoproteins and proteoglycans which have high ion-binding capacity and are thought to play an important role in the calcification process. The principle cells in bone are the osteoclasts and osteoblasts (including bone-lining cells and osteocytes). Osteoclasts, the cells responsible for resorption of bone, are derived from haematopoietic stem cells. Osteoblasts are derived from local mesenchymal cells. They are the pivotal bone cells, responsible directly for bone formation and indirectly, via paracrine factors, for regulating osteoclastic bone resorption.
2.11.2 Cartilage.

Articular cartilage is a specialised form of connective tissue that covers and protects the end of the bones in synovial joints. For the knee joint this includes the smooth surfaces covering the femoral and tibial condyles and the under surface of the patella. The surface is smooth and slippery with an extraordinarily low coefficient of friction, while the deeper layer merges with a calcified layer (the tidemark) that interlocks with the subchondral bone (Fig. 2.14) [168]. Cartilage is an elastic, resilient structure that acts as a shock absorber to protect the underlying bone. The properties of articular cartilage depend on the composition and structure of the extracellular matrix, and the synthesis and maintenance of this matrix is dependent on the chondrocytes. The functional integrity of the articular cartilage in a healthy joint depends on the chondrocytes synthesising the many different matrix components in the appropriate amounts and in the right sequence. During skeletal development, the articular cartilage forms very densely packed mesenchymal cells that differentiate into chondrocytes, which proliferates rapidly and synthesise the large amounts of...
extracellular matrix. The extracellular matrix is made up predominantly of water (up to 80%), collagen and proteoglycans, which are produced and maintained by the relatively sparse cells, the chondrocytes. Type II collagen is the predominant fibrous component making up 90-95% of the primary collagen and 40-70% of the total dry weight of articular cartilage. Proteoglycans trapped in the collagen fibrils make up 15-40% of the dry weight of articular cartilage. Proteoglycans play a crucial role in the ability to absorb loading forces in a reversible way.

Figure 2.14 Normal articular cartilage, illustrating the variability in density and orientation of the chondrocytes and collagen network. [168]
2.11 Summary of the literature review

The requirement for production of scaffolds for bone tissue engineering exhibiting 3D open porous structures with pore size of ~ 400 micron and porosity above 90% can be achieved by the foam replication technique. It has also been recognised that biocomposites made from Bioglass® foams and poly(DL-Lactide) or P(3HB) coatings are promising materials for bone tissue scaffolds in terms of their pore structure and bioactivity. For improvement of mechanical properties of ceramic 3D scaffolds, the limited literature available shows that this can be achieved through infiltration of polymers into the porous structure to achieve polymer-ceramic composite struts. This constitutes a broad, new and relatively unexplored field of research. Moreover, to obtain nanoscale features on the scaffold’s surface, which is also a requirement in the optimisation of the design of 3D scaffolds for bone tissue engineering, most work in the literature regarding nanostructured scaffolds has been on electrospinning of nanofibers. The development of biopolymer coatings of 3D-scaffold, where the polymer layer could also incorporate nanoparticles has been investigated. However, the combination of electrospinning methods and conventional 3D scaffold manufacturing techniques, e.g. foam replica method, has not been explored in the literature. This combination could offer the possibility of manufacturing layered scaffolds with adequate 3D geometry and surface topography. This technology approach will be investigated as part of the present project.
Chapter Three

3. Objectives and Aims of the project

The main aim of this PhD research project is the development of improved bioactive glass-based scaffolds for bone and osteochrondral tissue engineering. The objective is to fabricate highly porous scaffolds from melt-derived 45S5 Bioglass® powder using the sponge replication technique, aiming at enhancing the structural integrity (compressive strength, fracture toughness) of 45S5 Bioglass® based scaffolds by polymer infiltration and to gain understanding of the interaction of these two phases in the composite structure which mimics the composite structure of natural bone. A commercially available synthetic polymer, poly(D,L-Lactic acid) (PDLLA), should be incorporated as a coating onto the partially sintered Bioglass®-based scaffolds by a solution dipping technique. Natural polymers synthesised from bacteria which have different properties from PDLLA are investigated as well, in this case poly(3-hydroxybutyrate) (P(3HB)) and poly(3-hydroxyoctanoate) (P(3HO)) have been selected. Mechanical properties, in term of compressive strength and work of fracture, and bioactivity of these scaffolds coated with the different polymers were evaluated and compared. Coated 45S5 Bioglass® pellets sintered at the same condition as the scaffolds and immersed in SBF and 1.5 SBF were investigated to better evaluate the bioactivity mechanism and interfacial properties. It is emphasised that this is the first comprehensive investigation on the development and optimisation of Bioglass®-based 3D scaffolds combined with biodegradable polymers with intrinsic different properties, i.e. (PDLLA), P(3HB) and P(3HO). Another objective of this research work is to design and fabricate a bilayered scaffold structure in order to develop the first bioactive glass-ceramic scaffold coated with PDLLA nanofibers by combining the electrospinning method and the foam replica fabrication technique. An initial in vitro cell culture investigation on the bilayered scaffolds was carried out to assess the ability of this novel bilayered composite structure to support and foster cell proliferation on the PDLLA fibres layer (cartilage side). The final goal is to achieve an ideal bilayered scaffold for osteochondral tissue engineering that can provide
adequate mechanical support temporarily and degrade subsequently at a suitable rate in relation to the rate of new tissue formation.

### 3.1 Experimental Approach

A series of experiments was designed to achieve the several objectives of this project as summarised in the next paragraphs.

#### 3.1.1 Bioactive-glass/polymer composite scaffolds

In order to exploit the effect of interpenetrating network microstructures in the scaffold optimisation process, this research work investigated the infiltration of biodegradable polymer phases (i.e. PDLLA, P(3HB) and P(3HO)) into partially sintered Bioglass® glass-ceramic scaffolds prepared by the foam replica technique. With the objective of developing partially sintered, e.g. relative porous, strut structures, in different series of experiments sintering was stopped at a temperature of 1000°C, which is lower than the temperature normally used for complete densification of the structure [1], or sintering time was reduced. The partially sintered scaffolds were then dipped into the different polymer solutions in order to achieve polymer coatings on the 3D scaffold structure. The coated scaffolds were characterised in terms of their mechanical property, microstructure and bioactivity in SBF and compared with the behaviour of the dense scaffolds. In order to better evaluate the bioactivity mechanism and interfacial properties of the coated samples for different types of biodegradable polymer, polymer coated 45S5 Bioglass® pellets sintered at the same sintering condition were evaluated and characterised by XRD, SEM and EDS. The experimental details will be presented in Chapter 4. The schematic diagram of the scaffolds structures to be developed in this project is shown in Figure 3.1.
3.1.2 Bilayered scaffolds for osteochondral tissue engineering

In order to develop bilayered constructs/scaffolds for osteochondral tissue engineering applications, this research project investigated the electrospinning technique to deposit a PDLLA fibrous layer on the surface of the 3D porous bioactive glass-ceramic/PDLLA composite scaffolds. PDLLA was chosen due to its suitable properties and because it is a polymer approved by the US FDA for clinical use. It was anticipated that the use of PDLLA as coating of the scaffold would enhance the bonding at the interface between the coated scaffold and the electrospun PDLLA fibrous layer, which is a requisite to obtain adequate structural integrity and functionality of osteochondral bilayered constructs. The schematic diagram of the bilayered scaffolds designed in this project is presented in Figure 3.2. The PDLLA fibrous structure deposited by electrospinning technique was investigated in SBF to confirm that this layer is not bioactive and that no mineralisation occurs on the fibre surfaces in contact with simulated body fluid. Electrospinning technique was also used to deposit PDLLA fibrous structures on the flat surface of sintered 45S5 Bioglass® discs in order to introduce basic nanostructured topography for better bone cell attachment. In order to present an overview of the different tasks of the project...
and facilitate comprehension of the future chapters, the research methodology used is schematically shown in Figure 3.3.

Figure 3.2 Schematic diagram of the bilayered scaffold structure developed in this project for osteochondral tissue engineering.
Figure 3.3 Schematic diagram showing the research methodology used in this project.

- Scaffold coating with biodegradable polymer
  - PDLLA (commercially available)
  - P(3HB) and P(3HO) (bacteria grown in this project)

- 3D 45S5 Bioglass® based glass-ceramic scaffolds
  1. Sintering time and temperature (Optimisation).
  2. Selection of binders.
  45S5 Bioglass® based glass-ceramic sintered pellets.

- Nanofibrous surface
  - PDLLA
    Nanofibers on 3D 45S5 Bioglass® scaffolds. (Bilayered scaffolds)
  - PDLLA
    Nanofibers on 45S5 Bioglass® sintered pellets.

Characterisation:
- Microstructure
- Interfacial property
- Mechanical Properties
- Physical property
- Bioactivity in SBF and 1.5 SBF
- Cell culture assessment
Chapter Four

4. Materials and methods

4.1 Materials

The bioactive glass used in this project was a melt-derived 45S5 Bioglass® powder of composition (wt%): SiO$_2$(45%), Na$_2$O(24.5%), CaO(24.4%) and P$_2$O$_5$(6%). This powder was purchased from Novamin, Inc, Florida, USA. The particle size of the glass powder used in this experiment was in the range between 0.73µm to 17.59µm with the typical particle size (medium) being 8.66µm, determined using the Malvern Particle Size Analyser, as shown in Fig. 4.1. Poly(D,L-lactic acid) (PDLLA) that was used as a binder and coating material in this project, was purchased from Purac, (Purasorb PDLLA, Biochem, the Netherlands) with inherent viscosity of 2.15dL/g (chloroform as the solvent). Using the Mark-Houwink relations for PDLLA, ([η] = 1.33 x 10$^{-4}$ Mv$^{0.79}$), where Mv = viscosity average molecular weight, given by Van de Witte et al. [169] for poly-lactides in chloroform, the molecular weight of PDLLA used here is 200,000 g/mol. Purasorb (Purac, Biochem, the Netherlands) PDLLA was used without further purification. Dimethyl carbonate (DMC) of purity > 99% was used as a solvent. It was purchased from Sigma-Aldrich, UK. A different type of binder used was polyvinyl alcohol (PVA), 98-99% hydrolysed with molecular weight in the range of 85,000 to 146,000. PVA was purchased from Sigma-Aldrich, UK. A reticulated polyester-based polyurethane foam with 45ppi (pores per inch) was purchased from Recticel, England, and it was used as the template for the replica method developed to prepare Bioglass® scaffolds. A SEM image of the foam is shown in Figure 4.2. Deionised water was used in all the experimental preparations in this project. In a separate part of the project, P(3HB) and P(3HO) were produced from bacteria fermentation (as reported in detail below; section 4.3 and section 4.4). All chemicals used were purchased from Sigma-Aldrich, UK, except if stated otherwise. Table 4.1 summarises the physical properties of the biodegradable polymers used in this project.
Figure 4.1 Particle size distribution of 45S5 Bioglass® powder.

Figure 4.2 The macroporous structure of 45 ppi polyurethane sponge use to fabricate scaffolds by the replica technique. (Recticel, England)
Table 4.1 Physical properties of the biodegradable polymers used in this project.

<table>
<thead>
<tr>
<th></th>
<th>P(DLLA)[169]</th>
<th>P(3HB)[170]</th>
<th>P(3HO)(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mw (g/mol)</strong></td>
<td>200,000</td>
<td>885,000</td>
<td>225,000</td>
</tr>
<tr>
<td><strong>Contact Angle (°)</strong></td>
<td>80.04</td>
<td>87.9</td>
<td>64.5</td>
</tr>
<tr>
<td><strong>Tg (°C)</strong></td>
<td>55</td>
<td>2.7</td>
<td>-35.5</td>
</tr>
<tr>
<td><strong>Xc (%)</strong></td>
<td>amorphous</td>
<td>64.1</td>
<td>37.2</td>
</tr>
</tbody>
</table>

Where; Mw= molecular weight

\( Tg = \) glass transition temperature

\( Xc = \) percentage crystallinity

### 4.2 Fabrication of 45S5 Bioglass\(^\circledR\) based scaffolds

#### 4.2.1 Preparation of 45S5 Bioglass\(^\circledR\) powder slurry

The preparation of the 45S5 Bioglass\(^\circledR\) powder slurry was carried out according to a previously developed method [53] with slight modifications. 3 grams of PDLLA were dissolved in 100 ml DMC to give a polymer weight to solvent ratio of 3% \( \text{wt/v} \) PDLLA-DMC in solution. The mixture was then stirred using a magnetic stirrer for at least 12 hours to obtain a homogenous polymer solution. 68 grams of 45S5 Bioglass\(^\circledR\) powder were added to the 3% \( \text{wt/v} \) PDLLA-DMC solution to give a 66% \( \text{wt/v} \) Bioglass\(^\circledR\)-PDLLA solution and the mixture was stirred vigorously for 1 hour using a magnetic stirrer.

#### 4.2.2 Preparation of green bodies

The polyurethane (PU) foam (as shown in Fig. 4.2) was cut to give samples of final dimensions 5mm x 5mm x 10mm after sintering (considering a 50% linear shrinkage during sintering). Green bodies were prepared by slurry-dipping. The cut PU foams were immersed in the above prepared 45S5 Bioglass\(^\circledR\) slurry for 3 minutes; the foams were manually retrieved from the suspension as quickly as possible and the extra slurry was squeezed out vigorously by hand. The samples (green bodies) were then

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\(^1\) This experiment was carried out by Ms Ranjana Rai (University of Westminster, UK)
placed on tissue paper and dried under a fume hood at room temperature for at least 12 hours.

4.2.3 Heat treatment of the green bodies

The green bodies were sintered in an electric furnace using different heat treatment programs, as shown in Fig. 4.3. First, the green body was sintered at 550°C for 3 hours using a heating rate of 2°C/min to burn out the PU foam used as the replica template. Then, the temperature of the furnace was increased, in different series of scaffold production to 1100°C, 1000°C, 970°C, 950°C, 930°C, and 900°C at the rate of 2°C/min, respectively, and the dwelling time was set for 2 hours in order to sinter the 45S5 Bioglass® scaffolds. The effect of different sintering times on the mechanical properties of the 45S5 Bioglass® based scaffolds was also investigated by sintering the scaffolds at 1000°C at different sintering times of 2 hours, 1 hour and 0.5 hours, respectively. Finally, the temperature of the furnace was reduced to room temperature at 5°C/min before the sintered scaffolds were taken out from the furnace.

![Figure 4.3 Heat treatment program designed for burning-out polyurethane templates and sintering of 45S5 Bioglass® scaffolds.](image-url)
4.2.4 Selection of binders

In this part of the study, the preparation of 45S5 Bioglass® based scaffolds was carried out according to the method described in section 4.2.1, 4.2.2 and 4.2.3 with some modifications. A new method involves the use of a new type of binder, i.e. PVA, in the preparation of the Bioglass® scaffolds. For the preparation of the 45S5 Bioglass® slurry; 5 grams of PVA were weighted. This PVA amount was dissolved in 50 ml of water at 80°C using a hot plate stirrer for 1 hour. After complete dissolution of PVA, 30 grams of 45S5 Bioglass® powder were added and the mixture was stirred for 1 hour. For the preparation of the green body, the PU foams were immersed in the 45S5 Bioglass® slurry for 2 minutes and then they were manually retrieved from the suspension. The excess slurry was manually squeezed vigorously by hand. The green bodies were placed in the oven at 60°C for 24 hours to dry prior to being sintered at different sintering conditions following the heat-treatment programme in Fig. 4.3.

4.2.5 Functionalisation of 45S5 Bioglass® scaffolds with ALP enzyme

4.2.5.1 Cleaning of the scaffolds

This part of the experimental work was done at the Department of Material Science and Chemical Engineering, Polytechnic of Turin, Italy. First, 45S5 Bioglass® scaffolds were put in a clean beaker and acetone was added just to cover the whole scaffolds. Then, the beaker was placed in an ultrasonicator for 5 minutes. After 5 minutes, the scaffold was transferred into a clean beaker with 70 ml distilled water. The beaker was then put into an ultrasonicator for 5 minutes. The cleaning procedure for washing with distilled water was repeated three times. Finally, the scaffolds were dried on a clean tissue paper for few hours.

4.2.5.2 Silanisation of the scaffolds

A silanisation solution was prepared using 150ml ethanol and 35μl of silane (3-aminopropyltriethoxysilane). The mixture was stirred using a magnetic stirrer for
proper mixing. After that, dried scaffolds were put into the silane solution for 6 hours. Then, the scaffolds were transferred into a clean beaker for thermal treatment in an oven at 100°C for 1 hour and they were let to cool down for one day. Finally, the sample was washed in ethanol for 5 minutes in ultrasonicator.

**4.2.5.3 Preparation of enzyme (ALP) solutions**

Phosphatase alkaline from bovine intestinal mucosa (ALP) was chosen as the model enzyme for the functionalisation of the 45S5 Bioglass® scaffolds. The standard protocol for the preparation of 5 mg of enzyme requires 1 ml of PBS (phosphate buffer solution) to dissolve. For each scaffold, 10 ml of PBS is required for the functionalisation, therefore, in this experiment, 60 ml of PBS were required to dissolve 1.3 grams of ALP for six scaffolds. The ALP enzymes were stirred using a magnetic stirrer in PBS until they were completely dissolved. Then 10 ml of the enzyme solution were transferred into a 30 ml nalgene bottle containing the scaffold. The nalgene bottle was then put in an incubator at 37°C for 24 hours. The enzymatic activity of the functionalised Bioglass® scaffolds was measured using UV-VIS spectroscopy at 405nm absorbance.

**4.3 Synthesis of P(3HB) polymers using bacterial fermentation**

**4.3.1 Growing bacteria in nutrient broth (inoculum)**

*Bacillus cereus* SPV, used for the production of P(3HB), was available from the Department of Life Sciences, University of Westminster (UK). First, 0.039 grams of nutrient broth were dissolved in distilled water (30ml) and autoclaved at 121°C for 15 minutes. Then, a single colony of *Bacillus cereus* SPV strain was used to inoculate the autoclaved nutrient broth (30ml) and incubated at 30°C for 24 hours at 150 rev min\(^{-1}\) (Stuart Scientific Orbital Shaker, S150).

**4.3.2 Growing bacteria in nitrogen limiting medium (production medium)**

The production medium used was a specific nutrient limiting with glucose as the carbon source. First, glucose (20g/L), yeast extract (2.5g/L), potassium chloride (3.0g/L) and ammonium sulphate (5g/L) were dissolved in 270 ml water (production
medium). Then, 30 ml of soybean dialysate were added to the above dissolved solution of nitrogen limiting medium. The pH of the production medium was maintained at 6.8. The production medium solution was then autoclaved at 110°C for 10 min. Next, 30 ml of the inoculum prepared above (section 4.3.1), at an optical density of 4.5 was added into the production medium (300 ml). Finally, the inoculum in the production medium was incubated at 30°C for 72 hours at 150 rev min⁻¹ in a temperature controlled orbital shaker (Stuart Scientific Orbital Shaker, S150).

4.3.3 Isolation/harvesting the bacteria

After 72 hours incubation period, the bacterial cells were isolated from the production medium by centrifuging at 5682g for 10 min. After 10 min centrifugation, the supernatant was discarded while the cell pellets at the bottom of the centrifuge tube were kept. Then the harvested cell pellets were kept in the freezer at -80°C for at least 24 hours. Finally the frozen cell pellets were freeze-dried in a freeze-dryer for 24 hours.

4.3.4 Extraction of polymer

First, the freeze-dried cell pellet was weighed to determine its dry cell weight. Then the dry pellet was crushed and dissolved in 30% sodium hypochlorite solution and chloroform in a ratio of (1:1). The solution mixture was then incubated at 30°C for 2 hrs at 150 rev min⁻¹. Next, the mixture was centrifuged at 5682g for 18 minutes. Three different layers were formed. The first layer was a hypochlorite solution, the middle layer contained non-P(3HB) cell materials and undisrupted cells. The bottom layer was chloroform containing the P(3HB). After removal of the top and middle layer, polymer was precipitated from the CHCl₃ solution by introducing it into cold chilled methanol with continuous stirring. Finally, the polymer was dried in an oven at 50°C.
4.4 Synthesis of P(3HO) using bacterial fermentation

4.4.1 Growing bacteria in nutrient broth and production media

P(3HO) production with a two stage seed culture preparation was carried out using *Pseudomonas mendocina* obtained from the Department of Life Sciences, University of Westminster (UK). The first seed culture was prepared by inoculating sterile nutrient broth using single colony of *Pseudomonas mendocina* and growing it for 24 hours in a temperature controlled orbital shaker (Stuart Scientific Orbital Shaker, S150) at 30°C and at a speed of 200 rev min⁻¹. This was then used for inoculating sterile P(3HO) production medium to prepare the second stage seed culture. The organism was again grown under the same culture conditions of 30°C and 200 rev min⁻¹. The P(3HO) production medium used is a mineral salt medium (MSM) [171] which contained (g/l): (NH₄)₂SO₄, 0.4; Na₂HPO₄, 3.65; KH₂PO₄, 2.8; MgSO₄, 0.5. Sodium octanoate and MgSO₄ were autoclaved separately and then added aseptically to the rest of the medium. The medium also contained trace element solution, (1ml/L), whose composition was: CoCl₂, 0.218; FeCl₃, 9.7; CaCl₂, 7.8; NiCl₂, 0.118; CrCl₆.H₂O, 0.105; CuSO₄.5H₂O, 0.156 g in 1 L of 0.1 N HCl. The final pH of the medium was 7.

The second seed culture at an optical density (OD) of 3.3 was then used to inoculate the final PHA production medium (sterile, MSM media) and it was grown under the same culture conditions. The final working volume was 300 ml in 1L flasks and incubated for 72 hours in a temperature controlled orbital shaker (Stuart Scientific Orbital Shaker, S150) at 30°C and at a speed of 200 rev min⁻¹.

4.4.2 Extraction of polymer

Dried bacterial biomass was incubated in a dispersion containing 80% NaOCl and CHCl₃ in a 1:4 ratio at 30°C for 2.30 hours and 150 rev min⁻¹. It was then centrifuged at 5682g for 18 minutes following which three layers were formed. The topmost layer was that of hypochlorite, the middle layer contained the cell debris and the bottom layer was the CHCl₃ containing the dissolved polymer. Polymer was precipitated out by introducing the CHCl₃ layer into ice cold methanol with continuous stirring.

---

2 This experiment was conducted by Ms Ranjana Rai (collaborator from University of Westminster, UK)
Finally, the polymer was dried at room temperature (25°C). An established protocol has been developed by the collaborator at the University of Westminster to remove the bacterial component present in the polymers in order to reduce contaminations.

4.4.2.1 Removal of bacterial components.

Lipopolysaccharide, LPS is an integral component of the Gram negative bacteria cell wall and is pyrogenic in nature. As P(3HO) is produced from *P. mendocina* a Gram negative bacteria therefore the polymer was subjected to repeated steps of polymer purification to remove the co precipitated contaminating LPS.

The polymer was extracted from the lyophilised bacterial cells using the sodium hypochlorite dispersion method and subjected to sequential repeated steps of precipitation to remove the LPS as described below:

1st step precipitation: A non solvent, 50% each of methanol and ethanol was used.

2nd precipitation: The polymer was dissolved in CHCl₃ and again precipitated in chilled methanol.

3rd precipitation: The polymer was dissolved in acetone and again precipitated in methanol.

4th precipitation: The polymer was again dissolved in acetone and precipitated in 50% each of methanol and ethanol.

The endotoxin level in the purified polymer was then quantified using the FDA approved Limulus Amebocyte Lysate (LAL) test. The endotoxin level in the purified P(3HO) was found to be 7 Endotoxin units gram⁻¹. This complies with the endotoxin requirements of the FDA for biomedical applications such as implants and drug delivery systems [172].

4.5 Coating of 45S5 Bioglass® scaffolds with PDLLA

The scaffolds were coated with PDLLA by a solution dipping method. The dimensions of the sintered 45S5 Bioglass® scaffolds were measured using a digital calliper (Mitutoyo, UK) and the weight of the scaffolds was measured using an electronic analytical weighing balance (Ohaus, USA). Then, the scaffolds were slowly immersed in a solution of 5% wt/v PDLLA-DMC for 2 hours. After 2 hours
immersion time, the scaffolds were taken out from the PDLLA-DMC solution and put on tissue paper to dry at room temperature for at least 12 hours. Finally, the weight of the dried scaffolds was measured using an electronic analytical weighing balance and the dimensions were measured using a digital calliper (Mitutoyo, UK).

4.6 Coating of 45S5 Bioglass® based scaffolds with P(3HB) and P(3HO)

Bioglass® based scaffolds were coated with P(3HB) and P(3HO) by a suspension – dipping method. First, the synthesised P(3HB) polymer was dissolved in chloroform to make a 5% wt/v solution until the polymer was completely dissolved using a magnetic stirrer. Then the dimensions of the scaffolds were measured using a digital calliper and the weight of the scaffold was determined using an electric weighing balance. After the P(3HB) was completely dissolved, the scaffolds sintered at various sintering conditions were slowly immersed in the 5wt% P(3HB)-chloroform solution for 2 hours. After 2 hours immersion time, the scaffolds were taken out from the P(3HB)-chloroform solution with tweezers and were placed on a tissue paper to dry for at least 12 hours in normal air. Finally, the weight of the dried scaffold was measured using an electric weighing balance (Ohaus, USA) and the dimensions were measured using a digital calliper (Mitotuyo, UK). The same procedure was used for P(3HO) polymer coatings with slight different drying process which was of 1 week in normal air.

4.7 45S5 Bioglass® pellets with PDLLA polymer coating

To fabricate cylindrical Bioglass® pellets, 0.3 grams of 45S5 Bioglass® powder were poured into a clean stainless steel cylindrical die of 10mm diameter. Then, a plunger was inserted into the die and the 45S5 Bioglass® powder was uniformly compacted. Next, the die was positioned at the centre of a uniaxial hydraulic press and the valve of the press was closed. The pressure was increased to 30-40 MPa and the die was left at that pressure for 2 minutes. Then, the valve was opened to release the pressure. The pellet of 45S5 Bioglass® was taken out of the die by applying a low pressure.
After that, the 45S5 Bioglass® pellets were sintered using the same heat treatment program as the one used for 45S5 Bioglass® scaffolds which involved heating to 550°C for 3 hours at a heating rate of 2°C/min, and then increasing the temperature to 1000°C at 2°C/min for densification, the dwelling time were set at 2 hours. The temperature of the furnace was reduced to room temperature at 5°C/min prior to the pellets being taken out.

Finally, the sintered 45S5 Bioglass® pellets were coated with a solution of 5%wt/v PDLLA-DMC by a dip coating method (ratio of solid to coating solution volumes being about 1:50) for 2 hours. After 2 hours immersion time, the pellets were taken out from the solution with tweezers and were placed on tissue paper to dry for at least 12 hours in normal air.

### 4.8 45S5 Bioglass® pellets with P(3HB) and P(3HO) polymer coatings

The preparation 45S5 Bioglass® pellets and the sintering conditions were described in section 4.7. The sintered pellets were then coated with a solution of 5% wt/v P(3HB)-chloroform for 2 hours by a dip coating process. After 2 hours completely immersed in the P(3HB)-chloroform solution, the pellets were taken out using tweezers and were dried in air at room temperature for at least 12 hours. The same procedure was followed for coatings with P(3HO) coatings with a slight difference in the drying process which was 1 week in this case.

### 4.9 Preparation of PDLLA fibres on 45S5 Bioglass® pellets and scaffolds by electrospinning method

This part of the experiment was designed to develop polymer fibre coated Bioglass® derived sintered substrates. The process involved the production of polymer fibres by electrospinning, an electric field assisted process.

A custom made electrospinning apparatus (developed at Department of Mechanical Engineering, UCL (UK)) was used in this study, which is shown schematically in
Figure 4.4. First, a 5% wt/v PDLLA-DMC solution was prepared by dissolving 0.5 gram PDLLA in 10 ml DMC which was stirred for at least 12 hours using a magnetic stirrer. DMC was chosen as the solvent because of its suitable evaporation rate during electrospinning. Then, the above prepared PDLLA-DMC solution was poured into a 1 ml syringe attached to a needle (inner diameter 300 µm) and the polymer solution was pumped continuously using a specially designed perfusor pump (PHD4400, HARVARD apparatus) at various rates in the range 2-10 µl min⁻¹. The needle was coupled to a high power voltage supply (Glassman, Europe Ltd) capable of delivering 30 kV. The flowing solutions were subjected to an applied voltage between 8 and 15 kV. 45S5 Bioglass® pellets of 10 mm diameter and 2 mm height were processed by cold uniaxial pressing (pressure 30-40 MPa) and sintering at 1000°C for 2 hours (as described above). These sintering conditions are the same used to fabricate 3D porous scaffolds from the same Bioglass® powder. Sintered pellets were polished using silicon carbide paper of 1200 grit for 10 minutes.

In a typical experiment, the polished 45S5 Bioglass® pellet was positioned directly underneath the tip of the needle at a distance of 15 cm for collection of the PDLLA fibres during 5-10 minutes, as schematically shown in Figure 4.4. The 45S5 Bioglass® scaffolds sintered at 1100°C for 2 hours, sintered at 1000°C for 2 hours and coated with 5wt% PDLLA were also positioned underneath the tip of the needle for collection of the PDLLA fibres for 10 minutes, 30 minutes and 2 hours deposition time. Finally, the fibre coated 45S5 Bioglass® pellets and scaffolds were kept in a dessicator until characterisation was carried out, as detailed below (section 4.10).
4.10 Materials Characterisation

4.10.1 Physical characteristics of Bioglass® scaffolds

The density of the scaffolds (\( \rho_{\text{foam}} \)) was determined from the mass and volume of the scaffolds before and after coating with PDLLA. The porosities before \( (P_1) \) and after \( (P_2) \) coatings were determined as follows (Equations 4.1 and 4.2):

\[
P_1 = 1 - \frac{W_1}{\rho_{\text{BG}}V_1} \tag{4.1}
\]

\[
P_2 = 1 - \left( \frac{W_1}{\rho_{\text{BG}}} + \frac{W_2 - W_1}{\rho_{\text{PDLLA}}} \right) \frac{1}{V_2} \tag{4.2}
\]

where \( W_1 \) and \( W_2 \) are the weight of the scaffolds before and after coating, respectively; \( V_1 \) and \( V_2 \) are the volume of the scaffolds before and after coating with
PDLLA, respectively. The density $\rho_{BG} = 2.7 \text{g/cm}^3$ is the theoretical density of 45S5 Bioglass® and $\rho_{PDLLA} = 1.26 \text{g/cm}^3$ is the density of the solid PDLLA (provided by the supplier). The same equations were used to determine porosity of P(3HB) and P(3HO) coated scaffolds. In this case, $\rho_{P(3HB)} = 1.21 \text{g/cm}^3$ is the density of P(3HB)[170] and the density of P(3HO) is $\rho_{P(3HO)} = 1.06 \text{g/cm}^3$.

**4.10.2 Microstructural characteristics**

The microstructure of the Bioglass® based scaffolds was characterised in a JEOL 5610LV scanning electron microscope (SEM), before and after coating with 5wt% PDLLA, P(3HB) and P(3HO). Samples were gold-coated and observed at an accelerating voltage of 10-20 kV.

The energy dispersive X-ray (EDS) spectra (Kα) of Bioglass® pellets coated with 5wt% PDLLA, 5wt% P(3HO) and 5wt% P(3HB) after immersion in SBF and 1.5 SBF (see section 4.10.4) were collected at 10kV in a field emission gun (FEG) SEM (Leo15). The data were processed using an INCA (Oxford instruments) program. At least 3 measurements were taken for each condition investigated. Selected pellets were also characterised using X-ray diffraction (XRD) analysis with the aim to investigate the formation of HA crystals on coated Bioglass® pellets after different times of immersion in SBF. Data were collected using a Phillips PW1710 diffractometer over a range of $2\theta = 5-70^\circ$ using a step size of 0.04° and a counting time of 2s per step.

The microstructure of the PDLLA fibres deposited by electrospinning on Bioglass® pellets, scaffolds and on glass slides was also characterised using the JEOL 5610LV SEM. Samples were gold coated and observed at an accelerating voltage of 10-20kV. The EDS spectra of the selected PDLLA fibre coated pellets and scaffolds, before and after SBF immersion for 7, 14 and 28 days were collected at 10kV in the field emission gun (FEG) SEM (Leo 15). XRD analysis was also done using the above mentioned parameters with the aim to investigate the formation of HA crystals on the samples.
4.10.3 Mechanical properties

The compressive strength of the 45S5 Bioglass® scaffolds before and after coating with 5 wt% PDLLA, 5 wt% P(3HB) and 5wt% P(3HO) was measured using a Zwick/Roell Z010 universal testing machine. The compressive strength of the coated and uncoated selected scaffolds after 3 months in SBF was also determined. The samples were prismatic in shape, with dimensions: 10mm in height and 5mm x 5mm in cross-section. The cross-head speed was set at 0.5mm/min. During compression strength test, the load was applied until the compressive strain in (%) reached 70%. To make sure that the samples were correctly aligned perpendicularly to the bottom and top platens of the compression testing machine, the samples were carefully trimmed with a razor blade before being coated with PDLLA, P(3HB) and P(3HO) polymer (section 4.5 and 4.6). A Teflon film of 0.05mm thickness was also used to cover the bottom and top platens of the testing machine to prevent the samples from misalignment. To assess the parallelism of the samples, measurements of the sample size were taken at three different points with an error margin of ± 0.001mm. The error in measuring the load in this testing machine was assumed to be 5%, according to previous studies [53]. SEM images of the fracture surface were taken using a JEOL 5610LV SEM to evaluate the interaction of the polymer coating with the 45S5 Bioglass® scaffold strut structure during fracture.

4.10.4 SBF treatment of polymer coated 45S5 Bioglass® pellets and scaffolds

1.0 SBF and 1.5 SBF solutions were prepared according to the standard procedure introduced by Kokubo et al. [173] (see Table.4.2). The PDLLA, P(3HB) and P(3HO) coated 45S5 Bioglass® pellets (section 4.7 and 4.8) were immersed in 30ml of 1.0 SBF in a clean centrifuge tube, which had previously been washed with deionised water. The PDLLA coated 45S5 Bioglass® pellets were also immersed in the 1.5SBF (a more concentrated solution than SBF) to compare the difference in scaffold behaviour in the different media. The samples were placed inside an incubator at a controlled temperature of 37°C. The size of all cylindrical samples was 10mm in diameter x 0.2mm in thickness. Samples were extracted from the SBF solution after 1, 2 and 4 weeks. The SBF was replaced twice a week because the cation concentration
changed during the course of the experiment and also to simulate a dynamic flow of SBF, as shown in the literature [135]. Once removed from the incubator, the samples were rinsed gently with deionised water and left to dry at ambient temperature in a dessicator. The same procedure was followed for the PDLLA fibre coated 45S5 Bioglass® pellets and scaffolds (section 4.9).

Table 4.2 Amounts of reagents used for preparation of 1.0 SBF and 1.5 SBF [173].

<table>
<thead>
<tr>
<th>Order</th>
<th>Reagent</th>
<th>SBF (1000ml)</th>
<th>1.5 SBF (1000 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#0</td>
<td>Ultra-pure water</td>
<td>750</td>
<td>750</td>
</tr>
<tr>
<td>#1</td>
<td>NaCl</td>
<td>7.996g</td>
<td>11.994g</td>
</tr>
<tr>
<td>#2</td>
<td>NaHCO₃</td>
<td>0.350g</td>
<td>0.525g</td>
</tr>
<tr>
<td>#3</td>
<td>KCl</td>
<td>0.224g</td>
<td>0.336g</td>
</tr>
<tr>
<td>#4</td>
<td>K₂HPO₄.3H₂O</td>
<td>0.228g</td>
<td>0.342g</td>
</tr>
<tr>
<td>#5</td>
<td>MgCl₂,6H₂O</td>
<td>0.305g</td>
<td>0.458g</td>
</tr>
<tr>
<td>#6</td>
<td>1N HCl</td>
<td>40ml</td>
<td>60ml</td>
</tr>
<tr>
<td>#7</td>
<td>CaCl₂</td>
<td>0.278g</td>
<td>0.417g</td>
</tr>
<tr>
<td>#8</td>
<td>Na₂SO₄</td>
<td>0.071g</td>
<td>0.107g</td>
</tr>
<tr>
<td>#9</td>
<td>(CH₂OH)₃CNH₂</td>
<td>6.057g</td>
<td>9.086g</td>
</tr>
<tr>
<td>#10</td>
<td>1 kmol/m³ HCl</td>
<td></td>
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</tr>
</tbody>
</table>

4.10.5 Polymer/Bioglass® bonding strength in composite scaffolds

The bonding strength at the polymer/ceramic interface was evaluated qualitatively using a crack indentation method developed by Becher et al. [174] where cracks are generated by Vickers indentation and are propagated towards the interface at different impingement angles to discern whether they penetrate or are arrested at the interface. PDLLA and P(3HB) coated 45S5 Bioglass® pellets sintered at 1000°C for 2 hours were mounted in resin and polished to a 3µm finish. Vickers hardness indents were made in the sintered 45S5 Bioglass® pellets approximately 100µm from the interface using a microhardness tester (Zwick/Roell Indentec). Three to five indents were made on each sample with loads ranging between 100 and 500g in order to generate corner
cracks that would propagate towards the polymer/Bioglass® interface. Crack trajectories were examined using optical microscopy and characterised in the JEOL 5610LV (SEM) to record the incidence angle and the crack path. The crack path, i.e. the interfacial deflection or penetration into the polymer, and the interface debond length, ldb, were recorded. A schematic diagram showing the expected behaviour is shown in Figure 4.5

![Figure 4.5 A schematic diagram showing the crack propagation from a Vickers indentation on a sintered Bioglass® pellet (developed using the microhardness tester).](image)

**4.10.6 White light interferometry (Zygo®)**

The surface roughness introduced by the PDLLA fibres on the polished Bioglass® pellets was characterised by surface topography measurement using the Zygo® white light interferometer instrument. White light interferometry is a surface imaging technique that uses white light to obtain images of the sample surface [175]. This method was used to characterise the sample surfaces before and after PDLLA fibre deposition by electrospinning. The quantification of the surface roughness can be obtained by using dedicated software connected to the instrument. Rms is one of the useful quantitative roughness parameters, which is statistically given by the root-mean
square deviations from a central line on the surface, and it can be expressed by Equation 4.3:

$$\text{Rms} = \sqrt{y_1^2 + y_2^2 + \ldots + y_n^2/n} \quad (4.3)$$

where $y_1, y_2 \ldots y_n$ are deviations of $n$ discrete elements from a central line defined as the best fit surface selected with the remove control. The remove control is an option of the software that permits to remove from the data a surface figure and it is fundamental for the data to be meaningful. Rms is an average of the surface roughness analysed and therefore it can be considered representative of that particular surface. Ra is the average roughness or deviation of all points from a plane fit to the test surface, given by the following equation:

$$\text{Ra} = \frac{y_1 + y_2 + \ldots + y_n}{n} \quad (4.4)$$

Where $y_1, y_2 \ldots y_n$ are the deviations from the central line selected with the remove control and $n$ is the number of discrete elements.

### 4.10.7 Permeability study

The permeability test used to measure the intrinsic permeability of scaffolds ($m^2$), as shown in Fig. 4.6 was performed at the Aragon Institute of Engineering Research, Universidad de Zaragoza, Spain. The experimental procedure will be briefly explained. A pressure-induced permeability test was performed using deionized water ($\mu = 10^{-3} \text{ Pa.s}$). The fluid was moved with the use of a peristaltic pump and was taken from an open reservoir to the air (see Fig. 4.6). Permeability tests were performed following the Darcy’s law:

$$k = \frac{\mu t Q}{A \Delta p} \quad (4.5)$$

where $k$ = intrinsic permeability ($m^2$)
$t$ = specimen thickness (m)
$A$ = cross-sectional area ($m^2$)
Q = flow rate (m$^3$/s)
$\Delta p =$ pressure drop (Pa)
$\mu =$ dynamic fluid viscosity [Pa.s]

The pressure drop was measured between two points at the inlet and outlet of the reductor with a pressure meter Digitron 2080P (Digitron Instrumentation). Due to the set-up of the test, the measured pressure drop is attributed to the scaffold microstructure ($\Delta p$) and section change ($\Delta p_{sec}$). Therefore, the following equation can be written:

$$\Delta p_{Total} = \Delta p + \Delta p_{sec} \quad (4.6)$$

with $\Delta p_{Total}$ being the measured pressure drop by the pressure meter and,

$$\Delta p_{sec} = 2Q^2\rho/\pi \left[1/d_1^2 - 1/d_2^2\right] \quad (4.7)$$

Two samples, labelled (1) and (2), were tested. The samples were fabricated by the standard conditions (sintering temperature of 1100°C for 2 hours). Different fluid flow regimes were applied controlling the flow rate with the peristaltic pump on the two samples of Bioglass® foams. The thickness of the samples were 8.52 and 8.11mm for samples (1) and (2), respectively, being in both cases the cross-sectional area $A = \pi d_1^2/4$. The corresponding $\Delta p$ - Q curves were obtained for both samples and the intrinsic permeability values were estimated from the acquired data.
4.10.8 Capillary test using calf-serum

This part of the experiment was done at and in collaboration with the Department of Materials Science and Chemical Engineering, Polytechnic of Turin, Italy. Calf-serum, iron supplemented and originated from fed calves was stored at -20°C. It was purchased from Sigma-Aldrich. The calf serum was thawed at room temperature until
it was melted. A small amount of the calf-serum was then added into double-distilled water with a dilution ratio of 30:70. Then, 1.5 ml of the diluted calf-serum was transferred into a small weighing boat using a plastic pipette for the capillary test. 45S5 Bioglass\textsuperscript{®} scaffolds sintered at 1000\textdegree C and 1100\textdegree C for 2 hours of dimensions 5 mm x 5 mm x 10 mm were used to perform the capillary test. Red inks drops were added to the prepared solution to better observed the capillary-take of the fluid into the scaffolds porosity (see Fig. 4.7). The uptake of the calf-serum by the capillary action was recorded using a video-camera (see movie in supplementary CD).

![Figure 4.7 Photograph showing the experimental set-up for the capillary test.](image)

4.10.9 Characterisation of P(3HB) and P(3HO) polymers\textsuperscript{3}

4.10.9.1 Structural characterisation

\textit{i) Gas Chromatography-Mass Spectrometry, GC-MS}\n
The PHA monomer was identified by carrying out GC-MS analysis of the methanolysed polymer. Methanolysis was carried out as described by Furrer \textit{et al.} \cite{177}. The reaction mixture contained 10 mg of polymer, 1 ml of methylene chloride containing 10 mg/ml of 2-ethyl-2-hydroxybutyric acid. The polymer was left to dissolve at room temperature for 1 hour. 1 ml of Boron triflouride (BF3) in methanol (0.65 M) was also added, after which the tube was tightly sealed, vigorously shaken and then heated for 20 hours at 80\textdegree C. After the reaction, the tubes were cooled on ice for 5 min, 2 ml HPLC water was added and the tubes were vortexed for 1 min. After

\textsuperscript{3} Experiment was conducted by Ms Ranjana Rai (collaborator from University of Westminster, UK)
phase separation, the bottom organic phase was collected, dried over anhydrous Na$_2$SO$_4$ and neutralized by adding Na$_2$CO$_3$. It was then filtered and used for carrying out the GC-MS study.

**4.10.9.2 Physical characterization**

**i) Crystallinity**
XRD data were collected using a Phillips PW1710 diffractometer over a range 2$\theta$ = 5-70° using a step size of 0.04° and a counting time of 0.05s per step. Crystallinity (%) of the polymer was calculated from mathematical modal functions, Gauss and Lorentzian functions of the different aged polymer samples.

**ii) Contact angle study**
To evaluate the wettability of the polymer film, static contact angle measurements were carried out on 5 wt/v % solvent cast films. An equal volume of water (<10 ml) was placed on every sample by means of a gas tight micro-syringe forming a drop. Photos (frame interval: 1 s, number of frames: 100) were taken to record the shape of the drops. The water contact angles on the specimens were measured by analysing the recorded drop images using the Windows based KSVCam software. Six repeats for each sample were carried out. The experiment was done on a KSV Cam 200 optical contact angle meter (KSV Instruments Ltd).

**iii) Molecular weight analysis**
The molecular weight of the polymer was determined by carrying out gel permeation chromatography (GPC) analysis. The eluent used was tetrahydrofuran, THF ((CH$_2$)$_4$O), 10 mg/ml of polymer solution was introduced into the GPC system at a flow rate of 1 ml/min. The eluted polymer was detected with a differential refractometer. The data were collected and analysed using Viscotek Trisec 2000 and Trisec 3.0 software.

**iv) Thermal properties**
The thermal properties of the polymer, i.e. glass transition temperature ($T_g$) and melting temperature, ($T_m$), were studied by carrying out differential scanning calorimetry (DSC) using a Perkin Elmer Pyris Diamond DSC (Perkin Elmer Instrument). The amount of polymer used for the study ranged from 8 to 10 mg. The
sample was encapsulated in standard aluminium pans. All tests were carried out under inert nitrogen. The samples were heated/cooled/heated at a heating rate of 20°C min⁻¹ between -50 and 200°C.

4.10.10 In-vitro Assessment-Cell culture

4.10.10.1 Preparation of chondrocytes cells line (ATDC)

In vitro cell culture studies were carried out on various samples using ATDC chondrocyte cell line in-house stock in Eastman Dental Institute at University College London. Trypsin, phosphate buffer saline without Ca²⁺/Mg²⁺ (PBS) and low-glucose Dulbecco’s modified eagle medium (DMEM) was purchased from PAA The Cell culture Company (UK). Cell culture flasks were procured from BD Biosciences (Oxford, UK). Penicillin and streptomycin was purchased from Invitrogen (Paisley, UK).

The cells were resurrected from their frozen state by thawing them and transferring them quickly to centrifuge tubes containing 10 mL of warmed DMEM (supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL of streptomycin). The cell suspension was centrifuged at room temperature for 3 min at 1000 rpm. The cell pellet was carefully re-suspended using 5 mL of DMEM and 2 mL of the re-suspended cell suspension was added in the tissue culture flasks (gas permeable; surface area of 80 cm²), followed by adding 8 mL of fresh DMEM. The flasks were incubated in a humidified atmosphere, in 5% CO₂ and at 37 °C. Spent medium was changed every 2 days and the cells were regularly observed using phase microscopy. Once the cells were approximately 80% confluent, they were passaged to maintain steady growth.

For cell passage, medium was removed from the flask and washed with 5 mL of PBS followed by adding 2-3 mL (1 mL/cm²) of trypsin/EDTA onto the washed cell monolayer and incubated at 37 °C for 2 min. The cells were examined under inverted phase microscope to observe the detachment of the cells and 8 mL (3-4x the volume

---

4 Experiment was conducted in collaboration with Dr.V. Salih (Eastman Dental Institute, UCL, London, UK)
of trypsin) of fresh DMEM was added to the flask. The cell suspension was then centrifuged for 3 min at 1000 rpm. The cell pellet was resuspended using 5 mL of fresh DMEM. After that 3 mL of the re-suspended cell suspension was further divided into three new flasks (1 mL each) and 9 mL of fresh DMEM medium was added. The cells were then further allowed to grow until the required for new experiments. The cells were not passaged for more than 3-4 times and were frozen in ampoules and stored in liquid nitrogen at -196°C for further use.

4.10.10.2 Sterilisation of samples

All the samples (10 mm diameter of pellets and 3D scaffold (5mm in thickness x 5mm in width x 5mm in length) were sterilised using ultra-violet (UV) light. The samples were kept in 24-well microplates and placed under UV for 60 min, having been turned once after 30 min. This form of sterilisation was employed in order not to affect the chemical or, indeed, physical composition of the materials had more conventional methods been employed such as autoclaving, dry-heat sterilisation or alcohol treatment. The scaffolds architecture and surface properties seems not change due to the treatment with UV light. This is also a common technique of sterilisation for tissue engineering scaffolds. However, a more thorough study on the effect of the sterilisation to the materials properties will be study for future works. Sterilisation was followed by passivating the samples by immersing the samples in 1 mL of the cell culture medium (DMEM) and incubating (37 °C, 5% CO₂) for 24 h, and then followed by cell seeding.

4.10.10.3 Cell proliferation of ATDCs in porous (3D) scaffolds and pellets

i) Cell seeding on substrates
The semi-confluent ATDC cells were released from the flasks by trypsinisation and concentrated by centrifugation (1000 rpm, 3 min). A cell count was performed using trypan blue dye and haemocytometer. A seeding density of 20,000 cells/cm² and 50,000 cells/cm³ was used for the films and scaffolds, respectively. The samples were seeded with the ATDC cells and all experiments were performed in 24 well microplates. Passivated films on pellets were seeded with 25 µl containing 20,000
cells whereas, the 3D scaffolds were seeded with 5 µL (50,000 cells) of DMEM and left for 3 h to allow for cell attachment. After this time, 1 mL of fresh DMEM medium was added on to the wells containing the scaffolds. The plates were incubated in a humidified environment (37°C, 5% CO₂) for a period of up to 14 days. The medium of the wells was changed every second day and samples were analysed at various time points to measure the cell proliferation. Cell culture studies were carried out on triplicates samples per experiment. Standard tissue culture plastic was used as the control surface.

**ii) Assessment of proliferation of ATDC**

The proliferation of ATDCs in porous scaffolds was assessed with AlamarBlue at 1, 4, 7 and 10 days post seeding with the AlamarBlue dye reduction assay. Cell numbers were assayed by adding 100 µL (10%v/v) of AlamarBlue to each well for 4 h and measuring fluorescence intensity of culture supernatant using absorption at 560nm and emission at 590 nm at the selected time point.

### 4.10.10.4 Preparation of SEM samples

Specimens from Days 1, 7 and 14 were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer for a minimum of 24h at 4 °C. Subsequent dehydration using a series of graded ethyl alcohols (50%, 70%, 90% and 100%) was performed. Samples were then critical point dried by immersion in hexamethyldisilazane for 1 min and left in a fume cupboard for 2 h. The dried samples were then attached to aluminium stubs, gold/palladium coated and examined under SEM (JEOL 5610LV, JEOL, USA) at various magnifications.

### 4.10.10.5 Statistics

The in-vitro cellular proliferation tests were performed on six samples for each material. Raw data from the study were transferred to SPSS for Windows software (version 12.0.1, SPSS, Chicago, Ill) was used for statistical analysis.

**i) Individual Components on 2D surfaces (pellets)**

A three-way analysis of variance (ANOVA) was performed to compare the mean values of cell growth between the three days, four materials and six replicates.
Because the constant variance assumption was violated in this analysis, it was repeated after taking a logarithmic transformation of the data. The assumptions of this second ANOVA were checked by a study of the residuals and were satisfied. If a main effect (either days or materials) was significant in the ANOVA, a post hoc comparison using the Bonferroni correction was employed to assess which means differed. A significance level of $p < 0.05$ was used.

**ii) Cell proliferation on 3D surfaces (bilayered scaffolds)**
A hierarchical repeated measures ANOVA was performed to compare the mean values of cell growth with six replicates nested within each of the three materials having repeated measures over the four days. Because there was a significant interaction between materials and days, no overall conclusions could be drawn about the differences between the means for either materials or days. Instead, a one-way analysis of variance was performed to compare the mean values of cell growth for each day; if statistically significant, this was followed by post hoc comparisons with the Bonferroni correction to assess which two means differed from each other. A study of the residuals indicated that the assumptions of the hierarchical repeated measures ANOVA and each of the one-way ANOVAs were satisfied. Because of multiple testing, a significance level of 0.025 was used instead of the conventional 0.05 to avoid spuriously significant conclusions.
Chapter five

5 Optimisation of scaffolds by developing composite structures

5.1 Introduction

As discussed in Chapter 2, for bone tissue engineering scaffolds must be designed such that they mimic the structure and properties of the bone extracellular matrix. The strategy followed in this project is to combine biodegradable polymers and bioactive glass-ceramics (based on Bioglass®) to fabricate composite scaffolds. The intention is to mimic the natural bone composite structure which comprises about 60 wt% hydroxyapatite (inorganic phase) the rest being organic phase (collagen) and water. It is also well known that the fracture behaviour of mineralised tissue such as bone (and dentin) is influenced by the optimal interaction of the inorganic and organic phases, and toughening mechanisms induced by the presence of collagen fibrils in bone are starting to be elucidated [178;179]. Thus, the addition of a polymer phase to a porous bioactive glass (or ceramic) scaffold is expected to enhance the fracture toughness of the composite and to allow the functionalisation of the surface to induce bioreactivity.

Some of the Bioglass®-based foams fabricated in this work are extremely weak because they are only partially densified in order to leave residual micropores which should be filled by the polymer thus leading to improvement of their mechanical properties. It is expected that the fracture toughness of the brittle foams can be improved by coating the struts with a polymer layer and achieving penetration of the polymer into the residual pores and micro-cracks of the partially sintered struts. Previous investigations on dense materials have shown improved mechanical properties in interpenetrating polymer/ceramic composites, as compared to monolithic brittle ceramics or glasses [128]. However, very few investigations have been published on polymer coated ceramic scaffolds for tissue engineering [180]. Therefore, in the framework of this thesis, specific biodegradable polymer-coated 45S5 Bioglass® scaffolds and biodegradable polymer 45S5 Bioglass® scaffolds with interpenetrating network microstructures were developed. The purpose of this investigation was to produce coatings and interpenetrating network microstructures of
45S5 Bioglass® based glass-ceramic scaffolds using a commercially available biodegradable polymer (PDLLA) and synthesised natural polymers from *Basillus. Cereus SPV* bacteria (P(3HB)) and *Pseudomonas. Mendocina* bacteria (P(3HO)). The physical and mechanical properties, morphology, microstructure, interfacial properties and bioactivity assessment of PDLLA, P(3HB) and P(3HO) coated 45S5 Bioglass® derived glass-ceramic scaffolds intended for bone tissue engineering will be evaluated and compared in this chapter. The experimental details about the materials used and detailed information on scaffolds fabrication and polymer coatings procedures have been given in chapter four.

### 5.2 Results

Two series of sintering conditions were carried out to synthesize the partially sintered 45S5 Bioglass® based glass-ceramic scaffolds, as summarised in Table.5.1

<table>
<thead>
<tr>
<th>Series number</th>
<th>Sintering Condition</th>
</tr>
</thead>
</table>
| 900-1100      | Sintering Temperature: 900, 930, 950, 970, 1000, 1050 and 1100°C;  
                Sintering Time: 2 hours |
| 1000          | Sintering Temperature: 1000°C  
                Sintering Time: 0, 0.5, 1 and 2 hours. |

#### 5.2.1 Characterisation of the porous structure of 45S5 Bioglass® based scaffold (As-sintered)

The details of the fabrication of the porous scaffolds sintered at different sintering conditions (900-1100 and 1000 series) were given in chapter 4 (section 4.2)
Figure 5.1 Graph showing the porosities of 45S5 Bioglass® based scaffolds at different sintering conditions (900-1100 series).

Figure 5.2 Graph showing the porosities of 45S5 Bioglass® based scaffolds at different sintering conditions (1000 series).
The porosities of 45S5 Bioglass® based scaffold prepared by the replication method and sintered under different conditions are given in Fig. 5.1 and Fig. 5.2. For 45S5 Bioglass® based scaffolds sintered from 900°C to 1100°C at fixed 2 hours sintering time (900-1100 series), the porosities are in the range of 80-95%, except for scaffolds sintered at 1100°C for 2 hours which have porosities in the range of 75-85% (see Fig. 5.1). The trend showing that porosity decreased with increasing sintering temperature can be generally observed for the samples in the 900-1100 series, as expected. The figure shows a large scattering of data which is a well known issue when determining the porosity of such highly porous structures fabricated by sintering of glass or ceramic powders [1]. Certainly a higher number of measurements (samples tested) would be required to draw a meaningful quantitative conclusion of statistical relevance from this data. Nevertheless the expected trend of porosity decreasing with increasing temperature can be expected for the samples in the 900-1000 series.

The porosity of the 45S5 Bioglass® based scaffolds sintered for 1000°C at different sintering times of 0.5 hrs, 1 hr and 2 hrs (1000 series) should also decrease with increasing sintering time, as it is well known from sintering by viscous flow. The porosities of the scaffolds (1000 series) were in the range of 88%-95% (see Fig. 5.2). As mention above, a much higher number of samples should has been tested to identify a clear quantitative trend between sintering condition and porosity, however the established decreasing trend of porosity with increasing sintering time seems to hold also for this scaffolds series.

There was a scatter of results at each sintering condition for both series, which indicates that the porosity of scaffolds fabricated by the foam replica method is very sensitive to the parameters of the procedure. The scatter of results might be caused by 1) initial irregular thickness of the glass coating on the polymer foams during foam replica fabrication and 2) inhomogeneous temperature field inside the furnace. Typical pore structures of the 45S5 Bioglass® based scaffolds for the 1000 series are illustrated in Fig. 5.3. The pore size ranges between 300 and 500µm.

From the SEM images in Fig. 5.3, it can be confirmed that the porosity of scaffolds at different sintering times (1000 series) did not change substantially with increasing sintering time. Summarising, highly porous scaffolds were produced for both series of
sintering conditions, which is an important criterion for the application of these structures intended in bone tissue engineering. Moreover, it was confirmed that by changing sintering time and temperature, the porosity can be controlled but to a limited extent due to the intrinsic variability of the process.

Figure 5.3 SEM images showing the pore structure of 45S5 Bioglass® based scaffolds sintered at 1000°C for A) 0.5 hours, B) 1 hour and C) 2 hours. The magnification for all images is the same (X 50).

5.2.1.1 Capillary test
The experimental details of the capillary test are given in chapter 4 (section 4.10.8)

This test using a bovine calf serum was carried out on selected scaffolds. The test showed that porous 45S5 Bioglass® scaffolds sintered at 1000°C and 1100°C for 2 hours were able to absorb the serum by capillary action in 1-2 seconds, which was
recorded by a video camera (see Fig. 4.21). Red ink was added to the serum for better observation (see movie in supplementary CD). The measurements were repeated 3 times. The uptake of the bovine serum by capillary action was indicated by the colour of the scaffolds, which changed to red during uptake of the liquid. Figure 5.4 (A-D) shows summary of the results documented by the video camera. The whole and internal parts of the scaffolds were turned to red colour due to the capillary action (see Fig. 5.4 C-D). This capillary action indicates that the micropores of the 45S5 Bioglass® scaffolds sintered at 1000°C and 1100°C for 2 hours were highly interconnected. The results of this capillary test are similar to those of Verne et al. on similar silicate based scaffolds [181].

Figure 5.4 Photographs showing the 45S5 Bioglass® derived scaffolds (sintered at 1000°C for 2 hours) before capillary test: A) whole scaffolds and B) internal section of scaffolds. Bioglass® derived scaffolds after capillary test (2 seconds immersion in bovine calf serum): C) whole scaffolds and D) internal section of scaffolds.

5.2.1.2 Permeability test
The experimental details of the permeability test are given in chapter 4 (section 4.10.7).
Permeability is a key parameter for microstructural design of scaffolds, since it is related to their capability for waste removal and nutrients/oxygen supply. Darcy’s experiments were carried out in order to determine the relationship between the pressure drop gradient and the fluid flow velocity in Bioglass®-based scaffolds to obtain the scaffold’s permeability. Figure 5.5 shows the results for the variation of $\Delta P$ with $Q$ for two Bioglass® scaffold samples sintered at 1100°C for 2 hours. Using deionised water as working fluid, the measured average permeability value on scaffolds of 90-95% porosity was $k = 1.96 \times 10^{-9}$ m$^2$. This quantitative value lies in the published range of permeability values of trabecular bone. For cancellous bovine bone [182], values between $k = 2.0 \times 10^{-9}$ and $9.5 \times 10^{-9}$ m$^2$ have been reported for porosities in the range 80-90%. In addition, values of $k = 7.22 \times 10^{-9}$ and $5.13 \times 10^{-9}$ m$^2$ have been found for human cancellous bone of vertebral body and proximal femur, respectively, although high variation from these values may be obtained dependent on the site region and overall porosity [183]. Li et al. [184] established $k = 2.13 \times 10^{-10}$ m$^2$ for porous biomaterials of 70% porosity. Thus, it can be confirmed for the first time based on the present experiments that there is quantitatively strong resemblance between the Bioglass®-based scaffold pore interconnectivity and that of trabecular bone, via measurement of permeability.

![Figure 5.5 Relationship $\Delta p[Pa]–Q$ for two Bioglass® scaffold specimens (sintered at 1100°C for 2 hours) tested in deionised water (N= two samples tested).](image-url)
5.2.1.3 Surface functionalisation of 45S5 Bioglass® scaffolds

The details of this experiment are described in chapter 4 (section 4.2.5). Scaffolds sintered at 1000°C for 2 hours were used. Alkaline phosphatase was chosen as a model enzyme to functionalise the surface of 45S5 Bioglass® scaffolds via silane group in this study because it is quite simple and relatively low cost, being of interest because it promotes bone regeneration and mineralization [185]. The results of the ALP specific enzyme activity measurement (using UV-Vis absorbance at 405 nm after reaction with p-nitrophenylphosphate) showed that the enzyme ALP was successfully bond through chemical bonding, i.e. of the COOH group of the ALP to the NH₂ group of the silane (3-aminopropyltriethoxysilane) at 37°C, as shown in Fig. 5.6. It was also confirmed that the presence of ALP on the silanised 45S5 Bioglass® scaffolds was in an active state at 37°C. The enzymatic activity, where p-nitrophenylphosphate is hydrolysed by the alkaline phosphatase to produce p-nitrophenol giving a yellow colour, is shown by equation 5.1. Fig. 5.7 shows a photograph of the yellowish coloured functionalized scaffold after reaction with p-nitrophenylphosphate indicating qualitatively ALP enzyme was successfully attached.

![Figure 5.6 ALP specific enzymatic activity on silanised 45S5 Bioglass® scaffolds (BG-SIL-37-1) as compared to non-silanised scaffolds (BG 37) at 37°C. Values are presented by mean ± standard deviation (s.d) for N = 3 samples.](image-url)
5.2.1.4 Optimisation of the binder (i.e. poly-vinyl-alcohol (PVA)) for scaffold fabrication using the foam replica method

The possibility of using a cost-effective binder, as opposed to PDLLA, for the fabrication of Bioglass® based scaffolds was investigated. The goal of this part of the study was to achieve a reduction of the cost of the fabrication without compromising the quality of the scaffolds. Two types of binders which are of lower cost than the original binder (PDLLA) have been investigated. These binders are gelatine and PVA. 15wt/v% and 20wt/v% gelatine concentrations were investigated and it was found that the mechanical properties of scaffolds (compressive strength) were lower than those of scaffolds in which PDLLA was used as a binder. In addition, 0.5wt/v% and 10 wt/v% of PVA concentrations as binder in the fabrication process were investigated. A concentration of 10 wt/v% PVA was found to be the optimal concentration, and relevant results will be presented in this section.

Figure 5.7 Photograph showing the yellowish functionalised scaffold (above) as compared to the non-functionalised scaffold (below).
Fig. 5.8 shows a typical pore structure of a 45S5 Bioglass® scaffold fabricated using a 10wt/v% PVA binder. The pore structure of the scaffold is similar to that of scaffolds fabricated using PDLLA as a binder (see Fig. 5.3).

The porosities of the scaffolds sintered at various sintering conditions; 950, 1000, 1050 and 1100°C for 2 hours using 10wt/v% PVA as binder are shown in Fig. 5.9. The porosity of the sintered scaffolds decreased as the sintering temperature increased from 950 to 1100°C, as expected. The porosity values are in the range 80-95%.
depending on the sintering conditions. The porosity range of 80-95% was similar to the porosity range of sintered scaffolds using PDLLA as binder (section 5.2.2), which is suitable for bone TE applications, as discussed in Chapter 2.

The mechanical properties (compressive strength) of the scaffolds sintered at different sintering condition and coated with 5wt/v% PDLLA, as compared to the as-sintered samples, are shown in Fig. 5.10. The values of the compressive strength of PDLLA coated scaffolds using 10 wt% PVA as binder are between 0.2 and 0.7MPa for sintering temperatures between 950 and 1100°C and sintering time of 2 hours (see Fig. 5.10). This compressive strength values are comparable to the compressive strength values of the scaffolds fabricated using PDLLA as binder (similar sintering conditions) which will be discussed in the next section.

Figure 5.10 Graphs showing the compressive strength (MPa) of the 45S5 Bioglass® scaffolds sintered at different sintering conditions (temperature-time) using a 10 wt% PVA suspension as binder. Values are presented as mean ± standard deviation (s.d) where N = 5 samples for as-sintered and N = 5 samples for PDLLA coated samples.
Figure 5.11 SEM images showing the microstructure of strut cross-section of 45S5 Bioglass® scaffolds fabricated using 10 wt% PVA suspension as a binder sintered at 1000°C for 2 hours; A) before coating and B) after coating with 5 wt% PDLLA.

The microstructure of the as-sintered scaffolds and PDLLA coated scaffolds partially sintered at 1000°C for 2 hours using 10wt% PVA solution as binder (see Fig. 5.11 A, B) also show a similarity with that of scaffolds fabricated using PDLLA as binder (same sintering condition) (discussed in the next section). Thus, in the future and for the part of the results reported in this thesis, 10 wt% PVA as an optimum concentration for the binder in the fabrication of 45S5 Bioglass® scaffolds will be considered, which represents an improvement over previous developments [1] due to economic considerations.

5.2.1.5 Effects of concentration of the PDLLA coating solution and of immersion time

Details of the PDLLA coating procedures have been given in chapter four (section 4.5). Different polymer concentrations were used to evaluate the effects of the polymer infiltration on partially sintered scaffolds. 2.5 wt% and 5 wt% PDLLA concentrations of the coating solution were used. Fig. 5.12 shows the effect of the different concentrations of the PDLLA polymer solution to coat partially sintered scaffolds. It is obvious that the polymer layer is thinner when using 2.5 wt% PDLLA (see Fig. 5.12 A) than when using 5 wt% (see Fig. 5.12 B) which proves that higher concentrations can increase the amount of polymer which is coating and infiltrating the struts. A very high concentration polymer solution (20 wt%) was also tried but it was difficult to infiltrate the micropores with it, and it was seen to block the
macropores. The problem with high concentration of polymer is that a high polymer content increases the viscosity of the solution, reducing the fluidity making thus difficult to efficiently infiltrate the micropores and macropores of the 3D scaffolds.

![Image of SEM images of 45S5 Bioglass® scaffolds sintered at 1000°C for 2 hours and immersed in PDLLA solution with different concentration; A) 2.5wt% B) 5wt%]

Figure 5.12. SEM images of the 45S5 Bioglass® scaffolds sintered at 1000°C for 2 hours and immersed in PDLLA solution with different concentration; A) 2.5wt% B) 5wt%.

Different immersion time ranges were used to investigate their effects on the quality of the polymer infiltrations and coatings. Fig. 5.13 A represents a sample which was soaked for 2 hours while the sample in Fig. 5.13 B was immersed for 24 hours. No significant difference can be observed. The reason is that after immersing the sample in the solution for a while, the pressure inside the cracks reaches a balance with the outside pressure and thus the polymer stops flowing into the crevices even with a prolonged immersion time. Therefore, for the results reported in this thesis, 5 wt% PDLLA concentration and 2 hours immersion time are adopted for the fabrication of the coated scaffolds. The same procedure will also be applied with the coating procedure using different biodegradable polymers, i.e. P(3HB) and P(3HO), reported in this thesis, in section 5.2.4 and 5.2.5, respectively.
5.2.2 Characterisation of 45S5 Bioglass® based scaffolds coated with PDLLA film

5.2.2.1 Physical properties of scaffolds of the 900-1100 Series

The results of the compressive strength and porosity of 45S5 Bioglass® based scaffolds fabricated at various sintering temperatures of 900, 930, 950, 970, 1000, 1050 and 1100°C for 2 hours (900-1100 series) after coating with 5 wt% PDLLA polymer for 2 hours are shown in Table 5.2.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Compressive strength values (MPa) and porosity (%) of PDLLA coated samples. (900-1100 series)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>900-2hrs</td>
</tr>
<tr>
<td>1.</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>88%</td>
</tr>
<tr>
<td>2.</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>93%</td>
</tr>
<tr>
<td>3.</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>94%</td>
</tr>
<tr>
<td>4.</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>87%</td>
</tr>
<tr>
<td>5.</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>89%</td>
</tr>
<tr>
<td>6.</td>
<td>0.11</td>
</tr>
<tr>
<td>Sample No.</td>
<td>Compressive strength values (MPa) and porosity (%) of as-sintered (uncoated) samples of the 900-1100 series.</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Mean values of compressive strength (MPa)</td>
</tr>
<tr>
<td></td>
<td>Mean values of porosity (%)</td>
</tr>
<tr>
<td>900-2hrs</td>
<td>930-2hrs</td>
</tr>
<tr>
<td>1.</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>89%</td>
</tr>
<tr>
<td>2.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>-</td>
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<td></td>
<td>-</td>
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<tr>
<td>4.</td>
<td>-</td>
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<tr>
<td>5.</td>
<td>-</td>
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<td>6.</td>
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<tr>
<td>Mean values of compressive strength (MPa)</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Mean values of porosity (%)</td>
<td>90% ± 3</td>
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### Mean values of porosity (%)

<table>
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<tr>
<th></th>
<th>94%</th>
<th>93%</th>
<th>93%</th>
<th>90%</th>
<th>91%</th>
<th>79%</th>
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<tr>
<td></td>
<td>± 2</td>
<td>± 1</td>
<td>± 2</td>
<td>± 2</td>
<td>± 2</td>
<td>± 8</td>
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The effect of porosity on the mechanical properties (compressive strength) of PDLLA coated 45S5 Bioglass® based scaffold of the 900-1100 series is shown in the graphs in Figure 5.14.

The graphs of compressive strength against porosity of coated scaffolds fabricated at various sintering temperatures (from 900 to 1100°C) for 2 hours showed that, in general, the compressive strength values decreased as porosity increased (see Fig. 5.14), as expected. The porosity of the PDLLA coated scaffolds sintered at 900-1000°C for 2 hours is in the range of 80-90%. However, the PDLLA coated scaffolds sintered at 1100°C for 2 hours have a lower porosity range of 74-85% which could be due to higher volume shrinkage at higher sintering temperature. Thus, the compressive strength values of the 45S5 Bioglass® based scaffolds strongly depend on porosity for scaffolds sintered at: 900, 930, 950, 970, 1000, 1050 and 1100°C for 2 hours. The average porosity reduction of the PDLLA coated samples, as compared to the as-sintered scaffolds, for the 900-1100 series was around 7% (see Table 5.2 and 5.3). The average wt% of polymer incorporated in the struts structure was around 20wt% calculated based on the difference in the weight of the scaffolds before and after drying.
Figure 5.14 Graphs showing compressive strength values (MPa) against porosity of PDLLA coated 45S5 Bioglass® based scaffolds of the 900-1100 series.
Figure 5.15 Typical microstructures of 45S5 Bioglass® scaffolds sintered at 1100°C for 2 hours: A) before coating, B) after coating, C) strut surface before coating and D) strut surface after coating with 5wt% PDLLA. The inhomogenous coating due to strut’s roughness is indicated in (D).

Typical pore structures of the 45S5 Bioglass® based scaffolds before and after coating with PDLLA for 2 hours are illustrated in Fig. 5.15 (A-B). Highly porous scaffolds were produced as shown by the porosity values in Table 5.2 and 5.3 at all sintering conditions (900-1100 series). From the SEM images in Fig. 5.15, it can be concluded that the PDLLA coating of the scaffolds does not affect significantly the overall porosity. It was observed that extensive sintering of 45S5 Bioglass® scaffolds occur at 1100°C for 2 hours, as shown in Fig. 5.15 (C), revealing the partially crystallised microstructure of the struts with grain sizes between 0.5 to 2µm. The typical morphology of the scaffold strut surfaces coated with PDLLA is shown in Fig. 5.15 (D). The polymer phase was shown not to homogenously coat the struts surface, as indicated by the darker phase (PDLLA) and the lighter phase (45S5 Bioglass®), which is evident in Fig. 5.15 (D) due to the marked surface roughness of the as–sintered
scaffolds (Fig. 5.15 C). The coatings become thinner at the hill positions (H) and thicker at the valley positions (V), as shown in Fig. 5.15 (D).

5.2.2.2 Mechanical property - microstructure correlation of the 900-1000 series scaffolds

The relationship between microstructure and mechanical properties of the 900-1000 series scaffolds is presented in this section. The results of compressive strength (MPa) of PDLLA coated 45S5 Bioglass® scaffolds sintered at 900, 930, 950, 970, 1000, 1050 and 1100°C for 2 hours, compared to the as-sintered values, are summarised in the graph in Fig. 5.16.

![Figure 5.16 Graph showing the compressive strength values (MPa) of PDLLA coated Bioglass® scaffolds of the 900-1100 series compared with the values of as-sintered samples. All values are presented as mean ± standard deviations (s.d) where N = at least 10 samples (coated samples); N = at least 5 samples (as-sintered samples).](image)

The graph shows that, in general, there was a gradual increase in the compressive strength values of the coated samples as the sintering temperature increased from 900°C to 1100°C when compared to the non-coated (as-sintered) samples, respectively. The improvement in the compressive strength values after coating with PDLLA is probably due to the PDLLA effectively infiltrating into micropores and
microcracks of the partially sintered 45S5 Bioglass® based scaffolds, as mentioned above and suggested in the literature [180]. This behaviour will be illustrated by the SEM images of the struts microstructure, as presented next, and discussed in detail below.

For comparison, the average compressive strength value of the 45S5 Bioglass® based scaffolds sintered at 1100°C for 2 hours was the highest (0.58MPa) due to the fully densified strut, e.g. absence of residual porosity in the struts which would weaken the material. The microstructure of the scaffold struts at various sintering temperatures in the range 900°C to 1100°C at fixed 2 hours sintering time (900-1100 series) is illustrated by the SEM images in Figure 5.17.

From the cross-section view of the microstructure of the struts (as-sintered), it was observed that numerous micropores are present in scaffolds sintered at the lower sintering temperatures of 900, 930 and 950°C (Fig. 5.17 A-C). The microstructure of the struts became less porous at the higher sintering temperature of 970, 1000 and 1050°C (Fig. 5.17 D-F). It was also observed that the microstructure of the struts sintered at 1100°C for 2 hours (Fig. 5.17 G) was fully dense. It should be pointed out that the hollow nature of the struts is a common feature of sintered ceramic foams synthesised by the polymer-sponge method [186].

In addition, it was also observed from the microstructure of the struts (as-sintered) that extensive sintering of 45S5 Bioglass® particles did not occur at temperatures of 900 and 930°C; sintered for 2 hours. The scaffolds sintered at 900 and 930°C were indeed very fragile to handle before the coating treatments. However, the scaffolds were stronger to handle after coating with 5wt% PDLLA. It was concluded that after being infiltrated with the 5wt% PDLLA polymer, the mechanical strength of the 45S5 Bioglass® based scaffolds sintered at 900 and 930°C for 2 hours increased significantly when compared to the as-sintered samples (see also Fig. 5.16).

Similarly, the microstructure of the struts of as-sintered uncoated scaffolds sintered at 950, 970 and 1000°C for 2 hours indicated that 45S5 Bioglass® particles were partially sintered, as shown in Figure 5.17 A-G. It was also observed on the SEM images that the number of micropores in the struts decreased as the temperature
increased from 950 to 1000°C. It was concluded that the struts became significantly denser as the sintering temperature increased from 950°C to 1000°C. Moreover, after coating with 5wt% PDLLA; the compressive strength of the scaffolds sintered at 950, 970 and 1000°C for 2 hours gradually increased due to the infiltration of the polymer into the micropores of the struts of the scaffolds (see Fig. 5.16).

For comparison, the microstructure of the struts sintered at 1050 and 1100°C for 2 hours showed a fully densified structure (see Fig. 5.17 F-G). A fully sintered and crystallised strut will lead to increased mechanical properties of the scaffolds but it could possibly also decrease the bioactivity of the 45S5 Bioglass®, as discussed in the literature [51].

In conclusion, there is, as expected, a direct relationship between the microstructure of the struts and the mechanical properties (compressive strength) of the 45S5 Bioglass® based scaffolds for the 900-1100 series both in the as-sintered and coated scaffolds.
Figure 5.17 SEM images showing the microstructure of Bioglass®-based scaffold struts cross sections before and after coating with 5wt% PDLLA for the following sintering conditions: before coating: A) 900°C, B) 930°C C) 950°C, D) 970°C, E) 1000°C, F) 1050°C and G) 1100°C, sintered for 2 hours; after coating: A1) 900°C B1) 930°C, C1) 950°C, D1) 970°C, E1) 1000°C, F1) 1050°C and G1) 1100°C, sintered for 2 hours.
Figure 5.17 Cont.
Figure 5.17 Cont.

5.2.2.3 Physical analysis of the 1000 series scaffolds

The effect of sintering time at fixed sintering temperature of 1000°C on the compressive strength values of partially sintered 45S5 Bioglass® scaffolds was also investigated. The results of the compressive strength and porosity measurements of the PDLLA coated 45S5 Bioglass® based scaffolds sintered at 1000°C for 0.5, 1 and 2 hours (1000 series) are shown in Table 5.4.
Table 5.4 Compression strength values (MPa) and porosity (%) of PDLLA coated 45S5 Bioglass® based scaffolds of the 1000 series.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Compressive strength values (MPa) and porosity (%) of PDLLA coated samples (1000 series)</th>
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<tbody>
<tr>
<td></td>
<td>1000-2hrs</td>
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<tr>
<td>1.</td>
<td>MPa</td>
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<tr>
<td></td>
<td>Porosity</td>
</tr>
<tr>
<td>2.</td>
<td>MPa</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
</tr>
<tr>
<td>3.</td>
<td>MPa</td>
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<td></td>
<td>Porosity</td>
</tr>
<tr>
<td>4.</td>
<td>MPa</td>
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<tr>
<td></td>
<td>Porosity</td>
</tr>
<tr>
<td>5.</td>
<td>MPa</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
</tr>
<tr>
<td>6.</td>
<td>MPa</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
</tr>
<tr>
<td>7.</td>
<td>MPa</td>
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<tr>
<td></td>
<td>Porosity</td>
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<tr>
<td>8.</td>
<td>MPa</td>
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<tr>
<td></td>
<td>Porosity</td>
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<tr>
<td>9.</td>
<td>MPa</td>
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<tr>
<td></td>
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<tr>
<td>10.</td>
<td>MPa</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>11.</td>
<td>MPa</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
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Mean values of compressive strength (MPa) and porosity (%)

| MPa         | 0.34 ± 0.08 | 0.46 ± 0.24 | 0.35 ± 0.14 |
| Porosity    | 85% ± 3     | 84% ± 2     | 85% ± 2     |

Table 5.5 Compressive strength values (MPa) and porosity of as-sintered samples of the 1000 series

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Compressive strength values (MPa) and porosity (%) of as-sintered (uncoated) samples (1000 series)</th>
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<tbody>
<tr>
<td></td>
<td>1000-2hrs</td>
</tr>
<tr>
<td>1.</td>
<td>MPa</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
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<tr>
<td>2.</td>
<td>MPa</td>
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</table>

116
<table>
<thead>
<tr>
<th>Porosity</th>
<th>90%</th>
<th>92%</th>
<th>94%</th>
</tr>
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<tbody>
<tr>
<td>MPa</td>
<td>0.12</td>
<td>0.10</td>
<td>0.06</td>
</tr>
<tr>
<td>Porosity</td>
<td>90%</td>
<td>93%</td>
<td>94%</td>
</tr>
<tr>
<td>MPa</td>
<td>0.09</td>
<td>0.18</td>
<td>0.05</td>
</tr>
<tr>
<td>Porosity</td>
<td>92%</td>
<td>79%</td>
<td>95%</td>
</tr>
<tr>
<td>MPa</td>
<td>0.09</td>
<td>0.10</td>
<td>0.05</td>
</tr>
<tr>
<td>Porosity</td>
<td>88%</td>
<td>87%</td>
<td>94%</td>
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Mean values of compressive strength (MPa) and porosity (%)

<table>
<thead>
<tr>
<th>MPa</th>
<th>0.11 ± 0.03</th>
<th>0.11± 0.06</th>
<th>0.05 ± 0.01</th>
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<tr>
<td>Porosity</td>
<td>90% ± 1</td>
<td>89% ± 6</td>
<td>94% ± 4</td>
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</table>

The effect of porosity on the compressive strength of PDLLA coated scaffolds sintered at 1000°C for 2, 1 and 0.5 hours is shown in Fig. 5.18 (A-C).

**Figure 5.18** Graphs showing the relationship between compressive strength values and porosity of PDLLA coated scaffolds at different sintering conditions: A) 1000°C-0.5 hrs, B) 1000°C-1hrs and C) 1000°C-2hrs.

Fig. 5.18 shows the relationship between compressive strength values and porosity values of PDLLA coated scaffolds at different sintering condition (1000 series). The
porosity values for all scaffolds sintered at 1000°C fall in the range 80-90% (see Table 5.4 and 5.5). The graphs generally show the direct correlation between compressive strength values and porosity. It is very important to be able to control the porosity of the scaffolds in order to achieve smaller standard deviations, however, in the foam replica method being investigated here, it is very difficult to completely control the processing parameters, as mentioned earlier, and a certain scatter of porosity values is always expected [187].

### 5.2.2.4 Mechanical property-microstructure correlation in 1000 series scaffolds

The results of the compressive strength values of the 45S5 Bioglass® based scaffolds sintered at 1000°C at different sintering times (1000 series) is summarised by the bar graph in Figure 5.19.

![Bar graph showing compressive strength values of scaffolds of the 1000 series before and after PDLLA coating. All values are presented as mean ± standard deviations (s.d) where N = 10 samples (Coated samples); N = 5 samples (As-sintered samples).](image)

**Figure 5.19** Graph showing compressive strength values of scaffolds of the 1000 series before and after PDLLA coating. All values are presented as mean ± standard deviations (s.d) where N = 10 samples (Coated samples); N = 5 samples (As-sintered samples).
The compressive strength values of the samples coated with 5wt% PDLLA showed a significant improvement when compared to the non-coated (as-sintered) samples. In general, the compressive strength values increased as the sintering time decreased (see Figure 5.19). The increased in compressive strength values as the sintering time decreased indicates that higher amounts of polymer can be infiltrated into the partially sintered struts because there are more micropores and crevices formed in the partially sintered struts at the lower sintering time compared to the higher densification achieved for higher sintering time, which was confirmed by SEM investigations as discussed next.

SEM images of the microstructure of the scaffold struts, before and after coating, are illustrated in Figure 5.20. The cross-section images of the struts microstructure before and after coating show that the number of micropores increased as sintering time decreased. Therefore, as sintering time decreases, more polymer can infiltrate the micropores of the 45S5Bioglass® scaffold, which will lead to improved compressive strength values (see Fig. 5.19). SEM observations of the strut walls microstructure for all samples (see Fig. 5.21) indicate that the 45S5Bioglass® particles were partially sintered, suggesting that the glass particles were not completely softened to form a smooth surface by viscous flow. The PDLLA layer (darker phase) seems to infiltrate through the micropores of the partially sintered scaffolds which is indicated by the lighter phases, as shown by the arrow in Fig. 5.21.

In conclusion, there was a clear qualitative correlation between the microstructure of the 45S5 Bioglass® scaffolds sintered at 1000°C for 2, 1 and 0.5 hours and the scaffolds compressive strength values.
Figure 5.20 SEM images showing the microstructure of scaffold strut cross sections before and after coating with PDLLA at different sintering conditions of scaffolds of the 1000 series. Before coating: A) 0.5 hr, B) 1 hrs and C) 2 hrs; after coating: A1) 0.5 hr, B1) 1 hr and C1) 2 hrs.
Figure 5.21 SEM images showing the surface morphology of the 45S5 Bioglass® scaffold struts sintered at different sintering times before and after coating with 5wt% PDLLA. Before coating: A) 0.5 hr, B) 1 hr and C) 2 hrs; after coating: A1) 0.5 hr, B1) 1 hr and C1) 2 hrs. The arrow indicates infiltration of PDLLA into the micropores of the partially sintered scaffold.
5.2.2.5 Mechanical properties of PDLLA coated 45S5 Bioglass® scaffolds

Figure 5.22 Schematic diagram showing different stages (regimes) of the stress-strain curve for polymer coated scaffolds.

Figure 5.23 Typical compressive stress-strain curves of A) an as-sintered Bioglass® scaffold and B) a poly(D,L-Lactic acid) coated scaffold. Series 1000 scaffolds sintered at 2 hours.
A schematic diagram showing the expected compressive stress-strain curve of a coated scaffold is shown in Figure 5.22 while Figure 5.23 shows actual stress-strain curves for coated and uncoated scaffolds. The typical jagged curves show four stages with increasing strain: 1) stress increase (uncoated and coated scaffold), 2) stress decrease (uncoated and coated scaffold), 3) plateau (coated scaffold), and 4) densification stage (coated scaffolds), are shown in Figure 5.23. The first stage represents a linear elastic behaviour followed by a drop of the stress loading, which leads to a plateau stage during which the stress remains roughly constant while the strain reaches several milimeters depending on the infiltration method, before the densification process occurs for coated scaffolds. During loading, with increasing stress, the scaffolds are compressed layer by layer. Starting from about 50% compression strain, densification of the foams occurs, which is a typical behaviour of highly porous scaffolds [1].

The compressive stress-strain curve of the coated scaffolds was less jagged, compared with the curve of as-sintered Bioglass® scaffolds. The reduction in jagging could indicate that coated scaffolds had fewer microcracks on the surface of the struts. As mentioned above, the improvement in mechanical stability is thought to be due to the PDLLA coating infiltrating the micropores and microcracks on the strut surfaces. Fig. 5.24 shows photographs of the scaffolds after compressive strength test. It was confirmed PDLLA coated scaffolds can maintain their structure (shape) after failure.
while as-sintered scaffolds were broken into particulates, which qualitatively confirms
the increased in fracture toughness of the PDLLA coated scaffold.

The improvements in compressive strength and modulus with PDLLA coating were
significant and the area under the stress-strain curve, which represents the fracture
energy of the material, also increased with the coated samples. The increased of
fracture toughness is possibly due to the local fracture of the scaffold struts being held
together by the polymer layers which have penetrated the microcracks. As crack
opening progresses, the polymer layers stretch leading to the formation of fibrils
which can bridge the crack. The fibrils possibly appeared and developed along with
the crack opening displacement until this reaches a critical value where the polymer
fibrils will progressively deform (elongate) until their fracture. Thus, the presence of
the polymer and its deformation makes the material to absorb extra energy before
complete fracture and this phenomenon is usually more significant under tensile
loading. This effect is in fact similar to the toughening mechanisms in bone based on
crack bridging by collagen fibrils, which has been described in the literature [179].
The composite scaffold struts, representing thus an interpenetrating microstructure of
PDLLA and 45S5 Bioglass®, exhibits a similar mechanical behaviour to bone. Figure
5.25 shows the microstructures of the foam struts after fracture in which crack
bridging (A) and pull-out (C, D) of the polymeric fibrils can be observed and
compared with the fracture of bone, which exhibits toughening mechanisms based on
crack bridging induced by the collagen fibrils (B).
Figure 5.25 SEM images of the PDLLA coated 45S5 Bioglass® based scaffolds after fracture: (A) polymer bridging between the crack surfaces, (B) collagen fibrils bridging crack in bone [179], (C) polymer fibres pulled out at the fracture surface (D) partially magnified image of (C).

5.2.3 Characterisation of 45S5 Bioglass® sintered pellets coated with PDLLA film

5.2.3.1 Interface properties

This part of the investigation on 45S5 Bioglass® pellets coated with PDLLA film was carried out to characterise the interface between the polymeric layer and the sintered 45S5 Bioglass® surface. This interface is easier to characterise on planar surfaces than on the coated 3D scaffolds. Optical images of the coated 45S5 Bioglass® pellets indicate the presence of a very thin PDLLA film deposited on the pellets surface. The thickness of the polymer film coating was determined to be around 10 micron (see Fig. 5.26).
Figure 5.26 Optical image of PDLLA coating on a 45S5 Bioglass® pellet sintered at 1000°C for 2 hours.

Figure 5.27 SEM image showing crack propagation at the interface between the PDLLA coating and the Bioglass® substrate. The crack was formed by microindentation at a load of 200g applied at a 50µm distance from the interface, on the sintered Bioglass® substrate.

The adhesion strength of the interface between the PDLLA coating and the 45S5 Bioglass® substrate was determined by applying a load of 200g at a 50µm distance from the interface layer using a micro-indenter. Under these testing conditions, a microcrack was created which propagated in a controlled manner towards the interface. Detachment of the PDLLA layer at the interface, at a ~45° angle between the direction of the propagating microcrack and the interface, was observed to occur, as indicated by the arrow in Fig. 5.27. This detachment possibly indicates that the PDLLA polymer adhesion strength at the interface is not very strong which could also possibly explain the formation of bridging fibrils at the surface, as mentioned earlier (Fig. 5.25 A). The present assessment of the Bioglass®/PDLLA interface is only
qualitative. Nevertheless it provides information on the ability of the polymer to partially detach from the Bioglass® surface and thus to interpret the toughening mechanisms identified during scaffold fracture discussed above.

5.2.3.2 Bioactivity assessment of coated 45S5 Bioglass® pellets and scaffolds

i) Bioactivity analysis in SBF (45S5 Bioglass® pellets)

45S5 Bioglass® pellets were used for the quantitative assessment of the bioactivity of PDLLA coated 45S5 Bioglass® composites at different SBF concentrations assuming that the PDLLA coatings was homogenous on the planar surfaces. First of all, the bioactivity of the 45S5 Bioglass® pellets partially sintered at 1000°C for 2 hours and coated with 5 wt% PDLLA for 2 hours was investigated in SBF solution for 7, 14 and 28 days immersion period.

SEM observations and EDS analysis were carried out on the surface of the Bioglass® pellets after immersion times of 7, 14 and 28 days in SBF. SEM images of the selected Bioglass® pellets after immersion in SBF are shown in Fig. 5.28. The images showed a different morphology of the surface layer for 7, 14 and 28 days of immersion in SBF. The formation of HA-like cauliflower clusters on the surface of the coated Bioglass® pellets was clearly observed by SEM after 28 days in SBF (Fig. 5.28 C-D). The broken area (crack) of the coating, as shown in Fig. 5.28 B, should provide a channel for SBF to flow into and come in direct contact with the bioactive glass-ceramic substrate. With increasing immersion time in SBF, a large surface area of the bioactive glass is being exposed to SBF, and thus the HA-like phase will eventually covered the whole surface of the substrate which is indicated by the formation of the cauliflower like HA clusters, as shown in Fig. 5.28 (C). At higher magnification the HA crystals show the typical needle like structure, as shown in Fig 5.28 (D) [188].
Figure 5.28 SEM images of surfaces of PDLLA coated pellets sintered at 1000°C for 2 hours and immersed in SBF for: A) 7, B) 14 and C, D) 28 days, showing formation of hydroxyapatite (HA) layers on the surface of the pellets at different magnifications.

EDS analyses were done to confirm the elements present on the surface of the coated pellets after immersion in SBF. For EDS analysis, 3 measurements were taken and typical EDS spectra for each immersion time in SBF are shown in Fig. 5.29. Cr peaks in the spectrum were due to the coatings used to prepare the specimens for SEM.

From the EDS analysis, the presence of phosphorus (P) and calcium (Ca) peaks at around 2 keV and 3.8keV, respectively, was confirmed at 14 and 28 days. Although the analysis is not quantitative, it was determined that the relative intensity of the Ca and P peaks increased as the immersion time in SBF increased from 7 to 28 days. A high level of carbon was contributed by PDLLA on day 7, and this level decreased as the immersion time increased indicating that the PDLLA coating is being covered by the HA layer. The EDS analysis of the as-sintered (non-coated) 45S5Bioglass® pellets immersed in SBF for 28 days also showed the presence Ca and P peaks.
Thus, it can be assumed that the bioactivity of the PDLLA coated samples after 28 days immersion in SBF in terms of HA formation is similar to that of the non-coated samples, indicating that PDLLA did not negatively affect the bioactivity of the 45S5 Bioglass® substrates. This result is relevant considering the application of PDLLA coated Bioglass® based scaffolds in bone tissue engineering.
XRD analysis was done to confirm the formation of crystalline hydroxyapatite on the surface of the PDLLA coated pellets sintered at 1000°C for 2 hours after immersion in SBF for 7, 14 and 28 days. Results are shown in Figure 5.30. There was an increased in the height of peaks at $2\theta = 32^\circ$ and $26^\circ$, which correspond to HA peaks (commercial HA powder), when the immersion time in SBF increased from 7 to 28 days, as shown in Fig. 5.30.

![XRD spectra of PDLLA coated 45S5 Bioglass® pellets sintered at 1000°C for 2 hours and immersed in SBF for 0, 7, 14 and 28 days, compared to the spectrum of commercial crystalline HA. The major peaks of Na$_2$Ca$_2$Si$_3$O$_9$ phase and hydroxyapatite are marked by (Δ) and (●) respectively.](image)

**Figure 5.30** XRD spectra of PDLLA coated 45S5 Bioglass® pellets sintered at 1000°C for 2 hours and immersed in SBF for 0, 7, 14 and 28 days, compared to the spectrum of commercial crystalline HA. The major peaks of Na$_2$Ca$_2$Si$_3$O$_9$ phase and hydroxyapatite are marked by (Δ) and (●) respectively.

**ii) Bioactivity analysis in 1.5 SBF (PDLLA coated 45S5 Bioglass® pellets)**

The bioactivity of selected 45S5 Bioglass® pellets coated with PDLLA was investigated in 1.5 SBF for 7, 14 and 28 days immersion period to determine the formations of a surface HA layer in comparison with the results in normal SBF. SEM and EDS analyses were carried out on the surface of the PDLLA coated Bioglass® pellets sintered at 1000°C for 2 hours. SEM images of the selected Bioglass® pellets after immersion in 1.5 SBF for 7, 14 and 28 days are shown in Figure 5.31.
SEM images showed a different surface morphology for 7, 14 and 28 days of immersion in 1.5 SBF (see Fig. 5.31). The formation of HA-like crystalline apatite on the surface of the coated 45S5 Bioglass® was clearly observed by SEM after immersion in 1.5 SBF for 14 days and longer time. At 28 days immersion time, the presence of a thick layer of calcium phosphate on the substrates was observed (Fig. 5.31 C-D).

EDS analyses were carried out to confirm the elements present on the surface of the PDLLA coated 45S5 Bioglass® pellets. For EDS analysis, 3 measurements were taken and typical EDS spectra for each immersion time in 1.5SBF are shown in Figure 5.32. Au and Cr peaks in the spectrum were caused by the gold and chromium coating, respectively, used for the SEM-EDS sample preparation.
Figure 5.32 EDS spectra of 45S5 Bioglass® pellets sintered at 1000°C for 2 hours (PDLLA-coated) after immersion in 1.5SBF for A) 7, B) 14 and C) 28 days compared to a non-coated sample (D) and to an as-sintered sample (E).

From the EDS analysis, the presence of phosphorus (P) and calcium (Ca) peaks at around 2 keV and 3.8keV, respectively, was confirmed at 28 days. Although the analysis is not quantitative, it can be observed that the intensity of the Ca and P peaks increases as the immersion time in SBF increases from 7 to 28 days. A high level of carbon was contributed by PDLLA on day 7, and the level decreased as the
immersion time in 1.5 SBF increased. The EDS analysis of the as-sintered (non-coated) 45S5Bioglass® pellets immersed in SBF for 28 days also showed the Ca and P peaks. Thus it could be assumed that the bioactivity of the coated samples after 28 days immersion in SBF in terms of CaP formation is similar to that of the non-coated samples, indicating that the PDLLA polymer did not negatively affect the bioactivity of the 45S5 Bioglass® substrate when immersed in 1.5 SBF.

Figure 5.33 X-ray diffraction spectra of PDLLA coated 45S5 Bioglass® pellets sintered at 1000°C for 1, 0.5 and 0 hrs after immersion for 14 and 28 days in 1.5 SBF. The major peaks of Na$_2$Ca$_2$Si$_3$O$_9$ phase and hydroxyapatite are marked by (△) and (●) respectively.

XRD analyses of the PDLLA coated 45S5 Bioglass® specimens sintered at different sintering times; 0, 0.5, and 1hr, at 1000°C (1000 series) after immersion in 1.5 SBF for 14 and 28 days were carried out to investigate the kinetic of HA formation, as shown in Fig. 5.33. It was observed that as the sintering time decreased, the two major peaks of Na$_2$Ca$_2$Si$_3$O$_9$, at 2(0) = 34° and 35° decreased from 14 to 28 days and from sintering time of 1 to 0 hour. The presence of Na$_2$Ca$_2$Si$_3$O$_9$ is typical in bioactive glass-ceramics fabricated from Bioglass® by sintering at T>900 °C [53]. Thus, it can be assumed that the kinetic of apatite growth is faster as the sintering time is decreased from 1 to 0 hours. In addition, when comparing the HA formation on coated pellets sintered at 1000°C for 2 hours and immersed in 1Molar SBF solution
for 28 days, there was a clear increased of heights of peaks at $2\theta = 32^\circ$, which corresponds to HA peaks [53]. Thus it can be assumed that in PDLLA coated 45S5 Bioglass®-based scaffolds, the bioactivity, e.g. formation of the HA surface layer, will be delayed, as shown by XRD spectra in Fig. 5.33, but not suppressed.

Figure 5.34 X-ray diffraction spectra of PDLLA coated 45S5 Bioglass® pellets sintered at 1000°C for 0 hrs after immersion in 1.5 SBF for 0, 7, 14, 28 and 60 days as compared to the spectrum for commercial HA. The major peaks of $\text{Na}_2\text{Ca}_2\text{Si}_3\text{O}_9$ phase and hydroxyapatite are marked by (∆) and (●) respectively.

XRD analyses were done on PDLLA coated 45S5 Bioglass® pellets sintered at 1000°C for 0 hours and after 0, 7, 14, 28 and 60 days immersion in 1.5 SBF (see Fig. 5.34) in order to evaluate bioactivity at a longer period of time. There was an absence of peaks at $2(\theta) = 32^\circ$ corresponding to typical crystalline HA peaks (compared to commercial HA powder) from 7 to 14 days immersion in 1.5 SBF. However, there was an increased of peaks at $2(\theta) = 29^\circ$ corresponding to $\text{CaCO}_3$ and a decreased of the two major peaks of $\text{Na}_2\text{Ca}_2\text{Si}_3\text{O}_9$ at $2(\theta) = 34^\circ$ and $35^\circ$ from 0 to 60 days immersion time in 1.5 SBF. These peaks eventually disappear, leaving a typical halo (produced by an amorphous phase) overlapped by the sharp diffraction peaks of the HA phase. When immersion time in 1.5 SBF was increased to 28 days, there was an increase of the sharp peak at $2(\theta) = 32^\circ$, which corresponds to the HA phase. At a longer immersion period of 60 days, there was indication of an increase of the amorphous phase, overlapped with sharp diffraction peaks of HA phase. The
formation of HA layer observed after 60 days could indicate formation of secondary crystalline apatite layer on the surface of an amorphous calcium phosphate when immersed in 1.5 SBF in comparison to the formation of primary apatite layer when immersed in the normal SBF solution for the same immersion time. Therefore, the kinetic of HA formation is faster when immersed in 1.5 SBF as compared to SBF, also on PDLLA coated specimen, which is thus confirmed for the first time in the present study.

iii) Bioactivity analysis of 45S5 Bioglass® composite scaffolds in SBF

Fig. 5.35 shows SEM images of the 45S5 Bioglass® scaffolds sintered at 1000°C for 2 hours and coated with 5 wt% PDLLA after immersion in SBF for 7, 14 and 28 days. There was a clear formation of HA crystals on the surface of the scaffolds after 7 days immersion in SBF indicated by the cauliflower-like apatite clusters formed on the surface of the composite scaffolds (as shown in Figure 5.35 A), almost covering the entire 3D scaffolds structure. The formation of the HA clusters on the composite scaffolds surfaces increased with immersion time in SBF increasing from 7 to 28 days, as expected. At higher magnification, a needle-like crystalline morphology of HA was observed after 14 days immersion in SBF (see Fig. 5.35 B1-C1).

The results obtained by SEM were confirmed by EDS analysis as shown in Fig. 5.36. The morphology of the scaffold surfaces also indicates qualitatively that surface roughness has increased due to the HA layer formation from 7 to 28 days immersion in SBF (see Fig. 5.36 A-C).

EDS spectra of the scaffolds’ surface at different immersion times of 7, 14 and 28 days were obtained to confirm the formation of HA, which was indicated by the P and Ca peaks at 2keV and 3.8 keV, respectively (see Fig. 5.36 A1 to C1). The Ca/P ratio gradually increased, accompanied by a decreased of the Si, Na, C and O peaks with increasing immersion time. The Ca/P ratio was determined to be 1.67 at 28 days immersion time.
Figure 5.35 SEM images of PDLLA coated scaffolds sintered at 1000°C for 2 hours after immersion in SBF at A), A1) 7 days, B), B1) 14 days and C), C1) 28 days, at different magnifications showing formation of HA.
Figure 5.36 SEM-EDS results for PDLLA coated scaffolds sintered at 1000°C for 2 hours and immersed in SBF: A, A-1) 7 days, B, B-1) 14 days and C, C-1) 28 days, showing formation of hydroxyapatite layers on the surface of the scaffolds.
Figure 5.37 XRD spectra of the PDLLA coated 45S5 Bioglass® scaffolds sintered at 1000°C for 2 hours and immersed in SBF for 7, 14 and 28 days. The peaks of hydroxyapatite phase are marked (●) (The peaks of Na$_2$Ca$_2$Si$_3$O$_9$ are not visible).

Figure 5.38 XRD spectra of the as-sintered 45S5 Bioglass® scaffolds sintered at 1000°C for 2 hours and immersed in SBF for 7, 14 and 28 days. The major peaks of Na$_2$Ca$_2$Si$_3$O$_9$ phase and hydroxyapatite are marked by (Δ) and (●), respectively.
Figure 5.39 XRD spectra of the PDLLA coated 45S5 Bioglass\textsuperscript{®} scaffolds sintered at 1100\textdegree C for 2 hours and immersed in SBF for 7, 14 and 28 days. The major peaks of Na\textsubscript{2}Ca\textsubscript{2}Si\textsubscript{3}O\textsubscript{9} phase and hydroxyapatite are marked by (Δ) and (●), respectively.

Figure 5.40 XRD spectra of the as-sintered 45S5 Bioglass\textsuperscript{®} scaffolds sintered at 1100\textdegree C for 2 hours and immersed in SBF for 7, 14 and 28 days. The major peaks of Na\textsubscript{2}Ca\textsubscript{2}Si\textsubscript{3}O\textsubscript{9} phase and hydroxyapatite are marked by (Δ) and (●), respectively.
XRD analysis was done to confirm the formation of HA and to compare the kinetic of crystalline hydroxyapatite (HA) formation on the surface of the PDLLA coated scaffolds sintered at 1000°C and 1100°C for 2 hours after immersion in SBF for 7, 14 and 28 days. There was an increased in the peaks at $2\theta = 32^\circ$, which corresponds to HA peaks when the immersion time in SBF increased from 7 to 28 days for coated scaffolds sintered at 1000°C and 1100°C for 2 hours, as shown in Fig. 5.37 and Fig. 5.39 respectively. However, peaks corresponding to Na$_2$Ca$_2$Si$_3$O$_9$ at $2(\theta) = 34^\circ$ and $35^\circ$ are present in the spectra of the scaffolds sintered at 1100°C for 2 hours after 7 days in SBF (see Fig. 5.39). Comparison of Fig. 5.37 with Fig. 5.39 reveals that the kinetics of HA formation was faster in the coated scaffolds sintered at 1000°C for 2 hours than in those sintered at 1100°C for 2 hours after 1 week of immersion in SBF.

A comparison was also done using the non-coated scaffolds (as-sintered). Comparison of Fig. 5.37 with Fig 5.38 reveals that the kinetics of HA formation at day 7 was faster in the PDLLA coated scaffolds than in the non-coated scaffolds sintered at 1000°C for 2 hours. A similar trend was also followed for coated scaffolds sintered at 1100°C for 2 hours at day 7 and day 14. Summarising, XRD analysis shows that coated and uncoated scaffolds sintered at 1000°C for 2 hours exhibited HA formation after 2 weeks in SBF. However, the coated and uncoated scaffolds sintered at 1100°C for 2 hours exhibited HA formation only after 4 weeks in SBF. These results therefore confirm that PDLLA coated 45S5 Bioglass® based glass-ceramic scaffolds sintered at lower sintering temperature have a higher bioactivity when compared to the scaffolds sintered at higher sintering temperature of 1100°C for 2 hours. This finding is similar to previous studies [1]. In addition, coated scaffold also showed an improvement in the bioactivity as compared to the non-coated scaffolds. However, this finding is in contrast to the results of previous investigations [1]. The previous result may be due to the fact that PDLLA was used to coat the outer layer of the dense struts in the fabrication of scaffolds and the bulk degradation of PDLLA film is different from the degradation rate of PDLLA in a composite. Hence, it is difficult to directly compare the present results with previous studies since this is the first time that PDLLA/Bioglass® composites with interpenetrating network microstructure has being investigated. The results of this study could specifically be due to the interpenetrating network of PDLLA/Bioglass® scaffolds. A previous study by Okuno et al. has shown that the degradation rate of PLLA in a composite is faster than bulk PLLA. [189]
Thus it can be concluded from the SEM, EDS and XRD analyses, that PDLLA coatings do not impair the bioactivity of the scaffolds sintered at 1000°C for 2 hours and furthermore the PDLLA incorporated in the interpenetrating microstructure improves the kinetic of HA formation.

5.2.4 Characterisation of 45S5 Bioglass® scaffolds coated with P(3HB) films

5.2.4.1 Characterisation of synthesised P(3HB) from bacteria fermentation

Production of P(3HB) was carried out using bacteria Bacillus Cereus SPV strain [190]. The bacteria synthesise and accumulate the P(3HB) as inclusion bodies within the bacteria cell cytoplasm under nitrogen limiting conditions in the presence of excess carbon source. The synthesised polymer was characterised by GC-MS (Gas chromatography-mass spectroscopy) in order to determine the chemical structure of the components for each retention time. The chromatogram of methanolysed P(3HB) produced in this investigation gave a peak at retention times of 6.81 mins (see Fig. 5.41). The mass spectra of the compound gave m/z=117 corresponding to methyl ester of 3HB (Mw=118).

From the GC (Figure 5.41) and MS analysis (Figure 5.42) it can be confirmed that the polymer synthesised is mostly poly(3-hydroxybutyrate) P(3HB) using the library match search (Table 5.6). Table 5.6 shows the compounds identified for each retention time by the GC-MS analysis for the synthesised methanolysed P(3HB). When compared to results reported in the literature the present values showed the presence of 3HB monomer in the isolated polymer [190].
Figure 5.41. Chromatogram of the methanolysed P(3HB) produced in this investigation showing the peaks of each component separated at different retention times.

Figure 5.42 MS spectra of the methanolysed P(3HB) with a retention time of 6.81 min.
Table 5.6 Compounds in P(3HB) identified for each retention time (GC-MS measurement).

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Compound from GC-MS library match</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.76-7.0</td>
<td>Butyric acid, 3-hydroxy-, Methyl ester</td>
</tr>
<tr>
<td>9.44-9.54</td>
<td>Benzoic acid, methyl ester</td>
</tr>
<tr>
<td>11.27-11.33</td>
<td>Butyric acid, 4-methoxy-, methyl ester</td>
</tr>
<tr>
<td>13.58-13.63</td>
<td>5-Methyl-isoxazolidin-3-one</td>
</tr>
</tbody>
</table>

5.2.4.2 Physical analysis of scaffolds of the 900-1100 Series

The results of the compressive strength and porosity of 45S5 Bioglass® based scaffolds fabricated at various sintering temperatures of 930, 950, 970, 1050 and 1100°C for 2 hours (900-1100 series) after coating with 5 wt% P(3HB) polymer for 2 hours are shown in Table 5.7.

Table 5.7 Compression strength values (MPa) and porosity (%) of P(3HB) coated 45S5 Bioglass® based scaffolds of the 900-1100 series.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Compressive strength values (MPa) and porosity (%) of 5 wt% P(3HB) coated scaffolds (900-1100 series)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>930-2hrs</td>
</tr>
<tr>
<td>1.</td>
<td>MPa</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
</tr>
<tr>
<td>2.</td>
<td>MPa</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
</tr>
<tr>
<td>3.</td>
<td>MPa</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
</tr>
<tr>
<td>4.</td>
<td>MPa</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
</tr>
<tr>
<td>5.</td>
<td>MPa</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
</tr>
</tbody>
</table>

Mean values of compressive strength (MPa) and porosity (%)

<table>
<thead>
<tr>
<th>MPa</th>
<th>0.14</th>
<th>0.18</th>
<th>0.30</th>
<th>0.31</th>
<th>0.31</th>
<th>0.64</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>± 0.05</td>
<td>± 0.05</td>
<td>± 0.12</td>
<td>± 0.5</td>
<td>± 0.05</td>
<td>± 0.29</td>
</tr>
<tr>
<td>Porosity</td>
<td>89%</td>
<td>89%</td>
<td>89%</td>
<td>89%</td>
<td>88%</td>
<td>82%</td>
</tr>
<tr>
<td></td>
<td>± 9</td>
<td>± 2</td>
<td>± 2</td>
<td>± 1</td>
<td>± 1</td>
<td>± 9</td>
</tr>
</tbody>
</table>
Table 5.8 Compressive strength values (MPa) and porosity (%) of as-sintered (uncoated) samples of the 900-1100 series.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Compressive strength values (MPa) and porosity (%) of as-sintered (uncoated) scaffolds (900-1100 series)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>930-2hrs</td>
</tr>
<tr>
<td>1.</td>
<td>MPa</td>
</tr>
<tr>
<td>Porosity</td>
<td>96%</td>
</tr>
<tr>
<td>2.</td>
<td>MPa</td>
</tr>
<tr>
<td>Porosity</td>
<td>93%</td>
</tr>
<tr>
<td>3.</td>
<td>MPa</td>
</tr>
<tr>
<td>Porosity</td>
<td>94%</td>
</tr>
<tr>
<td>4.</td>
<td>MPa</td>
</tr>
<tr>
<td>Porosity</td>
<td>93%</td>
</tr>
<tr>
<td>5.</td>
<td>MPa</td>
</tr>
<tr>
<td>Porosity</td>
<td>94%</td>
</tr>
</tbody>
</table>

| Mean values of compressive strength (MPa) and porosity (%) |
| MPa     | 0.04 ± 0.01 | 0.05 ± 0.02 | 0.10 ± 0.05 | 0.11 ± 0.05 | 0.14 ± 0.05 | 0.53 ± 0.08 |
| Porosity| 94% ± 1     | 93% ± 1     | 93% ± 2     | 90% ± 2     | 92% ± 1     | 80% ± 8     |

The graphs of compressive strength against porosity of P(3HB) coated scaffolds fabricated at various sintering temperatures; from 900 to 1100°C, for 2 hours showed that, in general, the compressive strength values decreased as porosity increased (see Fig 5.43), as expected. The porosity of the P(3HB) coated scaffolds sintered at 900-1100°C for 2 hours is in the range 82-90%. Thus, the compressive strength values of the 45S5 Bioglass® based scaffolds strongly depend on porosity for scaffolds sintered at: 900, 930, 950, 970, 1000, 1050 and 1100°C for 2 hours. Moreover, the average porosity of the P(3HB) coated samples is slightly lower than the average porosity of the non-coated samples for the 900-1100 series due to the effects of thickening of the struts with polymer coating. (see Table 5.7 and 5.8).
Figure 5.43 Graphs showing the relationship between the compressive strength values and porosity for the 900-1100 series of P(3HB) coated scaffolds.
Figure 5.44 SEM images showing 45S5 Bioglass® scaffolds after coatings with P(3HB): A) scaffold pore structure, B) high magnification image showing P(3HB) polymer coating of a pore wall, C) strut surface and D) high magnification of strut surface. The arrow in (D) indicates Bioglass® particles can protrude from the P(3HB) coatings, leading to direct contact of the bioactive material and the local environment.

Typical pore structures of the 45S5 Bioglass® scaffolds after coating with P(3HB) are illustrated in Fig. 5.44 (A-D). Highly porous scaffolds were produced as shown by the porosity values in tables 5.7 and 5.8 at all sintering conditions (900-1100 series). From the SEM images (Fig. 5.44), it can be concluded that the P(3HB) coating does not affect greatly the porosity ((see Fig 5.44 (A)). However, it was also observed that the coating did not homogenously cover the struts of the scaffolds, as shown in Fig 5.44 (B). The non-homogenous coatings could also due to the hydrophobic nature of the polymer. The same observations were made in a previous study [135]. The SEM P(3HB) morphology observations show that the polymer exhibits a mesh-like structure and a rougher surface than the PDLLA coatings (e.g. see Fig. 5.21 B1), possibly due to the rigid and brittle nature of P(3HB) in comparison to PDLLA. The
P(3HB) coating on the struts is seen as the darker phase and the Bioglass® surface is observed as the lighter phase (see Fig. 5.44 C-D). It was observed that glass-ceramic particles can protrude from the P(3HB) coating due to the mesh-like structure of these coatings (see arrow in Fig. 5.44 (D)).

5.2.4.3 Mechanical property- Microstructure correlation in 900-1100 series scaffolds

The relationship between microstructure and mechanical properties of the 900-1000 series scaffolds is discussed in this section. The results of the compressive strength of P(3HB) coated 45S5 Bioglass® scaffolds sintered at 930, 950, 970, 1000, 1050 and 1100°C for 2 hours compared to the as-sintered values (Table 5.7 and 5.8) are summarised in a bar graph, as shown in Fig. 5.45.

![Graph showing the compressive strength values (MPa) of P(3HB) coated Bioglass® scaffolds of the 900-1100 series compared with the values of as-sintered samples. All values are presented as mean ± standard deviation (s.d) where N = 5 samples (Coated samples); N = 5 samples (As-sintered samples).](image-url)

Figure 5.45 Graph showing the compressive strength values (MPa) of P(3HB) coated Bioglass® scaffolds of the 900-1100 series compared with the values of as-sintered samples. All values are presented as mean ± standard deviation (s.d) where N = 5 samples (Coated samples); N = 5 samples (As-sintered samples).
The graph shows that, in general, there was an increased in the compressive strength values of the coated samples as the sintering temperature increased from 930°C to 1100°C when compared to the non-coated (as-sintered) samples, respectively. The improvement in the compressive strength values after coating with P(3HB) film is probably due to the (P3HB) infiltrating into micropores and microcracks of the partially sintered 45S5 Bioglass® based scaffolds. This behaviour will be illustrated by SEM images of the struts microstructure, as presented next.

At lower sintering temperature partially sintered scaffolds (sintered at temperatures between 930°C and 1000°C for 2 hours) exhibited considerable microporosity which has been completely infiltrated by the P(3HB), as shown in Fig. 5.46 A-D. The non-coated scaffolds (partially sintered) were very fragile to handle before coating. As a comparison, scaffolds sintered at higher sintering temperatures of 1050 and 1100°C for 2 hours showed denser struts and the P(3HB) phase appears as coating in the interior of the scaffolds (see Fig. 5.46 E- F).

In conclusion, there was a direct relationship between the microstructure of the struts and the mechanical properties (compressive strength) of the 45S5 Bioglass® based scaffolds for the 900-1100 series of P(3HB) coated and non-coated scaffolds, and this correlation, in the case of coated scaffolds, was related to the degree of penetration of the polymer into the microcracks and micropores of the partially sintered struts.
Figure 5.46 SEM images showing the microstructure of Bioglass® based scaffold struts (cross sections) after coating with 5wt% P(3HB) for the following sintering conditions: A) 930°C, B) 950°C, C) 970°C, D) 1000°C, E) 1050°C and F) 1100°C, sintered for 2 hours.
5.2.4.4 *Physical analysis of the 1000 series scaffolds*

The results of the compressive strength and porosity of 45S5 Bioglass® based scaffolds at different sintering times of 2, 1 and 0.5 hours (1000 series) after coating with 5 wt% P(3HB) for 2 hours are shown in Table 5.9

Table 5.9 Compressive strength values (MPa) and porosity of P(3HB) coated 45S5 Bioglass® based scaffolds of the 1000 series.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Compressive strength values (MPa) and porosity (%) of P(3HB) coated samples (1000 series)</th>
<th>1000-2hrs</th>
<th>1000-1hrs</th>
<th>1000-0.5hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>MPa 0.39</td>
<td>0.27</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porosity 89%</td>
<td>92%</td>
<td>89%</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>MPa 0.21</td>
<td>0.32</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porosity 91%</td>
<td>90%</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>MPa 0.41</td>
<td>0.57</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porosity 87%</td>
<td>85%</td>
<td>85%</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>MPa 0.27</td>
<td>0.39</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porosity 89%</td>
<td>88%</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>MPa 0.27</td>
<td>0.37</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porosity 87%</td>
<td>84%</td>
<td>89%</td>
<td></td>
</tr>
</tbody>
</table>

Mean values of compressive strength (MPa) and porosity (%)

| MPa | 0.31 ± 0.09 | 0.38 ± 0.09 | 0.30 ± 0.17 |
| Porosity | 89% ± 2 | 87% ± 4 | 89% ± 2 |

Table 5.10 Compressive strength values (MPa) and porosity (%) of as-sintered (uncoated) samples of the 1000 series.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Compressive strength values (MPa) and porosity (%) of uncoated samples (1000 series)</th>
<th>1000-2hrs</th>
<th>1000-1hrs</th>
<th>1000-0.5hrs</th>
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</thead>
<tbody>
<tr>
<td>1.</td>
<td>MPa 0.16</td>
<td>0.07</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porosity 89%</td>
<td>92%</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>MPa 0.13</td>
<td>0.08</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porosity 90%</td>
<td>92%</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>MPa 0.12</td>
<td>0.10</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porosity 90%</td>
<td>93%</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>MPa 0.09</td>
<td>0.18</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porosity 92%</td>
<td>79%</td>
<td>95%</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>MPa 0.09</td>
<td>0.10</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porosity 88%</td>
<td>87%</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>Mean values of compressive strength (MPa) and porosity (%)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>----------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPa</td>
<td>0.11 ± 0.03</td>
<td>0.11 ± 0.06</td>
<td>0.05 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Porosity</td>
<td>90% ± 1</td>
<td>89% ± 6</td>
<td>94% ± 1</td>
<td></td>
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</table>

The graphs of compressive strength against porosity of coated scaffolds fabricated at various sintering times (2, 1 and 0.5 hours) at sintering temperature of 1000°C showed that, in general, the compressive strength values decreased as porosity increased (see Fig. 5.47), as expected. The porosity of the P(3HB) coated scaffolds is in the range 82-90%. Thus, the compressive strength values strongly depend on porosity for scaffolds sintered at 1000°C. In addition, the average porosity reduction of the P(3HB) coated scaffolds as compared to the non-coated samples for the 1000 series is around 3% due to thickening of the struts with P(3HB) coating (see Table 5.9 and 5.10).

Figure 5.47 Graphs showing the relationship between the compressive strength values and porosity for the 1000 series of P(3HB) coated scaffolds.
5.2.4.5 Mechanical property- microstructure correlation in 1000 series scaffolds

The relationship between microstructure and compressive strength of the 1000 series scaffolds is discussed in this section. The results of the compressive strength (MPa) of P(3HB) coated 45S5 Bioglass® scaffolds sintered for 2, 1 and 0.5 hours at fixed temperature of 1000°C compared to the as-sintered values are summarised by the graph in Fig. 5.48.

![Graph showing the compressive strength values of scaffolds of the 1000 series before and after P(3HB) coating. All values are presented as mean ± standard deviations (s.d) where N = 5 samples (Coated samples); N = 5 samples (As-sintered samples).](image)

**Figure 5.48** Graph showing the compressive strength values of scaffolds of the 1000 series before and after P(3HB) coating. All values are presented as mean ± standard deviations (s.d) where N = 5 samples (Coated samples); N = 5 samples (As-sintered samples).

The graph shows that, in general, there was a significant increase of the compressive strength of the coated samples as the sintering time increased from 0.5 hours to 2 hours when compared to the non-coated (as-sintered) samples, respectively. The improvement in the compressive strength values after coating with P(3HB) is probably due to the P(3HB) at least partially infiltrating into micropores and mirocracks of the partially sintered 45S5 Bioglass® based scaffolds, as discussed above (section 5.2.2.5) for PDLLA coated scaffolds. This behaviour will be illustrated by SEM images of strut microstructures, as shown in Fig. 5.49.
There is, as expected, a direct relationship between the microstructure of the struts and the mechanical properties (compressive strength) of the scaffolds for the 1000 series both for coated and non-coated scaffolds.

Figure 5.49 SEM images showing the microstructure of scaffold struts cross-section before and after coating with 5wt% P(3HB) for the following sintering conditions: A) 0.5 hrs, B) 1 hrs and C) 2 hours sintered at 1000°C.

The cross-section images of the strut microstructure after coating shows that the number of micropores increased as sintering time decreased. Therefore, as the sintering time decreased, more polymer can infiltrate the micropores and macropores in the centre of the struts (see Fig 5.49 C) (formed from the foam replica fabrication) in order to improve the compressive strength. SEM observations of the strut walls microstructure for all samples indicate that the 45S5 Bioglass® structure was only
partially sintered since the glass particles were not completely softened to form a smooth surface by viscous flow at 1000°C.

Figure 5.50 Typical compressive stress-strain curves of A) an as-sintered Bioglass® scaffold and B) a P(3HB) coated scaffold. Series 1000 scaffolds sintered for 2 hours.

Figure 5.51 Photograph of the scaffolds after compressive strength test: A) as-sintered and B) P(3HB) coated scaffolds sintered at 1000°C for 2 hours.

Typical compressive stress-strain curves of uncoated and P(3HB) coated scaffolds are shown in Figure 5.50. The compressive stress-strain curve corresponding to coated scaffolds was seen to be slightly less jagged compared with the curve of as-sintered scaffolds. As mentioned above, the improvement in mechanical stability is thought to
be due to the P(3HB) polymer infiltrating the micropores and macropores on the strut surfaces. Fig. 5.51 shows photographs of scaffolds after compressive strength test for the as-sintered (Fig. 5.51 A) and the P(3HB) coated scaffolds (Fig. 5.51 B). The P(3HB) coated scaffolds sample is seen to retain some of its original structure after failure while the as-sintered scaffold was collapsed into particulates, which qualitatively confirms the increased in the fracture toughness of the P(3HB) coated scaffold.

The improvements in compressive strength and modulus with P(3HB) coating were significant and the area under the stress-strain curve, which represents the work of fracture of the material, also increased for the coated samples. As explained above (Section 5.2.4.5), the increased of toughness can be ascribed to the polymer phase which has infiltrated the micropores and microcracks of the partially sintered scaffolds and can induce toughening mechanism such as crack bridging and polymer fibril pull-out. Thus, the presence of the polymer will contribute to the material absorbing extra energy before fracture which is limited to the plastic deformation of the polymer phase. Figure 5.52 shows the microstructure of scaffolds after fracture in which polymeric elongated fibrils, bridging cracks can be observed.

Figure 5.52 SEM image of the fracture surface of a P(3HB) coated Bioglass® scaffolds after compressive strength testing, showing P(3HB) polymer fibrils formed due to P(3HB) infiltration into micropores.
5.2.4.6 Characterisation of 45S5 Bioglass® pellets coated with P(3HB) film

i) Interface properties

This part of the investigation on 45S5 Bioglass® pellets coated with P(3HB) film was carried out to study the interface between the polymeric P(3HB) layer and the sintered 45S5 Bioglass® surface. This interface is easier to characterise on planar surfaces than in the coated 3D scaffolds. The pellets were sintered following the same heating scheduled used for scaffolds, e.g. the microstructure of the pellets is expected to be similar to that of scaffolds, as demonstrated also in the literature [135]. SEM images of coated 45S5 Bioglass® pellets indicate a very thin P(3HB) film deposited on the pellets surface. The thickness of the polymer film coating was determined to be around 6 -7 µm (see Fig. 5.53).

![SEM image of a P(3HB) coated 45S5 Bioglass® pellet sintered at 1000°C for 2 hours.](image)

Figure 5.53 SEM image of a P(3HB) coated 45S5 Bioglass® pellet sintered at 1000°C for 2 hours.
The adhesion strength between the P(3HB) layer and sintered 45S5 Bioglass® substrate was determined qualitatively by microindentation, applying on the glass substrate a load of 200g at a 50µm distance from the interface. The crack propagation was seen to stop at the interface between the P(3HB) (see arrow in Fig 5.54) and 45S5 Bioglass®, indicating that, qualitatively, the P(3HB) polymer adhesion at the interface is stronger as compared to that found for PDLLA layers, as discussed earlier (section 5.2.3.1). The strong adhesion of P(3HB) to Bioglass® slightly altered the bioactivity of the coated pellets, which is discussed next.

**ii) Bioactivity analysis of the P(3HB) coated 45S5 Bioglass® pellets**

45S5 Bioglass® pellets were used for the quantitative assessment of the bioactivity of P(3HB) coated 45S5 Bioglass® pellets at different immersion times of 7, 14 and 28 days in SBF, considering that the P(3HB) coatings would be homogenous on the planar surfaces of the pellets.
Figure 5.55 SEM images of the surfaces of P(3HB) coated pellets sintered at 1000°C for 2 hours and immersed in SBF for A) 7, B) 14 and C,D) 28 days, showing the change of surface morphology and formation of hydroxyapatite (HA).

The bioactivity of the 45S5 Bioglass® pellets sintered at 1000°C for 2 hours and coated with 5 wt% P(3HB) for 2 hours was investigated. SEM and EDS analyses were carried out on the surfaces of the Bioglass® pellets after immersion time of 7, 14 and 28 days in SBF. SEM images of the selected Bioglass® pellets after immersion in SBF are shown in Fig. 5.55. SEM images showed a different morphology for 7, 14 and 28 days of immersion in SBF. The formation of HA-like crystals on the surface of the coated 45S5 Bioglass® pellets was indicated by SEM after 28 days (Fig. 5.55 C). The broken areas of the coating at day 7 and 14, as shown in Fig. 5.55 (A, B), should provide a channel for SBF to flow into and come in contact with the bioactive material. With increasing immersion time in SBF, a large surface area of the bioactive glass is being exposed to SBF (see Fig. 5.55 C), and thus HA-like crystals will
eventually cover the whole surface of the substrate. A similar behaviour was found for PDLLA coated specimens (section 5.2.3.2 (i)).

EDS analyses were done to confirm the elements present on the surface of the P(3HB) coated 45S5 Bioglass® pellets. For EDS analysis, 3 measurements were taken and typical EDS spectra of each immersion time in SBF are shown in Fig. 5.56. Cr peaks in the spectrum are due to the conducting coatings used to prepare the sample for SEM.

A high level of carbon was contributed by P(3HB) on day 7 and 14, and the level decreased as the immersion time increased. From the EDS analysis, the presence of phosphorus (P) and calcium (Ca) peaks at around 2 keV and 3.8keV, respectively, was confirmed at 28 days. The EDS analysis of the as-sintered (non-coated) 45S5 Bioglass® pellets immersed in SBF for 28 days also showed the Ca and P peaks. Thus it could be assumed qualitatively at least, that the bioactivity of the P(3HB) coated samples after 28 days immersion in SBF in terms of CaP formation is similar to that of the non-coated samples, indicating that the P(3HB) coating did not negatively affect the bioactivity of the 45S5 Bioglass®.

XRD analysis was done to confirm the crystallinity of the calcium phosphate formed on the surface of the P(3HB) coated pellets sintered at 1000°C for 2 hours after immersion in SBF for 7, 14 and 28 days. There was an increase in the peaks at $2\theta = 32^\circ$ corresponding to HA peaks (commercial HA powder) when the immersion time in SBF increased from 7 to 28 days, as shown in Fig 5.57. A comparison with the XRD analysis of PDLLA coated pellets (Fig. 5.30) on scaffolds produced at the same sintering conditions reveals that the peaks intensity at $2\theta = 32^\circ$ is higher than in the P(3HB) coated pellets (Fig. 5.57) at day 28, indicating that HA formation has occurred to a greater extent in PDLLA coated pellets than in P(3HB) coated pellets. This result can be explained by the stronger adhesion exhibited by the P(3HB) coating (section 5.2.4.6) on the sintered Bioglass® surface compared to PDLLA (section 5.2.3.1). The strongly adhered P(3HB) layer reduces the extent of SBF contact with the bioactive material leading to a comparatively lower level of HA formation under similar conditions.
Figure 5.56 EDS spectra of 45S5 Bioglass® pellets sintered at 1000°C for 2 hours (P(3HB)-coated) after immersion in SBF for A) 7, B) 14 and C) 28 days compared to a non-coated sample (D)
Figure 5.57 XRD spectra of P(3HB) coated 45S5 Bioglass® pellets sintered at 1000°C for 2 hours and immersed in SBF for 0, 7, 14 and 28 days, as compared to the pattern of commercial HA. The major peaks of Na$_2$Ca$_2$Si$_3$O$_9$ phase and hydroxyapatite are marked by (∆) and (●) respectively.

iii) Bioactivity analysis of the P(3HB) coated 45S5 Bioglass® scaffolds.

45S5 Bioglass® scaffolds sintered at 1000°C for 2 hours and coated with 5wt% P(3HB) were immersed in 30 ml solution of SBF for 7, 14 and 28 days in an incubator at 37°C. The bioactivity assessment of the coated scaffolds after immersion in SBF was characterised by SEM, EDS and XRD which is presented in this section.

Fig. 5.58 shows SEM images of 45S5 Bioglass® based scaffolds sintered at 1000°C for 2 hours and coated with 5 wt% P(3HB) after immersion in SBF for 7, 14 and 28 days. There was a clear formation of HA crystals on the surface of the scaffolds after 7 days immersion in SBF indicated by some cauliflower-like apatite clusters formed on the surface of the composite scaffolds (as shown in Figure 5.58 A), almost covering the entire scaffolds. The formations of the apatite clusters on the composite scaffolds increased as SBF immersion time increased from 7 to 28 days.
Figure 5.58 SEM images showing the formation of HA on the 5wt% P(3HB) coated 45S5 Bioglass® scaffolds sintered at 1000°C for 2 hours after immersion in SBF for A) 7 days, B) 14 days and C) 28 days.

The results obtained by SEM were confirmed by EDS analysis as shown in Fig. 5.59. The morphology of the scaffold also showed that the surface roughness increased due to HA formation from 7 to 28 days of immersion in SBF (see Fig. 5.59 A-C).

EDS spectra of scaffold surfaces at different immersion times of 7, 14 and 28 days were obtained to confirm the formation of HA, which was indicated by the P and Ca peaks present at 2keV and 3.8 keV, respectively (see Fig. 5.59 A1 to C1). The Ca/P ratio gradually increased, accompanied by a decreased of the Si, Na, C and O peaks with increasing immersion time. The Ca/P ratio was found to be 1.60 at 28 days of immersion in SBF.
Figure 5.59 SEM images and EDS spectra of the surface of P(3HB) coated scaffolds sintered at 1000°C for 2 hours and immersed in SBF for A, A1) 7, B, B1) 14 and C,C1) 28 days, showing formation of hydroxyapatite on the surface of the scaffolds.
Figure 5.60 XRD spectra of P(3HB) coated 45S5 Bioglass® scaffolds sintered at 1000°C for 2 hours and immersed in SBF for 0, 7, 14 and 28 days. The major peaks of Na$_2$Ca$_2$Si$_3$O$_9$ phase and hydroxyapatite are marked by (Δ) and (●), respectively.

XRD analysis was done to confirm the formation of crystalline hydroxyapatite on the surface of the P(3HB) coated scaffolds sintered at 1000°C for 2 hours after immersion in SBF for 7, 14 and 28 days. There was an increase in the height of peaks at 2θ = 32°, which correspond to HA peaks, when the immersion time in SBF increased from 7 to 28 days, as shown in Fig. 5.60. At 7 days, peaks corresponding to the Na$_2$Ca$_2$Si$_3$O$_9$ phase at 2(θ) = 34° and 35° were identified. The crystalline phase Na$_2$Ca$_2$Si$_3$O$_9$ transformed to an amorphous phase when the coated scaffolds were soaked in SBF for 14 days and longer, as expected also from the literature [1].

Thus, it can be concluded from SEM, EDS and XRD analyses, that P(3HB) coatings do not impede the bioactivity of the scaffolds sintered at 1000°C for 2 hours coated with P(3HB). This finding complements preliminary studies by Bretcanu et al. [135] on P(3HB) coated Bioglass® scaffolds sintered at higher sintering temperature of 1100°C for 1 hour.
iv) Compressive strength after SBF immersion

Figure 5.61 Compressive strength values of PDLLA and P3HB coated scaffolds compared to non-coated scaffolds after SBF immersion. All values are presented as mean ± standard deviations (s.d) where N = 7 samples for PDLLA and P(3HB) coated samples and N = 7 samples for AS (as-sintered) samples.

Compressive strength tests were carried out on dry scaffolds partially sintered at 1000°C for 2 hours, after immersion in SBF for selected time periods. It was found that the mechanical strength of both PDLLA and P(3HB) coated samples were gradually deteriorated after immersion in SBF for 7 days due to the transformation of the crystalline phase (Na$_2$Ca$_2$Si$_3$O$_9$) into an amorphous phase, as shown by the XRD analysis in Fig. 5.37 and 5.60, respectively. The reduction in mechanical strength is not unexpected, considering the fragility of the amorphous structure in comparison with the highly crystalline structure of as-coated samples before SBF immersion. Nevertheless the compressive strength of P(3HB) coated scaffolds was seen to be less deteriorated compared to that of PDLLA coated scaffolds, which could be due to the lower degradation rate of P(3HB) in SBF.
5.2.5 Characterisation of 45S5 Bioglass® scaffolds coated with P(3HO) films

5.2.5.1 Characterisation of poly-3-hydroxyoctanoate P(3HO).

Figure 5.62 A) The gas chromatogram for the methanolysis product of PHA produced from *P. mendocina* when grown in octanoate. (B) Mass spectra showing molecular ion related mass fragments of octanoic acid 3 hydroxymethyl ester.
Gas chromatography and mass spectrometry (GC-MS) was used to identify the monomer accumulated from the extracted polymer of *P. mendocina* grown on octanoate using MSM medium. The monomer in the accumulated polymer chain was identified as poly-3-hydroxyoctanoate, P(3HO). Mass spectrum of the GC peak at retention time 10.69 minutes (Figure 5.62 A) showed the molecular ion-related mass fragments due to methyl esters of 3HO with m/z =127 (Figure 5.62 B).

### 5.2.5.2 Characterisation of 45S5 Bioglass® pellets coated with P(3HO) film

**i) Bioactivity analysis of P(3HO) coated 45S5 Bioglass® pellets**

Figure 5.63 SEM images of the surfaces of P(3HO) coated pellets sintered at 1000°C for 2 hours and immersed in SBF for A) 0, B) 7 and C) 14 and D) 28 days, showing formation of hydroxyapatite (HA).
Sintered pellets were used for the quantitative assessment of the bioactivity of P(3HO) coated 45S5 Bioglass® sintered substrates at different immersion times of 7, 14 and 28 days in SBF, assuming that the P(3HO) coatings would be homogenous on planar surfaces.

SEM images showed a different surface morphology for 0, 7, 14 and 28 days after immersion in SBF (see Fig. 5.63). The formation crystalline HA on the surface of P(3HO) coated 45S5 Bioglass® pellets was not clearly observed by SEM after immersion in SBF for 7 and 14 days. However, the formation of hydroxyapatite clusters was clearly observed after immersion in SBF for 28 days.

EDS analyses were done to confirm the elements present on the surface of the P(3HO) coated 45S5 Bioglass® pellets sintered at 1000°C for 2 hours. For EDS analysis, 3 measurements were taken and typical EDS spectra of each immersion time in SBF are shown in Figure 5.64. Cr peaks in the spectrum were caused by the chromium coating, used for the SEM-EDS sample preparation. From the analysis, it was shown that there was a slight increase in Si, P and Ca after 7 and 14 days immersion time in SBF, indicating the surface is covered by the silica gel. The Ca and P peaks increased with a decrease of the Si peak after 28 days immersion in SBF, indicating the formation of HA on top of the silica gel. Thus these EDS results confirm the formation of HA agglomerates and the presence of silica gel layer underneath them.
Figure 5.64 EDS spectra of 45S5 Bioglass® pellets sintered at 1000°C for 2 hours (P(3HO) -coated) after immersion in SBF for A) 0, B) 7, C) 14 and D) 28 days
XRD analysis was carried out to confirm the formation of crystalline HA on the surface of the P(3HO) coated pellets sintered at 1000°C for 2 hours after immersion in SBF for 7, 14 and 28 days. There was an increase in the height of peaks at $2\theta = 32^\circ$ corresponding to crystalline HA when the immersion time in SBF increased from 7 to 28 days, as shown in Fig. 5.65. A comparison with the XRD analysis of PDLLA coated pellets (Fig. 5.30) fabricated at the same sintering conditions reveals that the peaks intensity at $2\theta = 32^\circ$ is higher than that of the P(3HO) coated pellets at day 28, indicating that HA formation is enhanced on PDLLA coated pellets in comparison to P(3HO) coated pellets. These results can be explained by the higher degradation rate of PDLLA in SBF, related to the amorphous nature of PDLLA. A comparison with the XRD analysis of P(3HB) coated pellets revealed that the peaks intensity at $2\theta = 32^\circ$ is about the same, which is related to the slower degradation rate of highly crystalline P(3HB) and P(3HO). The rate of degradation will determine the rate at which SBF will come in contact with the partially crystalline 45S5 Bioglass® sintered surface, determining thus the kinetic of HA formation.
5.2.5.3 Mechanical property -microstructure correlation in 1000 series scaffolds coated with P(3HO)

The relationship between microstructure and mechanical properties of the 1000 series scaffolds is discussed in this section. The results of the compressive strength (MPa) of P(3HO) coated 45S5 Bioglass® scaffolds sintered at 1000°C for 2 hours, 1 hour and 0.5 hours are summarised in Table 5.11 below.

### Table 5.11 Compressive strength values (MPa) and porosity of P(3HO) coated 45S5 Bioglass® based scaffolds of the 1000 series.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Compressive strength values (MPa) and porosity(%) of coated samples (1000 series)</th>
<th>1000-2hrs</th>
<th>1000-1hrs</th>
<th>1000-0.5hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MPa</td>
<td></td>
<td>0.30</td>
<td>0.19</td>
<td>0.18</td>
</tr>
<tr>
<td>Porosity</td>
<td></td>
<td>84%</td>
<td>84%</td>
<td>84%</td>
</tr>
<tr>
<td>2. MPa</td>
<td></td>
<td>0.30</td>
<td>0.14</td>
<td>0.12</td>
</tr>
<tr>
<td>Porosity</td>
<td></td>
<td>84%</td>
<td>82%</td>
<td>85%</td>
</tr>
<tr>
<td>3. MPa</td>
<td></td>
<td>0.35</td>
<td>0.15</td>
<td>0.06</td>
</tr>
<tr>
<td>Porosity</td>
<td></td>
<td>82%</td>
<td>84%</td>
<td>85%</td>
</tr>
<tr>
<td>4. MPa</td>
<td></td>
<td>0.30</td>
<td>0.20</td>
<td>0.08</td>
</tr>
<tr>
<td>Porosity</td>
<td></td>
<td>85%</td>
<td>83%</td>
<td>85%</td>
</tr>
<tr>
<td>5. MPa</td>
<td></td>
<td>0.20</td>
<td>0.24</td>
<td>-</td>
</tr>
<tr>
<td>Porosity</td>
<td></td>
<td>86%</td>
<td>82%</td>
<td>-</td>
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Mean values of compressive strength (MPa) and porosity (%)

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<tr>
<th></th>
<th>Mean values of compressive strength (MPa) and porosity (%)</th>
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<tr>
<td>MPa</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>Porosity</td>
<td>84% ± 1.5</td>
</tr>
<tr>
<td></td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>Porosity</td>
<td>83% ± 1</td>
</tr>
<tr>
<td></td>
<td>0.11 ± 0.05</td>
</tr>
<tr>
<td>Porosity</td>
<td>84% ± 0.5</td>
</tr>
</tbody>
</table>

Typical pore structures of the 45S5 Bioglass® scaffolds after coating with P(3HO) are illustrated in Fig. 5.66. From the SEM images it can be concluded that the P(3HO) coating does not affect greatly the porosity (see Fig. 5.66 A). However, it was also observed that the coating did not homogenously cover the struts of the scaffolds, as shown by Fig 5.66 (B) indicated by the arrow. Similar behaviour is observed for PDLLA coatings, as described previously in section 5.2.2.1. This non-homogenous coating is due to the uneven surface of the scaffolds. At higher magnification, the polymer (darker phase) is seen to infiltrate the macropores of the partially sintered
struts (lighter phase) as shown in Fig. 5.66 C. Fig. 5.66 D shows that P(3HO) filled the micropores of the partially sintered Bioglass® particles leaving however some open micropores on the struts.

Figure 5.66 SEM images showing the 45S5 Bioglass® scaffolds after coating with P(3HO) at different magnifications. The presence of a non-homogenous coating is indicated by the arrow in (B).
SEM images of the microstructure of the scaffold strut cross-section after coating with 5wt% P(3HO) are illustrated in Figure 5.67. The cross-section images of the struts microstructure after coating show that the number of micropores increased as sintering time decreased. Therefore, as the sintering time decreased, more polymer can infiltrate the micropores of the 45S5 Bioglass® scaffold, which will have a positive effect on the overall scaffold toughness (see Fig. 5.68). The P(3HO) polymer coatings seem to be thinner than the P(3HB) coatings discussed earlier because in this case the polymer has efficiently filled and covered most of the micropores and microcracks present in the partially sintered scaffolds, therefore leaving no excess polymer at the macropores level and on the surface of the struts.
Figure 5.68 Typical compressive stress-strain curves of A) as-sintered Bioglass® scaffold and B) a P(3HO) coated scaffold. Series 1000 scaffolds sintered at 2 hours.

Figure 5.69 Photographs of the scaffolds after compressive strength test: A) as-sintered and B) P(3HO) coated scaffolds sintered at 1000°C for 2 hours.

Typical compressive stress-strain curves of uncoated and P(3HO) coated scaffolds are shown in Figure 5.68. The jagged curves show the four regimes discussed above with increasing strain: 1) stress increase (uncoated and coated scaffold), 2) stress decrease (uncoated and coated scaffold), 3) plateau (coated scaffold) and 4) densification stage (coated scaffolds). Following the discussion above, the improvement in mechanical integrity of the scaffold is thought to be due to the P(3HO) coating efficiently infiltrating the micropores and microcracks in the struts. The compressive stress-strain
curve of the coated scaffolds was less jagged compared with the curve of as-sintered scaffolds. Moreover, comparing with the stress-strain curve of P(3HB) (Fig. 5.50) and PDLLA (Fig. 5.23) coated scaffolds, as presented above, the trace for P(3HO) coated scaffolds is seen to be less jagged than that of P(3HB) but has a similar pattern to that of PDLLA coated scaffolds. In addition, the work of fracture, i.e. the area under the stress-strain curve for P(3HO) is slightly higher than that for PDLLA and P(3HB) coated scaffolds due to higher plateau level of the P(3HO) coated scaffolds (Fig. 5.68) compared to those of PDLLA (Fig. 5.23) and P(3HB) coated scaffolds (Fig. 5.50) of similar compressive strength values (measured at 5% strain). This behaviour is suggested to be due to more efficient infiltration of the P(3HO) polymer into the micropores and crevices of the partially sintered scaffolds compared to PDLLA and P(3HB). This result could be due to both the elastomeric nature of the P(3HO) polymer and the higher wettability property (i.e. lower contact angle) of P(3HO) in comparison with PDLLA and P(3HB), as shown in Table 4.1. The level of plateau also depends on the quantity of polymer phase inside the scaffolds [83]. In addition, in the second stage of the curve, the drop in stress loading is not abrupt but progressive, indicating that continuous damage occurs in the structure, as compared to the sudden drop in stress with P(3HB) coated samples sintered at the same condition, which could be due to non-homogenous coatings and lack of extensive plastic deformation of P(3HB) (see Fig. 5.50), compared to P(3HO).

Fig. 5.69 shows photographs of scaffolds after compressive strength test for the as-sintered (5.69 A) and the P(3HO) coated scaffolds (5.69 B). The (P3HO) coated scaffolds is seen to retain its original structure (shape) after failure while the as-sintered scaffold was broken into particulates, which qualitatively confirms the significant increase in the toughness of the P(3HO) coated scaffold (see movie in supplementary CD). This behaviour is similar to PDLLA coated scaffolds, as described in section 5.2.2.5 and qualitative better than that of P(3HB) coated scaffold.

The improvement in compressive strength with P(3HO) coating was significant and the area under the stress-strain curve, which represents the work of fracture of the material, also significantly increased for the coated samples. As explained above, the expected increase of fracture toughness can be ascribed to the presence of the polymer phase which has infiltrated the micropores and microcracks of the partially sintered
scaffold. Thus, the presence of the deformable polymer will contribute to the material absorbing extra energy before fracture. Figure 5.70 shows the microstructure of a scaffold after fracture in which the polymeric fibrils bridging cracks can be observed. This effect is in fact similar to the toughening mechanisms in bone based on crack bridging by collagen fibrils [179] The fibrils bridging the crack in P(3HO) coated scaffolds are seen to be slightly longer than PDLLA (Fig. 5.25 A) and P(3HB) fibrils (Fig. 5.52), as described above, due to the elastomeric property (higher flexibility) of P(3HO).
Figure 5.71 SEM images and EDS spectra of the surface of P(3HO) coated scaffolds sintered at 1000°C for 2 hours and immersed in SBF at A, A1) 7, B, B1) 14 and C, C1) 28 days showing formation of hydroxyapatite layers on the surface of the scaffolds.
XRD analysis was done to confirm the formation of crystalline hydroxyapatite on the surface of the P(3HO) coated scaffolds sintered at 1000°C for 2 hours after immersion in SBF for 7, 14 and 28 days (see Figure 5.72). There was an increased in the height of the peak at \( \theta = 32^\circ \), which correspond to the HA peak, when the immersion time in SBF increased from 7 to 28 days, as shown in Fig. 5.72. At 7 days, peaks corresponding to \( \text{Na}_2\text{Ca}_2\text{Si}_3\text{O}_9 \) (at \( 2(\theta) = 34^\circ \) and \( 35^\circ \)) were identified, which indicate the crystallinity of the partially sintered scaffolds coated with P(3HO). The crystalline phase \( \text{Na}_2\text{Ca}_2\text{Si}_3\text{O}_9 \) transformed to an amorphous or weakly crystalline calcium phosphate phase when the coated scaffolds were soaked in SBF for 14 days and longer, according to the literature [53].

Thus, it can be concluded from the SEM, EDS and XRD analyses, that P(3HO) coatings do not impede the bioactivity of the Bioglass® based scaffolds sintered at 1000°C for 2 hours coated with P(3HO).

![Figure 5.72 XRD spectra of P(3HO) coated 45S5 Bioglass® scaffolds sintered at 1000°C for 2 hours and immersed in SBF for 0, 7, 14 and 28 days. The major peaks of \( \text{Na}_2\text{Ca}_2\text{Si}_3\text{O}_9 \) phase and hydroxyapatite are marked by (Δ) and (●), respectively.](image-url)
5.3 Discussion

In this section, the results of the investigation on the novel family of scaffolds developed in the framework of this project are discussed in relation to mechanical properties, microstructure and bioactivity in the wider context of available bioactive bone tissue scaffolds.

5.3.1 Comparison of the compressive strength of 45S5 Bioglass®/Polymer composite scaffolds with spongy bone and with previous investigations

Scaffolds produced by the foam replication method are very similar to spongy bone (also called cancellous bone) in terms of their pore structure. The partially sintered 45S5 Bioglass® scaffolds prepared using polymer sponge as porogen exhibit a highly porous structure with large pore sizes (300-500µm) combined with high porosity (85-90%) and high pore interconnectivity, as shown by the results of the capillary test in Section 5.2.1.1 and permeability test in Section 5.2.1.2. High porosity and pore interconnectivity should permit sufficient tissue ingrowth and vascularisation into the scaffolds in *in-vivo* applications. PDLLA, P(3HB) and P(3HO) coatings of the partially sintered 45S5 Bioglass® scaffolds did not significantly decrease the pore size, porosity or pore interconnectivity of the original scaffolds. In addition, XRD analysis indicated that PDLLA and P(3HB) coatings did not change the crystal phase composition of 45S5 Bioglass® scaffolds, as shown in Fig. 5.30 and 5.57, respectively.

In this investigation, PDLLA, P(3HB) and P(3HO) coatings were found to significantly improve the compressive strength and compressive modulus of the partially sintered 45S5 Bioglass® scaffolds, as compared to the non-coated scaffolds. The correlation between strain-stress behaviour and microstructure of scaffolds was discussed earlier in Sections 5.2.2.5, 5.2.4.5 and 5.2.5.3. There are many reports on the mechanical strength of cancellous bone [191]. The compressive strength of spongy bone (not the strut) is in the range of 0.2-4 MPa, when the relative density is ~ 0.1 [192]. The measured compressive strength values of the partially sintered composite scaffolds (0.3-0.4 MPa) and of the fully sintered composite scaffolds (0.5-0.6MPa) coated with PDLLA, P(3HB) and P(3HO) fall in this range, but lie closer to the lower
bound. These values are also consistent with data reported in the literature on similar coated scaffold materials [1;135]. Previous studies have shown that the compressive strength of PCL coated hydroxyapatite scaffolds of similar porosity range to those studied here (porosity 84%-89%) fabricated via sponge replica method was \( \sim 0.03 \text{MPa} \) [133]. Compressive strength data of porous HA-based scaffolds reported in literature [8;133] have been collected and the values are always lower than 0.3MPa. The compressive strength of the present scaffolds is higher than that of HA-based scaffolds of similar porosities. From this investigation, it is shown that a compressive strength of 0.3-0.4 MPa (partially sintered scaffolds coated with PDLLA, P(3HB) and P(3HO)) is sufficient for the scaffolds to be handled for SBF test and for safe cutting of the samples for mechanical tests, compared to scaffolds without polymer coatings, which are extremely weak and brittle (0.04-0.14 MPa). In addition, it has been reported that the compressive strength of HA scaffolds significantly increases [193] due to tissue in-growth in vivo. It has also been speculated that it might be not necessary to fabricate a scaffold with a mechanical strength equal to that of bone because cultured cells on the scaffolds and new tissue formation in vivo will create a biocomposite (e.g. by collagen secreted by cells) increasing the time-dependent strength of the scaffolds significantly [17].

An ideal scaffold should have at least a proper compression strength and fracture toughness to allow it to be manipulated adequately for tissue engineering applications. The present polymer coated 45S5 Bioglass® composite scaffolds possess such suitable mechanical competence, in term of toughness, when the polymer phase (i.e. PDLLA, P(3HB) or P(3HO)) has infiltrated the micropores and microcracks of the partially sintered scaffolds. A uniform and continuous polymer network can be introduced in the scaffold struts, which prevents it from collapsing due to an interlocking mechanism provided by the ductile polymer phase. In addition, our investigations showed that during fracture, a micron-scale crack-bridging mechanism occurs by polymer ligament stretching upon crack opening along the crack wake. This effect is similar to the toughening mechanism in bone based on crack bridging by collagen fibrils [179]. Hence, in principle, the composite systems investigated here mimic the natural bone in terms of fracture behaviour. To the author’s knowledge, there is no fracture toughness data available for cancellous bone. The proposed toughening mechanisms
for the partially sintered scaffolds coated with PDLLA, P(3HB) and P(3HO) are discussed next in Section 5.3.2.

### 5.3.2 Proposed toughening mechanisms

Three different mechanisms of toughening are proposed for the three different polymer phases used to infiltrate the partially sintered 45S5 Bioglass\textsuperscript{®} scaffolds based on the stress-strain curve and SEM fracture surface observations. The differences in the stress-strain curve of the scaffolds are proposed to be related to the physical property of the polymer used in each case since the method of infiltration applied was the same for all three polymers, i.e. immersion in 5 wt\% polymer solution for 2 hours. The physical properties of the polymers used are relatively different to each other, as shown in Table 4.1. According to Table 4.1, PDLLA and P(3HB) are of thermoplastic type while P(3HO) is a thermoplastic elastomeric polymer with very low Tg value (-35\(^\circ\)C). Moreover PDLLA and P(3HO) are ductile in comparison to P(3HB), which is a rather brittle polymer. Both PDLLA and P(3HB) are more hydrophobic than P(3HO) as revealed by static water contact angle measurements. PDLLA has a molecular weight (Mw) of 200,000 g mol\(^{-1}\), the Mw of P(3HO) is 225,000 g mol\(^{-1}\) and P(3HB) has a Mw of 850,000 g mol\(^{-1}\). These physical properties will influence the respective behaviours during polymer infiltration into the microcracks and micropores of the partially sintered scaffolds.

P(3HO) has the lowest Tg value (-35\(^\circ\)C) compared to PDLLA (55\(^\circ\)C) and P(3HB)(2.7\(^\circ\)C), P(3HO) is a thermoplastic elastomeric polymer [194]. Morphologically it consists of both crystalline and amorphous phases at room temperature and it has rubber-like elasticity. P(3HO) has a liquid-like behaviour at room temperature as compared to a glass-like behaviour of a thermoplastic polymer. This liquid-like behaviour of P(3HO) can improve the infiltration into the microcracks and micropores of the partially sintered scaffolds leading to increased depth of infiltration of the polymer due to better flow behaviour (hydrodynamic) at room temperature in comparison to PDLLA and P(3HB). Therefore, P(3HO) will fill the microcracks and micropores of the partially sintered scaffolds more efficiently than the other polymers.
On the other hand, PDLLA is an amorphous polymer while P(3HB) is a semi-crystalline polymer as shown in Table 4.1. The amorphous character of PDLLA will favour its hydrodynamic behaviour due to more flexible chains present, as compared to the more rigid chains present in the semi-crystalline P(3HB). This will increase the infiltration capability of PDLLA into the microcracks and micropores of partially sintered scaffolds, as compared to infiltration using P(3HB).

Based on the stress-strain curves, SEM observations of fracture surfaces and morphology the polymer infiltration behaviour for each biodegradable polymer can be proposed, as schematically shown in Figure 5.73:

**Figure 5.73 Schematic diagram showing the infiltration behaviour for 1) P(3HB), 2) PDLLA and 3) P(3HO) coating of partially sintered 45S5 Bioglass® scaffolds.**

Briefly, Fig. 5.73 (1) shows the infiltration behaviour proposed for P(3HB). P(3HB) coating does not infiltrate deeper down the micropores or microcracks of the partially sintered scaffold struts due to lower flowing ability (semi-crystalline structure) of
P(3HB). Therefore, more polymer will be present in the macropores and on the strut surfaces, as shown in Fig. 5.46. The PDLLA infiltration as proposed in Fig. 5.73(2) is better leading to a higher amount of polymer inside the micropores and microcracks probably due to the amorphous structure of PDLLA leading to higher flow ability, as compared to P(3HB). Therefore, only some polymer is still present in the macropores and on strut surfaces, as indicated in Fig. 5.17. Fig. 5.73 (3) shows the proposed mechanism for P(3HO) infiltration which indicates efficient infiltration of micropores and microcracks as a result of the elastomeric property of the polymer.

5.3.3 Comparison of 45S5 Bioglass®/polymer composites: assessment in simulated body fluid

The bioactivity of the scaffolds was confirmed after immersion tests in SBF for 7, 14 and 28 days. From the SEM, EDS and XRD analyses of PDLLA, P(3HB) and P(3HO) coated scaffold, it was confirmed that the bioactivity is maintained after a certain period of immersion in SBF. SEM and EDS analyses were used to evaluate the precipitation of HA while the crystallinity of HA crystals was studied using XRD. The mechanism of formation of HA on bioactive glass based substrates was discussed in detail in the literature review (section 2.6.1). Different physical properties of the polymers used have an effect on the bioactivity of the coated scaffolds. Molecular weight and adhesion strength are thought to have the greatest effect on the bioactivity of the coated pellets and scaffolds. PDLLA which has the lowest Mw showed the fastest formation of HA compared to P(3HB) and P(3HO). In addition, the adhesion strength of PDLLA to the sintered Bioglass® substrate (Fig. 5.48) is lower than that of P(3HB) (Fig. 5.75).

Based on the experimental results on PDLLA and P(3HB) coated Bioglass® pellets, the mechanism of bioactivity is proposed to be related to the formation of cracks in the coatings, which will provide a channel for SBF to come into direct contact with the bioactive glass for effective ion exchange and formation of HA on the surface of the pellets. The same mechanism is active in all scaffolds because they were fabricated under the same conditions, although the thickness of the polymer layer is slightly different in each. The thickness of the PDLLA coating on the scaffolds is in the range 1-5µm following calculated values in a previous study [1], while the thickness of the polymer coating on the present sintered pellets, by optical
observation, is around 10µm (see Fig. 5.26). The differences in the polymer layer thickness is considered to be the reason explaining the fact that the kinetic of formation of HA is faster in PDLLA coated scaffolds as compared to PDLLA coated pellets after 3 weeks of immersion in SBF, which was assessed by comparing the intensity of the XRD peaks.

In addition, the PDLLA coated scaffolds reached a stoichoimetric Ca/P ratio of the HA layer formed (Ca/P =1.6-1.7) after 28 days immersion in SBF while the PDLLA coated pellets did not reach such stoichoimetric ratio after the same period of immersion in SBF. This result could also be due to the highly porous structure and high specific area of the coated scaffolds, therefore, faster and more extensive ion exchange can occur than in the bulk specimen as has been shown also by previous studies [195]. Examining SEM images, the needle-like nanostructure of HA was observed after 1 week in SBF for scaffolds coated with PDLLA (see Fig. 5.35).

A new finding was made in this study for the partially sintered PDLLA coated scaffolds by XRD analysis (see Fig. 5.37, 5.38, 5.39 and 5.40), showing that HA formation is faster in coated samples, in particular at day 7 for both partially and fully sintered scaffolds. The explanation for this behaviour could be that PDLLA surfaces are hydrolysed by water molecules in SBF, which convert ester linkages into surface carboxylic groups which dissociate to provide negatively charged carboxylate anions (COO\(^{-}\)) for binding with calcium ions (Ca\(^{2+}\)) [146]. The binding of these ions stimulates surface nucleation with subsequent HA crystal growth, similar to the mechanism proposed by Murphy et al. [196]. Thus the kinetic of HA formation is increased at the initial stage of immersion in SBF. However, it is anticipated that for long-term immersion in SBF, the kinetic of HA formation on the uncoated scaffolds would match that of the coated scaffolds, as indicated by Fig. 5.37, 5.38, 5.39 and 5.40. This is because the crystalline phase in this material (partially crystallised Bioglass\(^{®}\)) can transform into an amorphous HA phase, as shown in previous reports in the literature [53].

Considering P(3HB), which has a higher Mw of 885,000gmol\(^{-1}\) than PDLLA (Mw = 200,000 gmol\(^{-1}\)), the kinetic of HA formation on P(3HB) coated scaffolds is lower than that on PDLLA coated scaffolds, which is found by comparing the intensity of
results in a previous study [135].

P(3HO) coated scaffolds, where P(3HO) has the lowest rate of degradation as compared to PDLLA and P(3HB), are less bioactive than P(3HB) and PDLLA coated scaffolds as shown by the lower peaks intensity of Ca and P at SBF immersion time of 28 days as revealed by SEM-EDS (see Fig. 5.71). This reduced bioactivity of P(3HO) coated scaffolds is documented also by delays in the HA formation, by XRD analyses.(see Fig. 5.72)

5.4 Conclusions

Results presented in this chapter suggest that PDLLA, P(3HB) and P(3HO) coatings on Bioglass® based glass-ceramic scaffolds sintered at a relatively low sintering temperature of 1000°C for 2 hours increased the mechanical properties (compressive strength) of the 3D scaffolds, as compared to the as-sintered (non-coated) scaffolds. The coatings, leading to an interpenetrating network microstructure in partially
sintered scaffold improved also the scaffold bioactivity (in the case of PDLLA coatings). Most importantly, the observation of the fracture surface of PDLLA, P(3HB) and P(3HO) coated scaffolds showed that a microscale crack-bridging mechanism is active by the polymer fibrils at the crack opening zone. This effect is similar to the toughening mechanism in bone, which is based on crack bridging by collagen fibrils [179]. The proposed toughening mechanism for the partially sintered scaffolds coated with PDLLA, P(3HB) and P(3HO) is thought to be related to the glass transition (Tg) values of the polymers, which will effect the viscosity of the solution and also the degree of infiltration into the microcracks and micropores of the partially sintered scaffolds. The degree of infiltration of the different polymers into the micropores and microcracks is proposed to vary as follows: P(3HO)> PDLLA> P(3HB). The results show that 45S5 Bioglass® derived scaffolds coated with PDLLA, P(3HB) and P(3HO) maintained their bioactivity when tested in an SBF solution, but the onset time of the hydroxyapatite formation process was seen to depend on the type of polymer used for the coating. The onset time of the bioreactivity process varies as follows: PDLLA>P(3HB)>P(3HO), which could be related to the rate of degradation and to the molecular weight of the polymer phase used in each case.
Chapter Six

6 Design of bilayered scaffolds: Electrospun PDLLA nanofiber coated 45S5 Bioglass® substrates

6.1 Introduction

As the micro- and nano- architectures of scaffolds have been shown to play a key role in cell-biomaterial interaction, the significance of surface science and topographic design increases and several techniques based on electric field assisted processing are being developed to produce specific structural patterns on biomaterial surfaces [197], as well as to fabricate novel nanostructured scaffolds which may have enhanced performance in bone regeneration applications. Electric field assisted processing consists of several variants, one of which is electrospinning, which has attracted considerable attention over the last decade largely due to its applicability in the biomedical materials field [198]. The principle of the process, which was patented in 1934 [199] and is capable of producing ultra-fine fibres, is based on electrically charging a suspended droplet of polymer melt or solution. Details of the process and its applications in TE have been presented in the literature review (Chapter 2). In tissue engineering applications, electrospinning has been used to prepare scaffolds directly, as a continuous collection of randomly generated fibres or, alternatively, as ordered structures by controlling the deposition pattern [200]. Nanofibrous structures manufactured by electrospinning, for example, have a high surface to volume ratio exhibiting the potential to provide high surface area for improved cell attachment. This design of fibrous structures can be applied to a variety of polymers including polyurethane, PLA, PS, polymethylmethacrylate, PLGA, collagen and polycaprolactone. In this part of the investigation PDLLA was selected as the polymer to introduce fibrous layers in a bilayered scaffold design.

An alternative approach, in particular for bone tissue engineering, is to deposit the fibrous nano- or micro-structures onto a substrate material exhibiting intrinsically bioactive properties, e.g. formation of biologically active hydroxyapatite crystals in contact with physiological fluids [201]. One of such surface reactive materials is
bioactive glass [202]. In this case, the approach takes advantage of the rapid rate of surface reactions of bioactive glasses, which leads to fast bone bonding without the formation of scar tissue as discussed in detailed in the literature review (section 2.6.1).

A novel structure of electrospun PDLLA fibers deposited on 45S5 Bioglass® (sintered discs) is introduced in this chapter, which is proposed as the basic material combination for the development of bioactive scaffolds. To explore the preliminary applications of these structures, surface roughness and in-vitro (acellular) bioactivity in simulated body fluid (SBF) were investigated.

In addition, a novel structure of electrospun PDLLA fibers deposited on 3D 45S5 Bioglass® scaffolds is also introduced in this chapter, which is proposed as the basic structure for the development of bilayered scaffolds/constructs for osteochondral tissue engineering applications. The suitability of bilayered constructs for osteochondral tissue engineering was discussed in the literature review section (Section 2.5). To explore the preliminary applications of this bilayered construct, morphology, bioactivity in simulated body fluid (SBF) and cell response on the bilayered scaffolds were investigated.

6.2 Results

6.2.1 PDLLA fibre deposition on 45S5 Bioglass® sintered pellets

6.2.1.1 Processing

A series of parameters related to different formulations of PDLLA polymer solutions and to the electrospinning apparatus were preliminary conducted on glass slides, used as substrate, to obtain the optimised conditions for PDLLA nanofibres deposition on polished surfaces of 45S5 Bioglass® pellets. The PDLLA solutions for electrospinning were prepared by mixing PDLLA granules in dimethylcarbonate (DMC) in concentrations of 2.5 and 5.0 wt/v%. The experimental procedure for the electrospinning set-up has been described in Chapter 4 (section 4.9). A summary of
the parameters investigated, including polymer solution concentration, flow rate, voltage applied, distance between needle and substrate as well as deposition time are tabulated in Tables 6.1, 6.2 and 6.3.

Table 6.1  Electrospinning parameters for deposition of PDLLA fibres on glass slide substrate using 2.5 wt% PDLLA solution in DMC.

<table>
<thead>
<tr>
<th>PDLLA in DMC (2.5 wt%)</th>
<th>Electrospinning parameters</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Flowrate: 10µl/min</td>
<td>Glass slide</td>
</tr>
<tr>
<td></td>
<td>Voltage: 8-10kV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance: 8 cm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deposition time: 1 minute</td>
<td></td>
</tr>
<tr>
<td>2)</td>
<td>Flowrate: 5µl/min</td>
<td>Glass slide</td>
</tr>
<tr>
<td></td>
<td>Voltage: 8-10kV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance: 8 cm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deposition time: 1 minute</td>
<td></td>
</tr>
<tr>
<td>3)</td>
<td>Flowrate: 5µl/min</td>
<td>Glass slide</td>
</tr>
<tr>
<td></td>
<td>Voltage: 10-12kV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance: 8 cm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deposition time: 1 minute</td>
<td></td>
</tr>
<tr>
<td>4)</td>
<td>Flowrate: 2 µl/min</td>
<td>Glass slide</td>
</tr>
<tr>
<td></td>
<td>Voltage: 10-12kV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance: 8 cm</td>
<td></td>
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<tr>
<td></td>
<td>Deposition time: 1 minute</td>
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</tbody>
</table>

Table 6.2  Electrospinning parameters for deposition of PDLLA fibres on glass slide substrate using 5wt% PDLLA solution in DMC.

<table>
<thead>
<tr>
<th>PDLLA in DMC (5 wt%)</th>
<th>Electrospinning parameters</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>5)</td>
<td>Flowrate: 10µl/min</td>
<td>Glass Slide</td>
</tr>
<tr>
<td></td>
<td>Voltage: 8-10kV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance: 8 cm</td>
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</tr>
<tr>
<td></td>
<td>Deposition time: 1 minute</td>
<td></td>
</tr>
</tbody>
</table>
| 6) | Flowrate: 5µl/min  
Voltage: 8-10kV  
Distance: 8 cm  
Deposition time: 1 minute | Glass Slide |
|---|---|---|
| 7) | Flowrate: 3µl/min  
Voltage: 10-12kV  
Distance: 8 cm  
Deposition time: 1 minute | Glass Slide |
| 8) | Flowrate: 2µl/min  
Voltage: 10-12kV  
Distance: 8 cm  
Deposition time: 1 minute | Glass Slide |
| 9) | Flowrate: 2µl/min  
Voltage: 12-14kV  
Distance: 8 cm  
Deposition time: 1 minute | Glass Slide |

Table 6.3 Optimum conditions for the electrospinning parameters of PDLLA fibers on Bioglass® pellets using 5wt% PDLLA solution in DMC.

<table>
<thead>
<tr>
<th>PDLLA in DMC (5 wt%)</th>
<th>Electrospinning parameters</th>
<th>Substrate</th>
</tr>
</thead>
</table>
| 10) | Flowrate: 5µl/min  
Voltage: 15 kV  
Distance: 15 cm  
Deposition time: 5 and 10 minute | 45S5 Bioglass® pellet |

6.2.1.2  **Microstructure analysis**

From SEM images of electrospun PDLLA nanofibers on glass slides and on polished 45S5 Bioglass® pellets, as shown in Figure 6.1, 6.2 and 6.3, it becomes clear that the morphology of PDLLA nanofibers depends strongly on the parameters chosen for the
electrospinning process. For example, a 2.5wt% solution of PDLLA at different flow rates of 10µl/min and 5µl/min, respectively (i.e. parameters (1) and (2) (Table 6.1)), but working at the same voltage (i.e. 8-10kV) at fixed collection distance (8 cm), led to different results. At a lower flow rate (5µl/min), the droplet sizes are smaller compared to 10 µl/min flow rate (see Fig. 6.1 (A) and (B)). As the voltage is increased (10-12kV) and the flow rate is reduced, the formation of both nanofibers and droplets was observed (see Fig. 6.1 (C)). From this observation, it is suggested that the emergence of fibers can occur when the flow rate is relatively low (i.e. < 5µl/min) while the voltage is increased (i.e. > 10-12kV) for the 2.5 wt% PDLLA solution.

The same pattern was observed for the 5wt% PDLLA suspension; e.g. the flow rate decreased while the voltage increased (i.e. parameter 5,6,7,8 and 9 in Table 6.2). SEM images of electrospun PDLLA fibres on 45S5 Bioglass® pellets and on glass slides are shown in Fig.6.3 and 6.2, respectively. It is confirmed that the morphology of PDLLA fibres depends strongly on the selection of the right combination of parameters for the electrospinning process and on the solvents used. There are several explanations as to why transitional structures such as beads arise in electrospinning, e.g. polymer content, applied voltage and flow rates [203]. As the flow rates were reduced from 10 µl/min (Figure 6.2(A)) to 3µl/min (Figure 6.2(B)) and 2 µl/min (Figure 6.2(C)) and the applied voltage was gradually increased from 8-10 kV (Figure 6.2(A)) to 10-12 kV (Figure 6.2(B)(C)) and 12-14kV (Figure 6.2(D)), a higher number of PDLLA fibres and fewer beads were formed for the 5%wt/v PDLLA-DMC solution. Fig. 6.2(D) shows the fibrous structure obtained at the lowest flow rate (2µl/min) and highest applied voltage (12-14 kV) investigated using glass slides as substrate.

Using the same solution, uniform PDLLA fibres (Figures 6.3(A) and 6.3(B)) were obtained on the Bioglass® substrate at a flow rate of 5µl/min and operating voltage of 15kV, selecting a collecting distance of 15 cm from the tip of the needle (see Figure 4.4). The density of the fibre network was seen to increase with deposition time, as shown in Figures 6.3(A) and 6.3(B), although the structural integrity of very dense mats was compromised with the collection of excess un-evaporated solvent. The diameter of the uniform PDLLA fibres is seen to vary between ~100nm and ~0.2µm. This result is comparable to other recent studies which have yielded fibres in the same
range [204]. However results from another investigation, which used a less volatile solvent system (DMF-THF) than the one utilised here (DMC), indicated that fibres with diameters as large as 1.5 µm could be produced [205]. The differences in fibre size are also related to the concentration of the polymer in the solvent. Polymer concentration values of ~5 wt/v% can also result in nano-fibres, as evident from literature results [204;206]

Figure 6.1 SEM images of PDLLA deposition on glass slides using a suspension of 2.5wt% PDLLA in DMC at different electrospinning parameters A = Flow rate (10µl/min), Voltage (8-10kV), B = Flow rate (5µl/min), Voltage (8-10kV) and C = Flow rate (2µl/min), Voltage (10-12kV), deposited for 1 minute. (See Table.6.1)
Figure 6.2 SEM images of PDLLA deposition on glass slides using a suspension of 5wt% PDLLA in DMC at different electrospinning parameters A = Flow rate (10µl/min), Voltage (8-10kV); B = Flow rate (3µl/min), Voltage (10-12kV); C = Flow rate (2µl/min), Voltage (10-12kV); D = Flow rate (2µl/min), Voltage (12-14kV) deposited for 1 minute. (See Table 6.2)

Figure 6.3 SEM images of PDLLA fibres deposited on 45S5 Bioglass® at optimum electrospinning parameters of Flow rate = 5µl/min and Voltage = 15kV: (A) Partially aligned fibres (5 minutes deposition time) and (B) Random fibres, using a suspension of 5wt% PDLLA in DMC (10 minutes deposition time). (See Table 6.3)
6.2.1.3 Surface topography measurement using white light interferometer

Three measurements were taken by white light interferometry (Zygo®) on the surface of PDLLA nanofibers deposited for 30 minutes on 45S5 Bioglass® substrates using the optimum conditions of electrospinning parameters discussed in the previous section. This measurement was carried out to investigate the surface roughness of the 45S5 Bioglass® substrate coated with nanofibers, as shown in Figure 6.4. The three measurements of the surface roughness gave results of rms values of 1.36 ± 0.05µm and ra values of 0.72 ± 0.05µm. The results indicate that the surface roughness increased due to deposition of PDLLA nanofibers which can be confirmed from the 3D plot (see Fig. 6.4 B), it can be also observed that there was a fairly homogenous distribution of the nanofibers deposited on the 45S5 Bioglass® surface in the area of 1.45mm x 1.09 mm (see Fig.6.4 A). As indicated by the colour scale, fibres which are in the micron size scale were able to be detected by the white light interferometer. Fibres which are in nanoscale cannot be detected by using this technique. For comparison, the rms value of the polished 45S5 Bioglass® pellets (Fig. 6.5) used as substrate was only 0.10 ± 0.05 µm and the ra values was 0.08 ± 0.05µm. From the 3D plot, the surface area is seen to be fairly smooth (see Fig. 6.5 B), as compared to the PDLLA fibre deposited surface (see Fig. 6.4 B).

![Figure 6.4](image)

**Figure 6.4** 3-D plot and scales in colour of the 45S5 Bioglass® surface coated with PDLLA fibers, deposited for 30 minutes obtained by white light interferometry (Zygo®).
Figure 6.5 Scheme showing (A) surface profile and scales in colour and (B) 3D representation of the polished 45S5 Bioglass® surface, obtained by white light interferometry.

6.2.1.4 SBF bioactivity studies.

The assessment of the bioactivity of PDLLA fibre coated 45S5 Bioglass® substrates was carried out in SBF (as described in Section 4.10.4). Briefly, disc of 10 mm in diameter (sintered Bioglass® pellets) coated with PDLLA fibers (deposited for 10 minutes) were immersed in 30 ml SBF for up to 28 days. Samples were characterised by SEM, EDS and XRD.
Figure 6.6 SEM images of PDLLA fibres deposited on 45S5 Bioglass® sintered discs after immersion in SBF: A, A1) 7 days, B, B1) 14 days and C, C1) 28 days at different magnifications, showing formation of HA indicated by the needle-like nanostructure.

From SEM images, it can be observed that there was formation of HA crystals on the disc substrates as well as on PDLLA fibres after 7, 14 and 28 days of immersion in SBF, as shown in Figure 6.6. At higher magnification, it was confirmed that hydroxyapatite crystals have formed a homogenous coating around the PDLLA fibres, as shown in Figure 6.6 A1, B1 and C1. HA crystals and clusters were also formed on the 45S5 Bioglass® sintered substrates after immersion in SBF for 7, 14 and 28 days, as shown in Figure 6.6 A, B and C. It was observed that the HA layer on the PDLLA fibres was thicker than that on the 45S5 Bioglass® substrates due to high volume to surface ratio (see Fig. 6.6 C) provided by the fibres. It was also observed that the size of the PDLLA fibres increased as the immersion time in SBF increased due to the formation of a thicker hydroxyapatite layer, as expected. The SEM image in Fig. 6.7 shows that the HA can also grew homogenously on PDLLA fibres, which were not in direct contact with the 45S5 Bioglass® substrates, as indicated by the arrow.
XRD spectra obtained on PDLLA fibre coated 45S5 Bioglass® sintered substrates after 7, 14 and 28 days immersion in SBF are shown in Figure 6.8. The peak at $2\theta = 32^\circ$ is the characteristic peak of crystalline hydroxyapatite (HA). The XRD spectrum of the material before immersion in SBF is also shown for comparison, where peaks corresponding to the Na$_2$Ca$_2$Si$_3$O$_9$ phase are indicated. The presence of Na$_2$Ca$_2$Si$_3$O$_9$ is typical in the present bioactive glass-ceramics fabricated from Bioglass® by sintering at $T>900^\circ$C [53], as discussed in Chapter 5. The PDLLA fibres are completely amorphous and cannot be detected by XRD (see Fig. 6.9). The HA formation on the samples is seen to occur after 7 days immersion in SBF, as shown by the XRD spectrum in Figure 6.8. There is an increased in the peak height at $2\theta = 32^\circ$ as the immersion time increased from 7 to 28 days.

The chemical composition of the HA formed on the PDLLA fibres was identified by EDS analysis, as shown in Figure 6.10. Calcium and phosphorus as the predominant elements identified in the EDS spectrum (in a ratio of approximately 1:50) confirm the presence of HA on the surface of PDLLA fibres as well as on the 45S5 Bioglass® substrate (in a ratio of approximately 1:52) (Figure 6.10).
Figure 6.8 XRD spectra showing the peaks corresponding to PDLLA fibre coated 45S5 Bioglass® specimens before and after immersion in SBF. The major peaks of $\text{Na}_2\text{Ca}_2\text{Si}_3\text{O}_9$ phase and hydroxyapatite (HA) are marked by ($\Delta$) and ($\circ$), respectively.

Figure 6.9 XRD spectra of PDLLA films: A) as fabricated and B) after immersion in SBF for 2 weeks. The PDLLA film after immersion in SBF shows no HA formation on the surface.
Figure 6.10 EDS analysis of the surface of a Bioglass® pellet coated with PDLLA fibers after 28 days in SBF, showing the presence of P and Ca, indicating the presence of a HA layer.

These results indicate that PDLLA fibres can be homogenously mineralised on the surface of 45S5 Bioglass® in contact with SBF. These results show thus for the first time the introduction of a fibrous HA topography on the polished surface of 45S5 Bioglass® sintered pellets using electrospun PDLLA fibres as a template. The rough fibrous topography should induce better cell attachment and strong bonding to new bone, following evidence in the literature [201]. On the contrary, PDLLA fibres (and scaffolds) on their own are not bioactive, as demonstrated by XRD analysis (Fig. 6.9) and in previous research [207].

Although rough surface topographies, produced for example by sandblasting methods, have been shown to significantly improve cell adhesion to biomedical implants [208], it is also possible that they can limit cell spreading and may lead to scar formation
It should be also mentioned that the literature is scarce regarding the detailed investigation of cell/surface interactions and cell adhesion on bioactive glass substrates with controlled surface topography [209;210]. In one of the few studies available, Levy et al. [209] used sandblasting technique to induce surface roughness on cast Bioglass® discs to create topographies of Rms ~ 794 nm. They showed that surface texture had an effect on cell (MC3T3 cells) spreading, this being lower on rough 45S5 Bioglass® surfaces during the initial stages of cell adhesion, however the rough surfaces provided numerous sites for cell attachment. In their work, however, the surface pattern morphology on the Bioglass® surface was not controlled, as opposite to the present investigation where a fibrous surface arrangement can be produced with high degree of control by tailoring the diameter and orientation of the electrospun PDLLA fibres.

In summary, amorphous PDLLA was electrospun on sintered Bioglass® based glass-ceramic surfaces forming fibrous structures. Processing conditions of the electrospinning method such as flow rate and voltage were the key parameters controlling fibre dimensions and morphology. These parameters were optimised to reduce the formation of undesired beads. PDLLA fibres deposited on 45S5 Bioglass® pellets were found to introduce a rough fibrous topography on the polished surface of the substrate and this controlled roughness should potentially encourage cell attachment. From the SBF studies, it was found that HA can grow on the PDLLA fibres after 7 days of immersion in SBF, with uniform HA covering the fibrous structure at day 14. This process provides a biologically active fibrous-like calcium phosphate patterned surface for the attachment of bone cells and for strong bonding to new bone. Recent investigations by Li et al.[211] have utilised a novel jet-based patterning technique, template-assisted electrohydrodynamic atomization spraying, to control the surface structure as well as dimensions of HA coatings on Ti substrates. A range of nano-hydroxyapatite (nHA) line-shaped patterns < 20 µm in width were successfully deposited and it was confirmed that the HA patterns were capable of regulating human osteoblast cell attachment and orientation.
This part of the investigation led also to study the deposition of PDLLA fibrous structures on 3D bioactive glass scaffolds. In this case, the novel bilayered scaffolds produced are intended for osteochondral tissue engineering, whose basic principles were described in Section 2.5 (literature review). The experimental results are presented in the next sections.

6.2.2 PDLLA fibre deposition on 45S5 Bioglass® scaffolds

6.2.2.1 Motivation

In this part of the project the research focus was on engineering the surface of 45S5 Bioglass® scaffolds by coating with PDLLA fibres using electrospinning in order to fabricate bilayered constructs suitable for osteochondral tissue engineering. The structure of the proposed bilayered scaffolds is schematically shown in Figure 6.11. The experimental detail of the electrospinning method has been presented in Chapter 4 (Section 4.9).

Figure 6.11 Schematic diagram showing A) bilayered structure scaffold covered with a thin fibrous mesh of PDLLA fibres obtained by electrospinning and B) the applications of the bilayered scaffolds in the osteochondral defect. (Diagram in (B) has been adapted from [212])

Using the optimised electrospinning parameters obtained from the PDLLA fibre coatings on the Bioglass® pellets as a substrate (section 6.2.1), the same parameters
were used to deposit PDLLA fibers on the 45S5 Bioglass® scaffolds. For the preliminary test, a fully sintered scaffold, i.e. sintered at 1100°C for 2 hours (section 6.2.2.2), was chosen as the substrate. For the optimum condition of PDLLA fibre deposition, sintered scaffolds (1000°C, 2 hours) which had been previously coated with 5wt% PDLLA and sintered at 1000°C for 2 hours were used as substrates considering that these coated scaffolds were developed to mimic the natural bone consisting of organic and inorganic phases (section 6.2.2.3). The mechanical properties of the scaffolds used were investigated and comprehensively discussed in Chapter 5. It was determined that mechanical properties and porosity of the 45S5 Bioglass® derived glass-ceramic scaffolds are acceptable for bone tissue engineering applications, as discussed in Chapter 5. Thus these scaffolds were selected for this part of the investigation, e.g. the PDLLA coated bioactive glass-ceramic scaffolds would represent the scaffold section forming bone tissue in an osteochondral tissue engineering strategy [35] (see Fig. 6.11)

6.2.2.2 Microstructure analysis of PDLLA fibres on 45S5 Bioglass® scaffolds by SEM.

Fig. 6.12 shows SEM images of the PDLLA fibrous deposition on 45S5 Bioglass® scaffolds. From the microstructure of the PDLLA fibres deposited on 45S5 Bioglass® scaffolds, it was observed that the fibrous mesh became denser when deposition time increased from 10 minutes to 2 hours, as shown in Fig. 6.12 A, B, and C. From the SEM images, it can also be seen that the PDLLA fibres were deposited on the struts of the Bioglass® scaffolds, as indicated by the red circle in Figure 6.12 (A-C). At higher SEM magnification, the increased density of the fibrous mesh on the struts with increasing deposition time becomes evident (see Figure 6.12 A1, B1 and C1). This observation can suggest that good bonding exists between the two layers, which is known to be a requisite to assure good integrity and functionality of the osteochondral constructs. Moreover, SEM examination of the 45S5 Bioglass® scaffolds revealed that pores are highly interconnected and possess a size in the range of 50 to 500 microns (6.12 A)
Figure 6.12 SEM images showing the PDLLA fibrous deposition on 45S5 Bioglass® scaffolds (uncoated) at different deposition times: A) 10 mins, B) 30 mins, C) 2 hours, and A1) 10 mins, B1) 30 mins and C1) 2 hours at higher magnification of the area’s showed by the circles.

Fig. 6.13 shows SEM images of the different orientations of the PDLLA fibers on the 45S5 Bioglass® scaffolds. Fig. 6.13 B suggests that the PDLLA fibre orientation on the struts was random. On the other hand, fibres deposited between strut points are
seen to be partially aligned (see Fig. 6.13 C). This pattern was formed due to the structure of the underlying porous 45S5 Bioglass® scaffolds and it was not observed in fibre depositions on planar surfaces, described above (section 6.2.1).

Figure 6.13 SEM images showing different fibre orientations at different positions of the PDLLA fibres on the surface of 45S5 Bioglass® scaffolds deposited for 2 hours showed by the circles.

Fig. 6.14A shows a SEM image of the interface in a PDLLA/45S5 Bioglass® scaffold deposited for 2 hours, where it is possible to observe the two distinct porous layers. Fig. 6.14 B shows the PDLLA fibrous layer at higher magnification. The estimation of the PDLLA fibrous layer thickness was obtained from SEM observation of the interface of the bilayered scaffolds (see Fig. 6.14 B). The fibrous layer thickness was estimated to be around 150 µm, as can be appreciated in Fig. 6.14 B. Fig. 6.15 shows a composite SEM image revealing the typical complete transverse section of the bilayered scaffolds.
Figure 6.14 SEM images showing the microstructure of a PDLLA/45S5 Bioglass® bilayered scaffold (coated): A) interface of the 45S5 Bioglass® scaffold coated with PDLLA fibre for 2 hours, B) layer of PDLLA fibres observed at higher magnification of the circled area in A).

Figure 6.15 SEM images showing the cross-section of the PDLLA/45S5 Bioglass® composites bilayered scaffolds. (The PDLLA fibre layer was deposited for 30 minutes)
6.2.2.3 SBF bioactivity studies

The bioactivity assessment of bilayered scaffolds constituted by PDLLA fibres deposited on the surface of 45S5 Bioglass® derived glass-ceramic scaffolds, obtained at different deposition times of 10 minutes, 30 minutes and 2 hours after immersion in SBF for 7, 14 and 28 days, is described in this section.

i) Microstructure and EDS analysis.

Fig. 6.16 shows SEM images of the PDLLA fibres on a 45S5 Bioglass® scaffold (coated) deposited for 10 minutes after immersion in SBF for 7, 14 and 28 days. There is formation of hydroxyapatite crystals on the PDLLA fibres after 7 days immersion in SBF, as shown in Fig. 6.16 A. The PDLLA fibres became coated with more HA crystals when immersion time in SBF increased from 14 to 28 days, as can be seen in Fig. 6.16 B and 6.16 C, as expected. EDS spectra on PDLLA fibres deposited for 10 minutes after immersion in SBF for 14 days (Fig. 6.16 B) show the presence of peaks corresponding to Ca and P, confirming calcium phosphate formation. Different Ca/P ratios at different locations indicate that the kinetic of HA formation was different, which is probably due to the different orientation and position of the fibres relative to the scaffolds substrate (see Fig. 6.17). The Au peaks are due to the gold coating used for SEM sample preparation. Thus it can be concluded that PDLLA fibres deposited for 10 minutes become mineralised after immersion in SBF for 7 days and longer periods. This result was obviously expected based on the evidence presented above (section 6.2.1), indicating the mineralisation of PDLLA fibres on sintered Bioglass® substrates upon immersion in SBF.
Figure 6.16 SEM images of PDLLA fibres on 45S5 Bioglass® based scaffolds (coated) deposited for 10 mins after immersion in SBF for A) 7, B) 14 and C) 28 days.
Figure 6.17 EDS analysis on PDLLA fibres deposited on 45S5 Bioglass® scaffolds (coated) electrospun for 10 minutes and immersed in SBF for 14 days. The spectra correspond to the positions indicated on the SEM image.

Fig. 6.18 (A-C) shows SEM images of PDLLA fibres on 45S5 Bioglass® scaffolds (coated) deposited for 30 minutes after immersion in SBF for 7, 14 and 28 days. From the SEM images it can be observed that on some PDLLA fibres no HA formation has occurred after 7, 14 and 28 days immersion in SBF. Thus it can be concluded that as the PDLLA deposition time increases from 10 to 30 minutes the thickness of the PDLLA fibrous layer increases (see section above) and therefore, hydroxyapatite formation on the PDLLA fibres located on the top layer was reduced as the fibres are not in direct contact with the bioactive glass surface.
Figure 6.18 SEM images of PDLLA fibres on 45S5 Bioglass® (coated) scaffolds deposited for 30 mins after immersion in SBF for A) 7, B) 14 and C) 28 days.

Fig. 6.19 shows SEM images of the PDLLA fibres on a 45S5 Bioglass® scaffold (coated with 5 wt% PDLLA) deposited for 2 hours after immersion in SBF for 7, 14 and 28 days. From the SEM images, it is seen that there was no HA formation on the PDLLA fibers. EDS spectra of the PDLLA fibers deposited for 2 hours and after immersion in SBF for 7, 14 and 28 days also confirmed that no HA has formed on the PDLLA fibres, showing only the C and O peaks corresponding to PDLLA fibres. Thus it can be concluded that PDLLA fibre layers deposited on Bioglass® based scaffolds for 2 hours do not mineralise after immersion in SBF for 7, 14 and 28 days. This is a very important result for the application of the present bilayered composites (Figure 6.11) in osteochondral tissue engineering, since it is required that the cartilage side does not mineralise, e.g. where the PDLLA mesh should support the attachment, growth and proliferation of chondrocytes only.
In summary, SEM observation and EDS analysis have confirmed that formation of HA (indicating bioactivity) on PDLLA fibres when immersed in SBF was reduced as the PDLLA fibre deposition time increased from 10 minutes to 2 hours, and the thickness of the fibrous PDLLA layer increased from 2 micron to 150 micron.

Figure 6.19 SEM and EDS results on PDLLA fibres deposited on 45S5 Bioglass® scaffolds (coated with 5 wt% PDLLA) deposited for 2 hours after immersion in SBF for A) 7, B) 14 and C) 28 days and EDS spectra after immersion in SBF for A1) 7, B1) 14 and C1) 28 days.
**ii) Final development of osteochondral scaffolds.**

Based on the previous results, it is proposed that the optimal scaffolds for osteochondral tissue engineering should have a structure as shown schematically in Figure 6.20.

![Figure 6.20 Schematic diagram showing the development of the interface of a bilayered scaffold A) as fabricated, B) after immersion in SBF for 14 days.](image-url)
Fig. 6.20 A shows schematically the expected structure of the bilayered scaffolds formed by PDLLA fibres deposited for 2 hours on the basic 3D Bioglass® scaffold coated with PDLLA. The PDLLA fibrous layer should reach in this case ~150µm in thickness. After immersion in SBF for 14 days, PDLLA fibres which are in direct contact with the 45S5 Bioglass® scaffold substrate will become mineralised thereby increasing the bonding strength at the PDLLA fibre / Bioglass® scaffold interface, as indicated Fig. 6.20 B. The mineralisation of the PDLLA fibres will decrease with increasing distance from the interface and it will be completely suppressed in the outer PDLLA fibre layers. From the EDS analysis reported in the previous section, it was confirmed that there is no HA formation on the fibres in the top layer after immersion in SBF for up to 28 days. This mechanism is very important to ensure: 1) strong bonding of the PDLLA layer to the bioactive glass substrate and 2) smooth transition at the interface given by the gradual change of HA layer formation dependent on the distance from the Bioglass® scaffold surface.

6.2.3 Cell culture assessment of the bilayered osteochondral scaffolds

This section presents a preliminary assessment of the cell compatibility of PDLLA fibre coated bilayered scaffolds for potential osteochondral tissue engineering applications. The main aim of this section is to report, for the first time, the cell-biomaterial interactions on these bilayered scaffolds in terms of chondrocyte attachment and spreading, which has been characterised using SEM, and the cell proliferation study characterised using the Alamar Blue Assay. The aim was to assess the material suitability for osteochondral applications on both 2D 45S5 Bioglass® sintered pellets and on 3D bilayered Bioglass®/PDLLA composite scaffolds surfaces. The ADTC5 cell line was chosen in this study due to its proven performance as an ideal cell line for development of tissue engineering strategies aimed at cartilage generation [213].
6.2.3.1 Cell proliferation on 2D surfaces (pellets).

Cell proliferation was quantitatively measured using the Alamar Blue assay on various components such as sintered 45S5 Bioglass® sintered pellets coated with PDLLA nanofibers, soda-lime glass slides coated with PDLLA nanofibres and soda-lime glass slides only. Tissue culture plastic (TCP) was used as a control surface. The soda-lime glass slide substrate was used to compare the cell behaviour on a planar, bioinert and favourable type of inorganic substrate for cell attachments other than TCP. The data are illustrated in Fig 6.21. Statistical analysis using ANOVA graph of the mean cell growth plotted against the days for each material gave no indication of an interaction between materials and days. In addition, the results of the statistical analyses indicated that there were significant differences between TCP and each of the individual materials tested (p ≤ 0.05) (refer to appendix table 1), showing that proliferation was lower than on TCP. This could be due to high cell affinity towards the TCP due to its high surface charge.

SEM observations reveal that cells attached and were able to spread differently on the different types of samples. For example, in Fig 6.22 A) the cells on a PDLLA fibre coated glass slide tend to spread broadly and in a flat fashion (as shown by the arrow). Conversely, the cells attached on a PDLLA fibre coated sintered 45S5 Bioglass® pellet tend to be more elongated, as shown in Fig 6.22 B). This effect could be due to the differences of the substrate surface components. However, there was no significant difference between cell proliferations on planar glass slides compared to glass slides covered with PDLLA fibres, suggesting that the proliferation on nanofibres is activated by the same mechanism from that on planar surfaces of glass slides. Some beads appeared to be present on the PDLLA fibres, which could be due to non-optimum condition of the electrospinning parameter during the fabrication of the fibrous PDLLA layer, as previously discussed in section 6.2.1.1. It should be pointed out that if even one of the parameters was less than optimal, irregular beads containing fibres could form.
Figure 6.21 Cell proliferation data up to 7 days on the different materials investigated. (Values are presented by mean ± standard deviation (s.d) where N = 6 samples)

Figure 6.22 SEM images of the ATDC-5 cell adherence and spreading on PDLLA fibres: A) on glass slide and B) on sintered 45S5 Bioglass® pellets (cells are indicated by arrows).
6.2.3.3 Cell proliferation on 3D surfaces (Bilayered scaffolds)

**Figure 6.23** Cell proliferation data up to 10 days for 45S5 Bioglass®/PDLLA composites with and without PDLLA fibres deposited on the scaffolds. (Values are presented by mean ± standard deviation (s.d) where N = 6 samples).

For this part of the investigation, 3D 45S5 Bioglass®/PDLLA scaffolds coated with PDLLA fibres (bilayered scaffolds) fabricated using the optimum conditions of electrospinning were used, as previously discussed in section 6.2.1.1. Scaffolds without the fibre deposition were also investigated for comparison purposes. The cells were cultured for up to 10 days. Cell proliferation was quantitatively measured using the Alamar Blue assay. The data are illustrated in Fig. 6.23. Statistical analyses showed that there were clear differences indicating an interaction between the test material used and the culture time point ($p \leq 0.001$). This is perhaps not surprising given the degradable nature of these dynamic materials with time in culture. Furthermore, the analysis suggests that the 45S5 Bioglass® scaffolds coated with PDLLA fibres supported greater cell growth by day 10 in culture compared with control surface ($p \leq 0.05$) (refer to appendix Table 2).
At days 1, 7 and 14, selected cell-seeded bilayered scaffolds were fixed and were observed by SEM. Fig. 6.24 A) shows that at day 1, several chondrocyte cells attached and proliferated on the surface of the PDLLA fibres of the bilayered scaffolds. Rounded cells suggest dividing cells during the process of cytokinesis. At higher magnification, it can be observed that cells are clearly attached to the fibres and formed a three dimensional cell-matrix network, as shown in Fig. 6.24 B). In particular at day 7, many cells are clearly attaching and spreading to and amongst the fibres and also communicating with one another using shared cytoplasmic filaments (cell-cell interaction), as shown in Fig. 6.25 A) such that they could only be recognised at high magnification (see Fig. 6.25 B). In comparison to day 1, the cells proliferated and spread into a larger area and formed a three dimensional cell-matrix network. The cells also showed guided growth according to the PDLLA fibre orientations, giving rise to a three dimensional and multi-cellular network guided by the architecture of the fibrous scaffolds, as shown in Fig. 6.25 B). At day 14, the cell spreading and proliferation increased as can be seen from the higher density of cells (darker phase), as shown in Fig. 6.26. The cells appeared to adhere to the fibres, and they seem to have started to migrate through the pores and grow within layers of fibres as indicated in Fig. 6.26. These results confirm that the PDLLA fibres deposited for 2 h using the optimum condition of the electrospinning parameters are suitable as a conducive substrate for chondrocyte cells and the cells were able to attach, proliferate and migrate within the 3D network of the fibrous PDLLA.
Figure 6.24 SEM images showing of the ATDC5-cell adherence and proliferation on PDLLA mesh of bilayered scaffolds at different magnifications at day 1: A) 100 µm, B) 30 µm scale. (Cells are indicated by arrows).
Figure 6.25 SEM images showing the ATDC5-cell proliferation and spreading on PDLLA mesh of the bilayered scaffolds at different magnification at day 7: A) 300 µm, B) 60 µm scale. (Cells are indicated by arrows)
Figure 6.26 SEM images showing the ADTC5-cells migrate through the pore and grew within layers of fibrous network at day 14.

6.3 Discussion

In this Chapter, we have assessed the potential application of a fibrous PDLLA matrix combined with Bioglass® substrates as suitable scaffold for osteochondral TE applications. By means of electrospinning, uniform PDLLA fibre meshes were produced with fibre diameters in the range between ~100nm and ~0.2µm to form a three-dimensional matrix architecture on 2-D sintered 45S5 Bioglass® pellets and on 3D 45S5 Bioglass® based composite scaffolds. Results showed that the PDLLA fibrous matrix deposited on 3D Bioglass® composite scaffolds, as a novel bilayered 3D composite construct, increased chondrocytes cell attachment and proliferation. Increased surface roughness due to electrospun fibres should lead to better cell attachment and proliferation of chondrocytes cells [79;214]. White light interferometry and SEM images confirmed qualitatively and quantitatively that the topography (roughness) of the 2D 45S5 Bioglass® sintered pellets and 3D bilayered 45S5 Bioglass® composite scaffolds have been altered due to the deposition of PDLLA fibres by electrospinning (see Fig. 6.4 and Fig. 6.12). The surface roughness
increased more than 13 times in terms of the rms value for 2D Bioglass® sintered pellets coated with PDLLA fibres. This result is an agreement with the study by Chen et al. [215] who modified the surface of poly(e-caprolactone) membranes via electrospinning. Topography (roughness) of the porous bilayered scaffolds could not be measured by white light interferometry because the surface was not flat. To produce a uniform fibrous layer, we have optimised the fabrication process and fine-tuning the electrospinning parameters, such as polymer viscosity, voltage, flow rate and electric field, as previously discussed in section 6.2.1.1. It should be pointed out that if even one of these parameters was less than optimal, irregular, bead-containing fibres could form, suggesting their coordinated requirement during the electrospinning process. One of the difficulties in the processing technique was to monitor the formation of fibres on the sintered pellets and scaffolds due to the opaque colour of the substrate, thus fibres were also deposited on glass slides to determine the optimal fibre formation and morphology. It was also observed that the fibre formation stability did not last for long period of time (less than 5 minutes) due to the instability of the jet formation at the needle tip. Therefore, deposition of PDLLA fibres needs to be done intermittently after 5 minutes. This process also allows enough time for solvent to be evaporated for the next PDLLA layer deposition. Nevertheless, the optimum electrospinning parameters were obtained using flow rate = 5µl/min, voltage = 15kV, 5wt% PDLLA concentration, distance of application of the electric field = 15 cm and 2 hours deposition time. Optimal fibre fabrication parameters are specific for a particular poly(α-hydroxy ester) [216].

A scaffold intended to be used in TE needs to have surface resemblance of the ECM found in the human body (literature review, section 2.4) and the incorporation of a fibrous layer aims at mimicking, to some extent, the natural matrix for the osteochondral TE applications. The fabrication of fibrous layers by electropinning for cartilage TE applications has been extensively investigated, as previously described in the literature review (section 2.10.2). Results in this chapter show that the deposition of a PDLLA fibrous layer on the surface of 3D Bioglass® composite scaffolds by electrospinning could be controlled reaching 150 µm in thickness which is sufficient to prevent HA mineralization from occurring. This effect is significant for the application of the bilayered constructs for osteochondral TE applications, which should not show mineralisation on the cartilage side. This is an agreement with
previous study by Reis et al. [217]. The risk of possible delamination between both the polymeric and composite constituents, respectively, was avoided since the bilayered constructs showed an integrated continuous interface assessed by SEM images and SBF in vitro assessment, as discussed in sections 6.2.2.2 and 6.2.2.3. It is hypothesized that the residue solvent could enhance the adhesion of PDLLA fibres to the PDLLA coated 45S5 Bioglass® scaffold substrate, forming a strong bond, according to previous results reported by Chen et al.[215]. The quantification of the interfacial adhesion strength has not been covered in the thesis and needs dedicated further investigation in the future. Nevertheless the bilayered scaffolds developed here were qualitatively robust and of adequate structural integrity for their handling for material characterisation and for cell culture studies. The micro features of biodegradable polymer scaffolds, in addition to the chemical composition and polymer molecular structure, are the primary determinants of the degradation profile of a 3D tissue-engineering scaffolds [214], although macro features such as the morphology and architecture of the scaffold (e.g. porosity) are also important factors. Apart from the materials aspects, the environment (biochemistry) in which the material is placed is vitally important. In relation to our scaffolds, ultra-fine electrospun fibres would be more susceptible to hydrolysis compared with larger fibres when used in vitro and in vivo. The results showed that PDLLA fibers can maintain their structural stability in SBF and in cell culture media for up to 14 days, as shown by the SEM images in Figure 6.19 and Figure 6.26, respectively.

Moreover PDLLA fibre roughness is hypothesized to act as anchorage for cells, to enhance cellular adhesion and spreading. A wide variety of cells, including chondrocytes, are able to detect changes in the surface topography and approaches to topographical control of cells have been reviewed by Wilkinson et al.[218]. Nevertheless, the present results showed that cells proliferated better on 3D 45S5 Bioglass® composite scaffolds compared to 2D sintered Bioglass® pellets deposited with PDLLA fibrous layers (see Fig. 6.21 and Fig. 6.23), respectively, which indicates that roughness might be a secondary factor in the control of cell behaviour when compared to the influence of ion release from the materials during their degradation in vitro. In addition, these results also show that chondrocyte cells proliferated better on 3D-scaffolds coated with PDLLA fibres compared to 3D-scaffolds without fibres, as indicated by the data in Fig. 6.23. Thus, it is hypothesized that in the present
composite scaffolds PDLLA fibre roughness is relevant in controlling cell behaviour when the ion release from the materials (e.g. material chemistry) during degradation is fixed. In this case the larger surface area achieved by nanofibrous modification provides more binding sites for the cells to attach and proliferate. The enhanced roughness and fibrous topology also contribute to stronger cell-cell interaction. It was reported that fibrous features with high surface area to volume ratio are able to absorb serum and ECM proteins for better cell adhesion. Woo et al. have reported that fibronectin and vitronectin preferentially absorbed to a nanofibrous scaffolds at the rate of 2-4 times higher than solid walled scaffolds [219]. Thus the present bilayered scaffolds exhibit both advantages by having the basic 3D bioactive porous Bioglass® based scaffold for releasing the relevant ions for bone regeneration and the PDLLA nanofibres for increased protein absorption leading to improved chondrocyte cell adhesion. The smaller pore size of the PDLLA fibrous layer compared to the larger pore size of the 3D Bioglass® based composite scaffolds (see Fig. 6.14) could also improved chondrocytes cell attachment. Indeed, investigations have shown that cells cultured in a 3D scaffold with large pore size (30 times the cell diameter, which is of the range 10-15µm) are more likely to behave as in a monolayer culture condition, which is known to promote dedifferentiation of chondrocytes [220]. On the other hand, too small a pore sizes may impair homogenous cell distribution within the scaffolds as well as access to nutrients [221]. To maintain the initial shape of the scaffold surface and the number of attached chondrocytes, adequate mechanical strength and highly cellular adhesivity are requirements for scaffolds in cartilage tissue engineering. Based on the current initial data, for cartilage tissue engineering, we may reasonable conclude that our novel bilayered construct based on PDLLA fibres on 3D 45S5 Bioglass® porous composite substrates can be potentially an ideal biomaterial to create a 3D scaffold with adequate strength (Chapter 5), high cellular adhesivity and excellent support for chondrogenesis for osteochondral tissue engineering applications (Chapter 6).

The other consideration is that chondrocytes exhibit a profound change in their phenotype after isolation from the ECM. They show the development of a fibroblastic morphology and a switch in production from type II collagen to type I collagen. To maintain the chondrocyte phenotype through the process of cartilage regeneration, scaffolds must have the potential to support chondrogenesis while maintaining the
chondrocyte phenotype. These aspects were not yet covered and will be considered for future studies. Another limitation of this study is that the results were derived from an *in vitro* experimental model. Therefore, the biocompatibility of the current bilayered scaffolds in living joints is still unclear and remains an important task for future investigations.

Although there are limitations as mentioned above, the initial data derived from this study suggest great potential for the future of bilayered PDLLA fibre coated 45S5 Bioglass® composite scaffolds for osteochondral defect applications.

### 6.4 Conclusions.

The present bilayered PDLLA fibre coated 45S5 Bioglass® composite scaffolds have been shown to have excellent chondrocyte cell-support ability. Cells infiltrate and migrate effectively into the porous network structure of the PDLLA fibre layer. Bilayered scaffolds showed better cell proliferation than 3D Bioglass® scaffolds without PDLLA fibre coating, while cell proliferated better on the PDLLA fibrous matrix deposited on 3D Bioglass® scaffolds than on 2D Bioglass® sintered pellets. An increase in surface roughness due to deposition of PDLLA fibres by electrospinning improved cell attachment and proliferation. However, compared to the ion release effect from the Bioglass® based scaffold, surface roughness can be a secondary factor in the control of cell proliferation. The PDLLA fibre stability was seen to be maintained up to 14 days in culture solution, probably due to the attachment of cells and extracellular matrix produced by the cells, but this hypothesis needs further investigation.
Chapter Seven

7 Conclusions and Further Work

7.1 General remarks

In the framework of this research project, the fabrication and characterisation of novel 3D porous bioactive composite scaffolds based on 45S5 Bioglass® and biodegradable polymers intended for bone and osteochondral tissue engineering have been investigated. The experiments were designed to achieve the several objectives of this project, including: 1) optimisation of the Bioglass® based scaffold processing technology and their microstructure, 2) coating of the basic scaffold with suitable polymer layers, 3) coating of Bioglass® substrates with nanofibers by electrospinning to form bilayered scaffolds for osteochondral tissue engineering and 4) in vitro trial of the bilayered scaffolds via cell culture.

The results of this research project have demonstrated that a better combination of mechanical and biological properties can be achieved with a composite using PDLLA, P(3HB) or P(3HO) polymer phases in combination with 45S5 Bioglass® derived glass-ceramic scaffolds for tissue engineering applications. Coated 45S5 Bioglass® pellets fabricated under the same conditions as the scaffolds proved the bioactivity and biodegradability of the material. The initial in vitro trials via chondrocyte cell culture, have suggested that bilayered 45S5 Bioglass®/PDLLA nanofibrous composite scaffolds constitute a suitable matrix for osteochondral tissue engineering, but it is also clear that to achieve clinical applications further work is needed in terms of both basic biomaterial engineering and in-vivo testing. This Chapter will summarize the key areas investigated during the course of this research, namely: optimisation of Bioglass® scaffold microstructure by developing composites, PDLLA nanofibers coating to form bilayered scaffolds and in vitro testing of the bilayered constructs.
7.2 **Optimisation of scaffolds microstructure by developing composites**

1. Highly porous, bioactive and biodegradable 45S5 Bioglass®-based scaffolds for bone engineering have been successfully synthesised using the replication technique in particular series 1000 (different sintering time; same sintering temperature) and series 900-1100 (different sintering temperature; same sintering time). The uncoated 45S5 Bioglass®-based scaffolds sintered under the optimum conditions of 1000°C for 2 hours have a similar macroporous structure to spongy bone: 1) they are highly porous (~ 80%-90%), 2) they have completely interconnected open porosity, 3) they have appropriate pore size to deliver nutrients, remove waste and to vascularise (300-500 micron) and 4) they can be functionalised with ALP enzymes.

2. A significant progress in the optimisation of the scaffold microstructure is the possibility to use a cost-effective binder as opposed to PDLLA, as investigated in this work, and 10wt% PVA was selected as optimum condition.

3. PDLLA polymer coating has significantly increased the mechanical strength (compressive strength) of 45S5 Bioglass® based glass-ceramic scaffolds by effective infiltration of the polymer phase into the macro and micro-cracks of the scaffold struts when compared with the as-sintered (non-coated) scaffolds. The mean compressive values of samples after coating with PDLLA became three times higher than that of the sintered scaffolds at the optimum sintering condition of 1000°C for 2 hours. The mean compressive strength values of samples after and before coating with PDLLA are 0.34 ± 0.08MPa and 0.11 ± 0.03MPa respectively.

4. The bioactivity of the PDLLA coated scaffolds was maintained in SBF and this was proven by characterising PDLLA coated pellets sintered at the same conditions of the scaffolds and applying a wide range of techniques (SEM, EDX, and XRD).

5. P(3HB) and P(3HO) polymers have been also used to coat Bioglass® scaffolds and they significantly increased the mechanical strength (compressive strength) of the coated 45S5 Bioglass® scaffolds for series 1000 (different sintering time; same sintering temperature). The strong relationship between strut microstructure and scaffold mechanical strength was confirmed. The
mean compressive strength values of P(3HB) and P(3HO) coated scaffolds were 0.31 ± 0.09MPa and 0.29 ± 0.05MPa, respectively. The mean compressive strength values were three times greater after polymer coating confirming the suitability of the present approach.

6. P(3HB) coatings infiltrated to a lesser extent the microcracks of the scaffold struts compared to PDLLA and P(3HO), due to the highly crystalline structure of P(3HB). P(3HO) efficiently infiltrated the microcracks and macrocracks of the scaffold struts due to lowest Tg value of this polymer. The degree of infiltration of the coating varied as follows: P(3HO)>P(DLLA)>P(3HB).

7. The bioactivity of P(3HB) and P(3HO) coated scaffolds was maintained and this was proved by assessing P(3HB) and P(3HO) coated sintered pellets upon immersion in SBF. The onset time for the HA formation is as follows: PDLLA>P(3HB)>P(3HO).

8. The significant finding in relation to the optimisation of the scaffold microstructure is the formation of polymer fibrils bridging the cracks, in the struts, which seems to induce a similar toughening mechanism to the one acting in bone, based on crack bridging by collagen fibrils.

7.3 PDLLA nanofiber coatings on the 45S5 Bioglass® pellets and scaffolds: bilayered scaffold development

1. PDLLA nanofibers of diameter ranging from 100 nm to 0.2µm were deposited on 45S5 Bioglass® substrates at optimum electrospinning parameters of flow rate = 5µl/min and voltage = 15kV, deposited at 15 cm distance from the substrate using a suspension of 5wt% PDLLA in DMC.

2. The surface roughness of the 45S5 Bioglass® flat substrates increased by the PDLLA nanofiber deposition, which was verified by white light interferometry.

3. HA grew homogenously on the PDLLA nanofibers deposited on 45S5 Bioglass® sintered substrates after immersion in SBF for 7 days and longer time periods.

4. For osteochondral applications, PDLLA nanofibers were deposited on 3D 45S5 Bioglass® scaffolds and the deposition time was varied to form bilayered
constructs. The density of the PDLLA fibrous layer increased with increasing deposition time. The thickness of the PDLLA fibrous layer reached 100-150µm on the 3D scaffolds after deposition for 2 hours using the optimum condition of electrospinning.

5. From the SBF immersion study, it was confirmed that PDLLA fibres deposited for 10 minutes were mineralised after 7 days and for longer immersion periods, while fibres deposited for 2 hours did not become mineralised after immersion in SBF for 7 days and longer. This is a very important result for the application of the present bilayered composites in osteochondral tissue engineering, since it is required that the cartilage side does not mineralise, e.g. where the PDLLA mesh should support the attachment, growth and proliferation of chondrocytes only.

6. Initial in vitro studies of the bilayered scaffolds via cell culture techniques using chondrocyte cells (ATDC5) suggested that the bilayered scaffolds have an excellent cell-supporting ability. Cells can proliferate and migrate into the 3D porous fibrous network structure of the PDLLA layer. Compared to the effect of ion release, surface roughness is a secondary factor in controlling the cell proliferation based on comparison of cell proliferation on 2D 45S5 Bioglass® sintered pellets and on 3D 45S5 Bioglass®-based scaffolds coated with PDLLA fibres.

### 7.4 Suggested Further Work

Based on the results presented and discussed in the framework of the present project, a number of investigations remain to be carried out for the full assessment of the scaffolds suitability for tissue engineering of bone and cartilage.

#### 7.4.1 Composite scaffolds

1. The biodegradability of the Bioglass®-based scaffolds can be tailored according to the application requirement, depending on the bone anatomic position. On the other hand, the bioreactivity of the scaffolds is influenced by several factors, such as strut crystallinity and density, overall porosity of the
foam, surface chemical modification and the presence of biopolymer coatings. In the case of coated scaffolds, the intrinsic degradability properties of the polymer coatings, their thickness and homogeneity will also affect the general biodegradability of the construct. Therefore, it is essential to build a database on the bioreactivity of the scaffolds dependence of these individual factors, for supporting the potential suitability of the scaffolds for clinical applications. This will provide a broader scope than the limited approach investigated in this study.

2. Kinetic studies of Bioglass® powder sintering remain to be carried out. There is very limited viscosity data available for Bioglass® powder at relevant temperatures and variables such as heating rate, sintering temperature, holding time, particle size and green body density need to be investigated in detail. The interaction of densification (by viscous flow) and crystallisation should be investigated quantitatively at different heating rates, providing a broader scope than the limited variables investigated in this study. These sintering kinetic studies will be relevant to tailor the sintering schedule required to fabricate Bioglass® based glass-ceramic scaffolds with improved structural integrity.

3. The mechanical properties of coated scaffolds sintered at a relatively lower temperature of 1000°C should be further investigated, for example by the impact test and three point bending test for a complete mechanical assessment of the scaffolds, since the polymer infiltration may have different effects under different loading modes. Moreover the time-dependant variation of mechanical properties, e.g. compression strength, of scaffolds immersed in relevant environments, e.g. SBF, remains to be investigated.

4. To further increase the mechanical property of the partially sintered scaffolds, improved infiltration techniques and different concentrations of polymers should be further investigated. The goal should be to develop fully interpenetrating polymer/Bioglass® microstructure of the struts for enhanced scaffold toughness. In this regard, it will be necessary to measure the viscosity of PDLLA and P(3HB) solutions under different conditions, including,
possibly, the development of vacuum infiltration method to ensure that the polymer penetrates fully the open cracks (or pores) in the struts leading to a truly interpenetrating strut microstructure.

5. The interfacial properties of the polymer /Bioglass® scaffolds (both PDLLA and P(3HB) coated scaffolds) need thorough investigations with a range of appropriate characterisation techniques. Currently, the interfacial properties have only been investigated qualitatively on sintered Bioglass® discs coated with PDLLA. Quantitative investigations need to be carried out and the link between interfacial strength and effective mechanical properties, in particular intrinsic strut properties, must be established.

6. The fracture mechanics of the composite scaffolds needs to be investigated in detail using the results presented in this study as starting information. The fracture characteristics of the composite depend not only on the relative volume fraction of the Bioglass® and the polymer phases, but also on the microstructural arrangement of these phases and their relative size and orientation. Obviously the interfaces play a key role in determining the mechanical properties (see point (5) above).

7. Stronger composite scaffolds might be achievable by increasing the organic/inorganic interfacial bonding by using surface functionalised phases of either the Bioglass® scaffolds or the polymer phases. The inclusion of nanoparticles into the biopolymer matrix with the dual objective of improving the mechanical properties as well as of incorporating nanotopographical features that mimic the nanostructure of natural bone can be considered.

8. It is essential to increase the number of samples used for mechanical property characterisation in order to reduce the standard deviation and to arrive to quantitative and statistically meaningful conclusions regarding the correlation between process parameters and compressive strength data.

9. Different dimensions of composite scaffolds should be considered in future investigations of the mechanical properties and bioactivity reaction
characterisation. Different dimensions of the scaffolds, relevant for a range of applications in tissue engineering, will influence the overall properties of the composite scaffolds and thus the effect of scaffold dimension remains to be investigated.

10. It is essential to carry out a comprehensive biocompatibility test of the composite scaffolds coated with natural biodegradable polymer synthesised from bacteria fermentations, in this case the P(3HB) and P(3HO) coated 45S5 Bioglass® scaffolds. These studies should directly clarify the possible effect of bacteria derived polymers on biocompatibility.

11. In order to reduce P(3HB) polymer brittleness, and thus the lack of toughening effect on the composite scaffolds, future experiments could combine P(3HB) with the more elastomeric polymer (P(3HO)) to form a hybrid polymer type for the coatings of the Bioglass® scaffolds. These new scaffolds should be compared in their structural integrity and compression strength with PDLLA coated scaffolds.

12. To improve the biological function of the scaffolds, an obvious future development, exploiting our technology of scaffold coating with biodegradable polymers, will be the incorporation of growth factors and other bioactive molecules in the polymer phase, which should include bone growth factors and angiogenesis growth factors. In addition, multifunctional scaffolds with a drug delivery capability could be also developed using as platform the polymer coated Bioglass® based glass-ceramic scaffolds developed here.

### 7.4.2 Bilayered scaffolds

1. Alternative combinations of Bioglass® scaffolds and biopolymers should be considered for osteochondral tissue engineering applications, in particular the use of P(3HO) is promising based on the high flexibility of
this polymer, which should impart increased fracture toughness to the bone side of the scaffold.

2. The fundamental experimental aspects of the electrospinning technique applied to the development of PDLLA fibres needs a thorough investigation in order to develop a reliable method to fabricate robust and homogenous nanofibrous mats on scaffolds that exhibit the optimal properties for cartilage regeneration.

3. The thickness of the PDLLA fibrous layer should be increased to ~1 mm taking special consideration not to greatly reduce the pore size and porosity of the fibrous matrix. The thickness of the natural cartilage layer is around 1 mm thickness, thus further improvement of the electrospinning method is required.

4. A higher molecular weight PDLLA for the electrospinning technique can be considered in order to improve the polymer biodegradation stability and the mechanical properties of the PDLLA fibres.

5. To facilitate viability, attachment and phenotypic maintenance of the chondrocyte cells, PDLLA fibres mesh can be treated with NaOH to increase the surface hydrophilicity. Other possible surface functionalisation methods for PDLLA fibrous substrates should be considered.

6. The interfacial properties of the bilayered constructs developed in the present project needs more detailed investigations. Currently, only SEM observation was used for characterisation of the interfacial properties. In future work a complete mechanical property characterisation will be required, with emphasis on testing the shear strength at the interface. The possible degradation of bilayered scaffolds in a relevant environment, e.g. SBF, needs to be investigated to assess the possible synergetic effect of the degradation products of the bioactive glass-ceramic scaffold on the PDLLA fibre degradation, e.g. effect of possible local change of pH.
7. The mechanical properties of the bilayered scaffolds during cell culture need to be evaluated and investigated in detail. It is hypothesised that the mechanical properties of scaffolds will increase with increasing cell culture time. Therefore, a long term cell culture investigation is recommended.

8. The material chemistry property of the bilayered scaffolds during the cell culture studies needs to be evaluated and investigated in detail. The bioreactivity of the scaffolds is influenced by several factors, such as crystallinity, densification of the struts, porosity and surface coating, as discussed in the present work. Therefore, it is essential to build a database of relevant material property variation during cell culture studies.

9. A long term cell culture investigation of the bilayered scaffolds is essential to assess in a comprehensive manner the response of chondrocyte cells (ATDC 5) in contact with the bilayered scaffolds and to better understand the implications for osteochondral tissue engineering.

10. Mechanical forces play a central role in the physiology of a wide variety of tissues. Cells and tissues react to external mechanical stimuli that can include gravitational and hydrostatic pressures, shear stresses caused by fluid flow, acoustic waves and contractile forces exerted from one cell to another. Therefore, a bioreactor based characterisation of the materials developed here would be the obvious next step in their further development to optimise the scaffolds performance for osteochondral tissue engineering.

11. Further investigations in vitro and in vivo are required to assess the phenotype of the chondrocyte cells (ATDC 5) in contact with the bilayered scaffolds to better understand the implication for osteochondral tissue engineering. The results of further in-vitro studies and, specially, in vivo assessments, will enable to realistically consider the bilayered constructs for future osteochondral tissue engineering strategies.
12. From the point of view of the material development, in order to improve the biological function of the bilayered scaffolds, growth factors such as TGF-ß family (TGF-ßs), insulin-like growth factor (e.g., IGF-1) and bone morphogenetic proteins (BMPs) or drugs can be grafted to the PDLLA fibre mesh or they can be mixed together to produce nanofiber meshes loaded with relevant biomolecules by electrospinning.

13. Recently, the possibility of producing encapsulated cells in fibres and meshes by electrospinning has been explored. There is also a possibility to apply this approach to the development of the bilayered scaffolds investigated in this project.

14. A major goal in osteochondral tissue engineering is to be able to promote tissue synthesis derived from progenitor cells, preferentially from a single cell source, that will be differentiated in the construct to chondrocytes and osteoblasts. This will require the incorporation of cell adhesion molecules and specific growth factors in different region of the construct for tissue engineering chondrogenesis and osteogenesis. Our scaffolds can be the matrices of choice to test different cell culture based methods in the field of osteochondral regeneration.

15. Further investigation of the effect of sterilisation on the bilayered scaffolds required systematic study.
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**APPENDIXES**

Table 1. **Individual Components (2D sintered pellets) Summary Data – MULTIPLE COMPARISONS**

Response: Cell Growth (Bonferroni)

<table>
<thead>
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<th>Material vs. Material</th>
<th>Mean difference</th>
<th>Std. error</th>
<th>Sig.</th>
<th>95% CI Lower</th>
<th>95% CI Upper</th>
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*Based on observed means*

*The mean difference is significant at the 0.05 level*

Table 2. **Bioglass ± PDLLA fibres (3D scaffold) Summary Data – MULTIPLE COMPARISONS**

Response: Cell Growth (Bonferroni)

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<th>Std. error</th>
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*Based on observed means*

*The mean difference is significant at the 0.05 level*