Bioactive and Biodegradable Scaffolds for Hard Tissue Engineering

by

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This thesis is submitted for the

requirement of the award of degree of

MASTER OF ENGINEERING (MEng)

by research from

DUBLIN CITY UNIVERSITY

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2009

DECLARATION

I hereby certify that this material, which is now submit for assessment on the programme of study leading to the award of Master of Engineering is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ (candidate) ID no.: **57127930** Date: _____

To my family

AKNOWLEDGEMENTS

Hereby I would like to acknowledge first of all my supervisors, Dr. Joseph Stokes and Dr. Tim Prescott for their assistance and guidance during my work.

I would like to thank my husband, Filip, for his faith, love, support, patience and humour which helped me all the way.

Furthermore I would like to thank my NOVELSCAFF colleagues: Engin Vrana, Yurong Liu, Diana Garcia Alonso, Szilvia Eösöly, Tamás Szűcs, Joanna Podporska, and Marcin Lipowiecki; and Vittoria Flamini, for their help, patience and professional advice.

Also, I would like to thank to Mr. Liam Domican, Mr. Michael May, Mr. Chris Crouch and Mr. Maurice Burke for their technical assistance.

This research has been supported by a Marie Curie Early Stage Research Training Fellowship of the European Community's Sixth Framework Programme under the contract number MEST-CT-2005-020621.

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ABSTRACT

Bone tissue engineering offers interesting and unique challenges to those studying scaffolds design. First, a porous structure with irregular three dimensional shape is formed, called scaffold. Scaffolds should provide structural integrity, strength, transport properties such as permeability and an ideal micro-environment for cell and tissue ingrowth and healing, be biocompatible, osteoconductive and bioactive. Ensuring adequate mechanical performance under loads is another scaffold requirement, if a scaffold cannot provide a modulus in the range of hard tissue (10–150MPa), then any nascent tissue formation will probably also fail due to excessive deformation. Blending of polycaprolactone (PCL) and hydroxyapatite (HA) has demonstrated great potential for bone tissue regeneration applications. PCL is noted for its biocompatibility and it is a slowly degrading poly(α –hydroxy acid) which will allow more time for regeneration tissue to establish. PCL films have been used for the growth of bone cells and exhibit low tensile modulus and strength, properties that can be improved by blending the polymer with hard, biocompatible filler, like HA. HA is part of the natural bone (70%) which makes it a perfect candidate for stimulating bone growth, due to its biocompatibility and bioactivity.

The present study aims to build a porous scaffold for bone regeneration, combining the biodegradability of PCL with the osteoconductive properties of HA for cell attachment, growth and differentiation. Porous structures were produced by several innovative combinations of different conventional techniques: salt leaching, gas forming, solvent evaporation and phase separation. Samples of different ceramic content were obtained and characterized using scanning electron microscopy (SEM/EDX), FTIR, mechanical, hydrophylycity, roughness and degradation tests. The prepared scaffolds present different porous patterns throughout the matrix and the porosity was controlled by altering the initial volume fraction of the porogen agent (salt particles, effervescent agents, filler material and solvent). The tensile strength of the HA/PCL composites decreased with increasing HA content, porosity and technique being used. The bioactive and biocompatible features were investigated through immersion in simulated body fluid (SBF) and bone cell culturing (MC3T3). The best composites found contained 4% HA: 96%PCL formed at a thickness of 1.2 mm using solvent evaporation technique, and a thickness of 10mm using phase separation technique.

CHAPTER 1. INTRODUCTION

When Gloria Clausen, a middle school teacher from Mendham, N.J., was told by her doctor that she had a rare, cancerous tumor in the tibia of her left leg, she was presented with two options: have the leg amputated below the knee or have a bone transplant that would save her leg. Like many patients faced with similar situations one of her first questions was, "Where will you get the bone?" [1]

Bone tissue engineering offers interesting and unique challenges to those studying scaffolds design. First, a porous scaffold with irregular three dimensional shapes is build. In the same time the scaffold must have high strength to replace the structural function of the bone, temporarily until it has regenerated [2]. Also, for this purpose the material must be biocompatible, osteoconductive and bioactive. The porous structure should enable cell attachment, growth and differentiation.

Synthetic materials have been studied over the past few years as scaffolds for cell and tissue ingrowth. Blending of polycaprolactone and hydroxyapatite (HA) have demonstrated great potential for bone tissue regeneration. Polycaprolactone (PCL) is an important member of the aliphatic polyester family. It had been used efficiently as a drug delivery system, but also has been introduced to enhance bone ingrowth and tissue regeneration [3]. The PCL degradation process involves simple mechanisms organized into two stages: random hydrolytic ester cleavage and weight loss through the diffusion of oligomeric species from the bulk. It is a polymer with a very slow degradation rate, depending on the chosen molecular weight [3].

On the other hand 70% of bone consists of hydroxyapatite, a form of calcium phosphate. As the main mineral of the bone, HA has the capacity of stimulating bone ingrowth. The ceramic takes the form of polygonal sintered coarse particles, resembling the apatite in the natural bone. Composites combining these two materials have found applications for bone tissue engineering. In the present study biomimetically porous scaffolds were prepared combining different conventional techniques, including salt leaching, gas forming and phase separation.

1.1. Aims of the Study

The aim of this research is to produce porous and bioactive scaffolds for bone tissue regeneration, using one or more combination of the already known conventional techniques (salt leaching, phase separation, gas forming, or freeze drying). At the same time methods of improving these techniques will be studied, with the aim of producing a porous structure using a simple composite material (a composite that resulted from the homogeneous dispersion of one dispersed phase throughout a matrix).

The best way of building a bone scaffold is to copy the natural bone structure and morphology as much as possible. For this reason the targeted structure will not have a predesigned porous structure with pores of identical sizes, as the solid freeform fabrication techniques produce. The main features to be studied will be pore size, open porosity, adequate mechanical strength (depending on the place of implantation -facial, long bone, cranium reconstruction or just ex vivo scaffold), biocompatibility and bioactivity.

1.2 Thesis Outline

The thesis is divided into a number of sections. Chapter one introduces the research work and the aims. Chapter two describes human bone composition and its healing process. A brief introduction to what tissue engineering is and how scaffolds can be processed is also presented. How natural and synthetic polymers have been used in tissue engineering is considered.

Chapter three describes the process adopted to produce the scaffolds in this study, via different combination of conventional techniques. An overview of the equipments used to characterize the structures and the materials is also outlined. An investigation to evaluate the in vitro behaviour of the produced scaffolds is presented with a description of the cell studies and procedures outlined.

Chapter four presents the experimental results and discussions for the various samples obtained using various combinations of conventional techniques.

CHAPTER 2. LITERATURE REVIEW

This chapter is an overview of Tissue Engineering science and contains information about bone structure and function, about scaffolds for bone tissue engineering, bone substitutes and producing techniques.

2.1 Review of Tissue Engineering

Biomedical engineering involves the application of engineering science and technology to problems arising in medicine and biology. The integration of each engineering discipline (electrical, mechanical, chemical, and so on) with each discipline in medicine (pathology, cardiology, neurology) or biology (biochemistry, pharmacology, molecular biology, cell biology, and so on) is a potential area of biomedical engineering application [2].

Tissue engineering is the use of a combination of cells, engineering and materials methods, and suitable biochemical and physico-chemical factors to improve or replace biological functions. While the application of engineering expertise to the life sciences requires an obvious knowledge of contemporary technical theory and applications, it also demands an adequate knowledge and understanding of relevant medicine and biology. It has been argued that the most challenging part of finding engineering solutions lies in the formulation of the solution in engineering terms. From a biomedical engineering point of view also this demands a full understanding of the life science substrates as well as the quantitative methodologies [2].

Langer and Vacanti [3] stated that tissue engineering is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ".

Also most definitions of tissue engineering cover a broad range of applications, in practice the term is closely associated with applications that repair or replace portions or whole tissues (bone, cartilage, blood vessels, bladder, and so on). Often, the tissues involved require certain mechanical and structural properties for their human applications.

2.2. Bone: Structure and Function

The bone is a hard connective tissue. It is the major component of almost all skeletal systems in adult vertebrate animals. Bone appears to be nonliving—in fact, the word skeleton is derived from a Greek word meaning dried up. However, bone actually is a dynamic structure composed of both living tissues, such as bone cells, fat cells, and blood vessels, and nonliving materials, including water and minerals [4].

An adult human has 206 bones, which account for 14 percent of the body's total weight. The longest and strongest bone is the thighbone, which at maturity is about 50 cm (20 in) long and 2.5 cm (1 in) wide. The smallest bone, the stirrup bone, is one of three tiny bones buried within the middle ear: it is only 0.18 cm (0.07 in) long [4].

Bones are multipurpose structures that play diverse, vital roles in vertebrates. They provide a framework for the body, supporting it and giving it shape, and also provide a surface for the attachment of muscles and act as levers, permitting many complex movements. Many bones protect softer internal organs, for example: skull bones protect the brain, and rib bones form a cage around the lungs and heart. In addition to these structural and mechanical functions, bones also participate in the body's physiology. Bones store calcium, a mineral essential for the activity of nerve and muscle cells. The soft core of a bone, the bone marrow, is the site of formation of red blood cells, certain white blood cells, and blood platelets.

Bone is a lightweight and relatively hard natural composite mainly made up from collagen fibril, hydroxyapatite and a small amount of non-collagen proteins. The matrix material of this composite primarily consists of type I collagen that is formed as chains, which twist into triple helices. These triple helices form batches bonded together into fibrils. The fibrils are ordered into layers and mineral crystals deposited between them. This nanocomposite has an anisotropic structure where the collagen fibrils are responsible for the tensile strength and the minerals provide the compressive strength. The anisotropic structure of the bone results in superior mechanical properties that combine toughness and stiffness, but they are only present in particular directions [5].

The components of the bone include approximately: 60wt% mineral, 30wt% matrix and 10wt% water, where the matrix comprise about 15wt% living tissue of cells [6], including osteocytes (bone cells), osteoclasts (bone resorbing cells), osteoblasts (bone building cells) and bone lining cells [7]. The mineral components are mainly crystalline mineral salts and calcium, present in the form of hydroxyapatite ($Ca_{10}(PO_4)(OH)$) containing many other substitutions, such as: magnesium, sodium, potassium, fluorine, chlorine, and carbonate ions.

Bones in human and other mammal bodies are generally classified into two types: 1) cortical bone, also known as compact bone and 2) trabecular bone, also known as cancellous or spongy bone (Figure 2.1). These two types are classified on the basis of porosity and the unit microstructure.

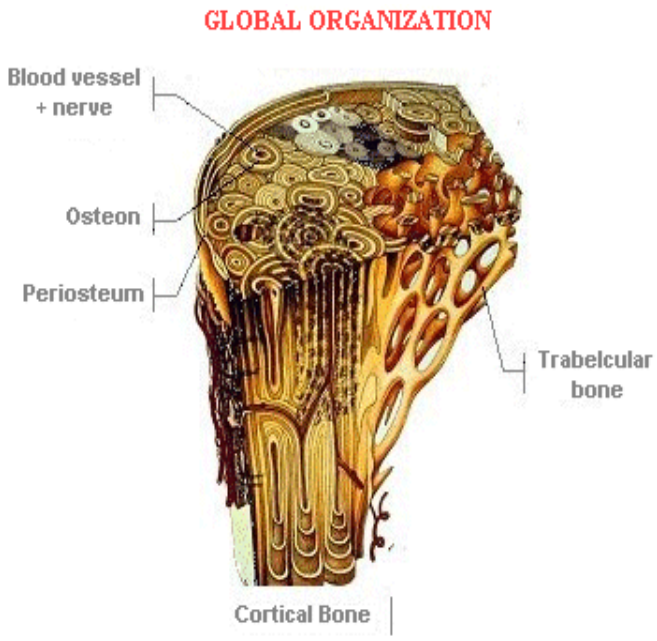


Figure 2.1: Structure of the bone [8]

2.2.1 Cortical Bone

Cortical bone (Figure 2.2) represents nearly 80% of the skeletal mass [6]. It is also called compact bone, because it forms a protective outer shell around every other bone in the body. Cortical bone has a slow turnover rate and a high resistance to

bending and torsion. It provides strength where bending would be undesirable as in the middle of long bones. Cortical bone is dense with a porosity ranging between 5% and 10%. Cortical bone is found primarily in the shaft of long bones and forms the outer shell around cancellous bone at the end of joints and the vertebrae. The basic first level structures of cortical bone are osteons or Haversian systems.

Each osteon is composed of a central vascular channel surrounded by a kind of tunnel, called the Haversian canal. The canal can contain capillaries, arterioles, venules, nerves and possibly lymphatics. Between each osteon are interstitial lamellae (concentric layers of mineralized bone). Lamellar bone gets its strength from its plywood-like construction: parallel layers of bone alternating in orientation by 90 degrees.

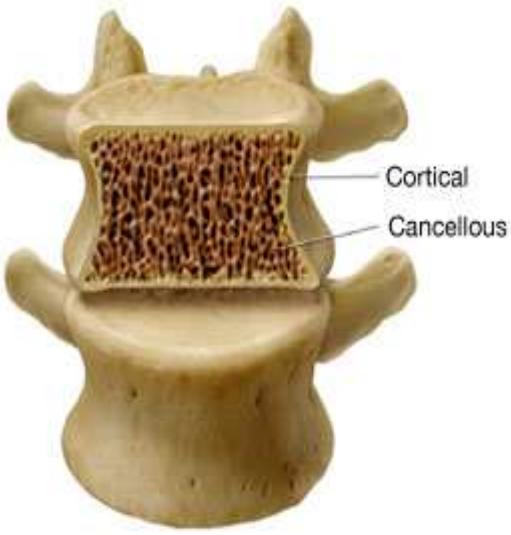


Figure 2.2: Cortical and trabecular bone [8]

2.2.2 Trabecular or Cancellous Bone

Trabecular bone is much more porous with porosity ranging anywhere from 50% to 90% [6]. It is less dense, more compliant and has a higher turnover rate than cortical bone. It is found in the epipheseal and metaphysal regions of long bones and throughout the interior of short bones. It constitutes most of the bone tissue of the axial skeleton: bones of the skull, ribs and spine. It is formed in an intricate and structural

mesh. It forms the interior scaffolding, which helps bones to maintain their shape despite compressive forces.

Also it is believed to dissipate the energy from cortical contact loads, due to its spongy appearance, as it is composed of bundles of short and parallel strands of bone fused together [6]. In the middle the bone contains red, yellow marrow, bone cells and other tissues. Its basic first level structure is the trabeculae.

2.2.3 Physical Properties of the Natural Bone

Chemical composition and physical properties of the natural bone depend on species, age and the type of bone. Mechanical properties as compressive strength, Young's modulus, tensile strength, hardness and fracture toughness have been studied greatly. The orientation of bone specimen, which can be defined as longitudinal (parallel to the predominant osteon ligaments), or transverse (through the osteon section), affects the mechanical properties. Compact bone has a compressive strength in the longitudinal direction (parallel to the axis) ranging from 131-224MPa, and a Young's modulus between 17-20GPa [9], that is twice that of the transverse direction. It exhibits also good fracture toughness, which is much higher in the transverse direction than in the longitudinal one.

The mechanical properties of the trabecular bone are highly dependent on its density. Compressive strength varies with the second power of density, where as Young's modulus scales as the second or third power, with values ranging between 0.5-10 MPa and 50-100 MPa for strength and modulus, respectively [10] (Table 2.1). It may appear that the trabeculae are arranged in a random manner, but they are organised to provide maximum strength, similar to braces that are used to support a building. The trabeculae of cancellous bone follow the lines of stress and can realign if the direction of stress changes [10].

Table 2.1: Mechanical properties of human bone, adapted from [9]

Mechanical property	Test direction related to bone axis	
	Parallel	Normal
Tensile strength (MPa)	124-174	49
Compressive strength (MPa)	170-193	133
Young's modulus (GPa)	17-18.9 20-27 [random]	11.5
Micro Hardness (VPN)	30-60	-
Fracture Toughness (MPa-$m^{1/2)}$	2-12	8
Bending strength (MPa)	160	-
Shear strength (MPa)	54	-
Ultimate Tensile Strength (UTS)	0.014-0.031	0.007
Ultimate Compressive Strain	0.0185-0.026	0.028
Yield Tensile Strain	0.007	0.004
Yield Compressive Strain	0.010	0.011

2.2.4. Bone Healing

Bone healing or fracture healing is a proliferative physiological process, in which the body facilitates repair of bone fractures. The bone healing process includes three major phases of fracture healing, two of which can be further sub-divided to make a total of five phases [11]:

1. Reactive phase
   1.1 Fracture and inflammatory phase
   1.2 Granulation tissue formation

2. Reparative (modeling) phase
   2.1. Callus formation
   2.2. Lamellar bone deposition

3. Remodeling phase
   3.1 Remodeling to original bone contour

The modeling and the remodelling phases are based on the separate actions of bone resorbing cells, called osteoclasts (multinucleated cells that form by fusion of mononuclear precursors of haematopoetic origin), and bone forming cells, called osteoblasts (that derive from mesenchymal stem cells found in the bone marrow, periosteum and soft tissues).

Reactive phase: after fracture, the first sign is the presence of blood cells within the tissues which are adjacent to the injury site (this is visible using light and scanning microscopy). After fracture, the blood vessels constrict and stop any further bleeding. Within a few hours after fracture, the extravascular blood cells will form a blood clot or hematoma [12]. All of the cells within this blood clot will degenerate and die. Also some of the cells outside it, but adjacent to the injury site, degenerate and die. Only the fibroblasts survive and replicate, forming a loose aggregate of cells with small blood vessels, known as granulation tissue.

Reparative (modeling) phase: days after fracture, the cells of the periosteum replicate and transform. The periosteal cells proximal to the gap develop into chondroblasts (cartilage like cells) and form hyaline cartilage. The periosteal cells distal to the fracture gap develop into osteoblasts and form woven bone. The fibroblasts within the granulation tissue also develop into chondroblasts and form hyaline cartilage. These two new tissues grow in size until they unite with their counterparts from other

pieces of the fracture. This process forms fracture callus [12]. The fracture gap is bridged by the hyaline cartilage and woven bone, restoring some of its original strength.

Another subphase is the replacement of the hyaline cartilage and woven bone with lamellar bone. This process is known as endochondral ossification.

The hyaline cartilage and "bony ossification" with respect to the woven bone. The lamellar bone begins forming soon after the collagen matrix of either tissue becomes mineralised. Substitution of the woven bone with lamellar bone precedes the substitution of the hyaline cartilage with lamellar bone. The osteoblasts form new lamellar bone onto recently exposed surfaces of the mineralized matrix. This new lamellar bone is the form of trabecular bone. At some stage all of the woven bone and cartilage of the original fracture callus is replaced by trabecular bone, restoring much or all of the bone's original strength.

Remodeling: this process substitutes the trabecular bone with the compact bone. The trabecular bone is first resorbed by osteoclasts, creating a shallow resorbtion pit known as "Howship's lacuna" [11, 12]. Then osteoblasts deposit compact bone within the resoption pit. The fracture callus is remodelled into a new geometry which closely duplicates the bone original shape and strength.

2.3 Tissue Engineering Scaffolds

2.3.1 Bone Tissue Engineering

Beginning in the late 1980s, the field of tissue engineering has made rapid advances as a new discipline. Tissue engineering holds promises of [13]:

1. Eliminating re-surgeries by using biological substitutes;
2. Using biological substitutes to solve problems of implant rejection, transmission of diseases associated with xenografts and allografts and shortage in organ donation;
3. Providing long-term solutions in tissue repair or in the treatment of diseases;
4. Offering potential treatments for medical conditions that are currently untreatable.

The advent in tissue engineering has been motivated by the challenge of producing tissue substitutes that can restore the structural features and physiological functions of natural tissues in vivo [14-16], despite the limitations of current therapies for tissue loss or malfunction.

Ideally a tissue engineering scaffold must comply with a large number of requirements. Apart from being biocompatible and biodegradable, scaffolds should possess other properties such as the appropriate mechanical properties, as to provide the correct stress environment for the new tissue [15,17], adequate degradation rate to assure that the strength of the scaffolds is retained until the newly grown tissue takes over the synthetic support [18], adequate porosity and permeability in order to allow the ingrowth of cells and circulation of nutrients, and the appropriate surface chemistry for enhanced cell attachment and proliferation [15,18]. The primary criterion for selecting materials for bone tissue engineering is that they should be osteocompatible. Also the material has to be bioactive (osteoconductive/osteoinductive).

The development of bone tissue engineering is directly linked to changes in materials technology. Standard material requirements already exist in the design process of engineered bone substitutes. However it is critical to include the clinical requirements in order to offer an optimum engineered device. There are multiple clinical reasons to develop bone tissue–engineering alternatives, including the need for better filler materials that can be used in the reconstruction of large orthopaedic defects and the need for orthopaedic implants that are mechanically more suitable to their biologic environment [19, 20]. The traditional biological methods for bone–defect repair include autografting and allografting cancellous bone, applying vascularised grafts of the fibula and iliac crest, and using other bone transport technique [20]. Although these are standard treatments, shortcomings still exist.

Autografts are grafts that were harvested from the same person and transplanted into another location in the body. Although the best results are achieved with autografts this solution has several drawbacks like the limited amount of harvested material. It also requires more operation time, and complications at the donor site are possible.

Allografts are harvested from a human cadaver. Allografts eliminate the previously mentioned drawbacks but they do not heal as fast and as well as the autografts. Not only is the operating time required for harvesting autografts expensive, but often the donor tissue is scarce, and there can be significant receiver site morbidity associated with infection, pain and hematoma [21].

Another method for bone defect repair is via *bone cement fillers*. Bone cements are prepared in the operating room and therefore can be susceptible to infection.

Bone marrow replacement is another possible tissue-engineering application for the treatment of patients following high-dose chemotherapy and/or radiation treatment [22]. This technique requires the sterile aspiration of the marrow from the posterior iliac crest. Marrow can be used in tissue-engineering culturing techniques and also as a basis for marrow expansion.

Alloplastics are the grafts made by man. The key advantage of these solutions is that they are available in the desired size and form, and also the lack of donors is not an issue. Nowadays there are two common types of approaches. One approach is to apply bioinert materials as permanent replacement. This technique is widely applied in case of knee, hip, or ligament replacement. The other solution is to use the patient's own cells to build up an implant that can augment or replace a tissue function including a complete organ. The fundamental problem is that the cells can proliferate into larger colonies; however they are incapable of forming three-dimensional tissues/organs in vitro [23]. To form a three-dimensional organized tissue the cells need complex mechanical, chemical, and electrical signals which are present in their natural environment [24]. For this reason it is necessary to use a porous matrix called a scaffold to which the cells can attach, proliferate and differentiate in vitro and afterwards this can be inserted to the anatomical defect. This matrix is only necessary until the cells form the desired anatomical shape and gain sufficient mechanical properties to withstand the physiological loading. Therefore scaffolds are ideally made from bioresorbable materials [25].

Bone tissue engineering can provide better alternatives that possess better mechanical properties than those that are currently used. In this way the mechanical properties of a bone tissue-engineered construct can be modified in order to resemble

the natural tissue properties.. A related application is the use of a tissue-engineered surface to permanently stabilize implants by coating the prosthesis with cells or tissue before implantation. This can be extremely useful in reconstructive orthopaedic surgeries that potentially have high incidences of failure secondary to large bone defects [26].

For bone tissue regeneration four components are required: a morphogenetic signal, responsive host cells that will respond to the signal, a suitable carrier of the signal that can deliver it to specific sites then serve as scaffolding for growth of the responsive host cells, and a viable, well vascularised host bed [27, 28]. Also, another important thing that must be mentioned is that almost 40years ago, researchers became aware that the osteoconductive properties of the synthetic absorbable polymers were dependent on their location and the structure of the material that they were made of [29].

Natural tissues are three-dimensional (3-D) structures composed of cells surrounded by extracellular matrix (ECM). The ECM forms the supporting matrix for the cells to reside and the cell-cell and cell-ECM contact plays an important role in maintaining cell differentiation and function. Bone tissue engineering can be viewed as the use of a scaffolding material to either induce the formation of bone from the surrounding tissue or to act as a carrier or template for implanted bone cells or other agents. Materials used to construct bone tissue- engineered scaffolds may be injectable or rigid, the latter requiring an operative implantation procedure. Until now, the areas of materials research can be divided into accellular and cellular, with drug delivery included in both areas [30].

2.4 Bone Substitutes Used in Tissue Engineering

Synthetic biodegradable polymers have been used extensively as supports for cell growth, but attempts to supplement these materials with bioactive molecules to stimulate or modulate the remodelling process has only been a recent venture [30].

Biomaterials can be divided into four major classes of materials: polymers, metals, ceramics (carbon, glass-ceramics and glasses) and natural materials (both from plants and animals). Sometime different class of materials are combined together to

form a composite materials, such as hydroxyapatite particle-reinforced poly (lactic acid). These materials, composites, are the fifth class of materials. The properties of biomaterials must match the ones of the tissue that they replace. As a general rule they have to be biocompatible. For bone regeneration, in particular choosing the right biomaterials must answer demands like: adequate porosity, mechanical strength to support load, flexibility to withstand shocks and appropriate rate of degradation as to offer time for cells attachment, proliferation and division [31].

The diversity and sophistication of materials used currently in medicine and biotechnology is the proof of significant scientific and technological advances that have occurred over the past 50 years. Starting with the World War II to the early 1960s, few pioneer surgeons were taking commercially available polymers and metals, fabricating medical devices implants and components from them, and applying them clinically [30,31]. Also there were some failures that led the surgeons to recognise the need of having more diverse research using physical, biological and materials scientists and engineers.

This stimulated the development of many new materials in the 1970s. Materials were designed specifically for medical use, such as biodegradable and bioactive ceramics. Some of them were derived from existing materials with new technologies, such as polyester fibres, that were woven or knit into the form of tubes for use as vascular grafts, or cellulose acetate plastic that was processed as bundles of hollow fibres for the use in artificial kidney dialysers. Also there were some materials borrowed from some unexpected sources such as pyrolytic carbons or titanium alloys that had been developed for use in air and space technology. Other materials were modified to provide special biological properties, such as immobilization of heparin for anti-coagulant surfaces. More recently biomaterials scientists and engineers have developed a growing interest in natural tissues and polymers in combination with living cells. For these new techniques of isolation, purification and application of many different natural materials appeared [30, 31]. Bone tissue – engineering systems have included demineralised bone matrix, collagen composites, fibrin, calcium phosphate, polylactide, poly(lactide-co-glycolide), polylactide-polyethylene glycol, hydroxyapatite, dental plaster, and titanium [27].

2.4.1 Calcium Phosphate (Ceramics) and Sulphate

There are several calcium phosphate ceramics that are considered biocompatible. Of these, most are bioresorbable and will dissolve when exposed to physiological environments. The earliest application of calcium phosphate salts was in the form of powders. The most commonly used calcium phosphate ceramics are hydroxyapatite (coral based or synthetic) and tricalcium phosphate, used in form of implant coatings and defect fillers. These materials require high temperature and high pressure processing to produce dense, highly crystalline, bioinert ceramics, which are not moldable intraoperatively; however they often have poor fatigue characteristics [32]. The order of these calcium phosphates, depending on their solubility, is as follows [33, 39]:

Tetracalcium Phosphate ($Ca_4P_2O_9$) > Amorphous calcium Phosphate > alpha-Tricalcium Phosphate ($Ca_3(PO_4)_2$) > beta-Tricalcium Phosphate ($Ca_3(PO_4)$ 2) >> Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$).

Unlike the other calcium phosphates, hydroxyapatite does not break down under physiological conditions. In fact, it is thermodynamically stable at physiological pH and actively takes part in bone bonding, forming strong chemical bonds with surrounding bone. This property has been exploited for rapid bone repair after major trauma or surgery. While its mechanical properties have been found to be unsuitable for load-bearing applications such as orthopaedics, it is used as a coating on materials such as titanium and titanium alloys, where it can contribute its 'bioactive' properties, while the metallic component supports the load applied by the body. Such coatings are applied by plasma spraying. Careful control of the processing parameters is necessary to prevent thermal decomposition of hydroxyapatite into other soluble calcium phosphates due to the high processing temperatures [39].

Gypsum, also referred to as ‘Plaster of Paris’, owes its name to a village just north of Paris. Although its external use for the creation of hard setting bandages dates back to the seventeenth century, its first internal use to fill bony defects was reported in 1892 by Dressmann [33]. The Plaster of Paris has been used as a bone void filler, and as

an antibiotic-laden plaster in the treatment of infected bony defects [34-37]. Calcium sulphate ($CaSO_4$) has long been used in its partially hydrated form. Medical grade calcium sulphate is crystallized in highly controlled environments producing regularly shaped crystals of similar size and shape. It possesses a slower, more predictable solubility and reabsorption. One such material is OsteoSet (Wright Medical Technology, Arlington, TN), which was approved by FDA in 1996 [35]. The material comes in the form of 30 and 48 mm pellets that typically dissolve in vivo within 30 to 60 days depending on the volume and location [36]. The main advantages are that it can be used in presence of infection and it is comparatively cheap. Since it is bioabsorbable, it has inherent advantages over other antibiotic carriers, such as polymethylmethacrylate (PMMA), which become a nucleus for further infection after elution of the antibiotics, thus requiring a separate operation for removal from the surgical site. When this is combined with the eradication of dead space and the acidic environment created during $CaSO_4$ resorption, the compound can be an extremely effective treatment for acute bony infections with bone loss. However, some cases of inflammatory response and a single case of allergic reaction have been reported with the use of this compound [38].

2.4.2. Natural polymers

Naturally derived protein or carbohydrate polymers have been used as scaffolds for the growth of several tissue types [45-47]. By far the most popular natural polymer used for tissue engineering scaffolds is collagen [50].

A. Collagen

Collagen is the main protein of connective tissue in animals and the most abundant protein in mammals, making up about 25% to 35% of the whole-body protein content. It is naturally found exclusively in metazoa including sponges [50]. The collagen in the tissues of a vertebrate occurs in at least 10 different forms, each predominant in a specific tissue. All the forms share the triple helix structure and variations are restricted to the length of the molecule. Collagen constitutes 1% to 2% of muscle tissue, and accounts for 6% of the weight of strong, tendinous muscles. The gelatin used in food and industry is derived from the partial hydrolysis of collagen [50].

B. Starch

Starch, also called asylum, is polysaharide carbohydrate composed of a large number of glucose unites. It is produced by all green plants and it is used as energy store and is a source of food for humans. Work conducted by Lam et al. [45] demonstrated the feasibility of using natural biopolymers (starch, dextran and gelatin) and distilled water as the binder. This aqueous system eliminated the problem linked to the use of an organic solvent. However, when a scaffold is bound by water, it is therefore water-soluble, necessitating a lengthy postprocessing step to 'waterproof' the product [45].

C. Chitosan

Chitosan is produced by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans, like crabs or shrimps. Chitosan purified from shrimp shells is used in a granular hemostatic product, Celox, made by Medtrade Biopolymers Inc. of Crewe, England [62] and in the chitosan dressings made by HemCon Medical Technologies Inc. of Portland, OR, USA [63]. Scaffolds with various pore sizes and porosities were produced by selecting the appropriate solvent and optimizing processing conditions, as shown in Hua Wu et al. work [61]. The work showed that chitosan does not change the polymer crystal structure.

2.4.2. Synthetic Polymers

Since the approval of biodegradable sutures by the FDA in the 1960, medical products based on lactic acid, glycolic acid, poly (dioxanone), poly (trimethylene carbonate) copolymers and polycaprolactone homopolymers and copolymers have been accepted for use as medical devices [50]. The development of synthetic biodegradable polymers has in recent years benefited the design and development of three-dimensional templates or scaffolds for tissue engineered products to support, reinforce and in some cases organise the regenerating tissue [40]. These functions require a porous scaffold with interconnected porosity and desirable chemical properties [41]. As the polymer degrades over time in the body tissue grows hence the need for a secondary surgery to remove the implant [42]. In addition, synthetic polymers have the advantage over

natural biodegradable polymer in that they can be easily mass-produced. The properties, in particular, the degradation rate, can be tailored to suit specific applications [43]. The sterilisability and biocompatibility of these polymers have also been well-documented [44].

A. Poly-caprolactone

Polycaprolactone (PCL) is a biodegradable polymer, part of the polyesters family, with a low melting point of around 60° and a glass transition temperature of -60°. This polymer is derived by chemical synthesis from crude oil, by ring opening polymerization of ε- caprolactone using a catalyst such as stannous octanoate. This polymer has a good resistantce to water, oil, solvent and chlorine. Its role is as an additive for resins, to improve their processing characteristics and their end use properties. It is a biocompatible material and for this reason it can be mixed with starch to lower its cost and increase biodegradability or it can be added as a polymeric plasticizer to PVC.

Polycaprolactone degrades by hydrolysis of its ester linkages in physiological conditions and for this reason it has received a great deal of attention for use as an implantable biomaterial. It has proved interesting for the preparation of long term implantable devices, because it has a very slow degradation rate, even slower than that of polylactide. PCL is a FDA approved material that is used in the human body as a drug delivery system, suture (sold under the name Monocryl) or as adhesion barrier. It is now being investigated as a scaffold for tissue repair via tissue engineering. It also has been used as a hydrophobic block of amphyphilic synthetic block copolymers used to form the vesicle membrane of polymersomes [49, 52, 53].

It has also been used to encapsulate a variety of drugs, for controlled release and targeted drug delivery. Also it is being used in Odontology/ Dentistry in root canal fillings. It acts like gutta-percha, having the same handling properties, and for re-treatement purposes it may be softened with heat, or dissolved with solvents like chloroform [49].

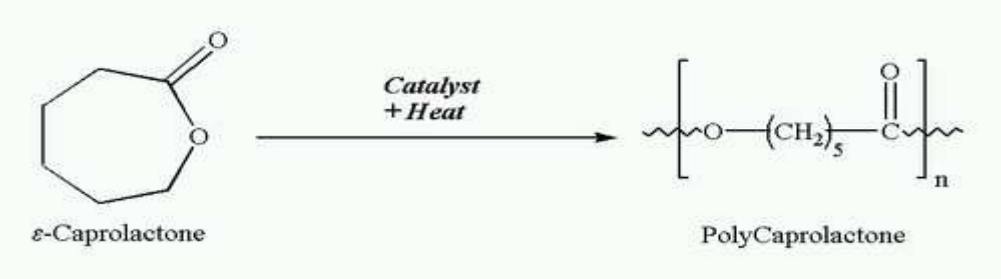


Figure 2.3: Ring open polymerization of ε- caprolactone to polycaprolactone [62]

B. Poly-Lactic-Acid (PLA)

Polylactic acid or Polylactide (PLA) is a biodegradable, thermoplastic, aliphatic polyester derived from renewable resources. Corn starch (in the U.S.) or sugarcanes (rest of the world) are its common feedstock. Bacterial fermentation is used to produce lactic acid, which is oligomerized and then catalytically dimerized to make the monomer for ring-opening polymerization, as shown in figure 2 [65]. It can be easily produced in a high molecular weight form through ring-opening polymerization using most commonly a stannous octoate catalyst, but for laboratory demonstrations Tin (II) chloride is often employed.

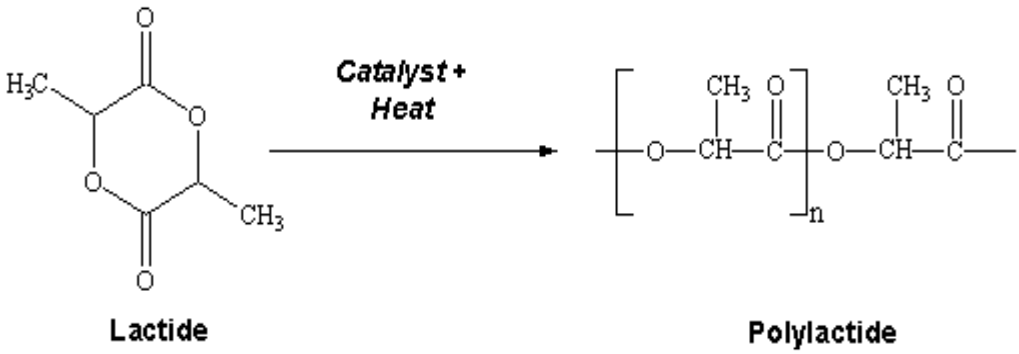


Figure 2.4. Ring open polymerization of lactide to polylactide [65]

Due to the chiral nature of lactic acid, several distinct forms of polylactide exist: poly-L-lactide (PLLA) is the product resulting from polymerization of L, L-lactide (also known as L-lactide). PLLA has a crystallinity of around 37%, a glass transition temperature between 50-80° C and a melting temperature between 173-178° C. The

polymerization of a racemic mixture L- and D-lactides leads to the synthesis of poly-DL-lactide (PDLLA) which is not crystalline but amorphous [53].

C. Poly-Glycolyc-Acid (PGA)

Polyglycolide or Polyglycolic acid (PGA) is a biodegradable, thermoplastic polymer and the simplest linear, aliphatic polyester. It can be prepared starting from glycolic acid by means of polycondensation or ring-opening polymerization. PGA has been known since 1954 as a tough fiber forming polymer, however, owing to its hydrolytic instability its use has initially been limited [40]. Currently polyglycolide and its copolymers, poly (lactic-co-glycolic acid) with lactic acid, poly (glycolide-co-caprolactone) with ε-caprolactone and poly (glycolide-co-trimethylene carbonate) with trimethylene carbonate, are widely used as a material for the synthesis of absorbable sutures and are being evaluated in the biomedical field.

Polyglycolide has a glass transition temperature between 35-40°C and its melting point is reported to be in the range of 225-230° C. PGA also exhibits an elevated degree of crystallinity, around 45-55%, thus resulting insoluble in water [41]. The solubility of this polyester is somewhat unique, in that its high molecular weight form is insoluble in almost all common organic solvents (acetone, dichloromethane, chloroform, ethyl acetate, tetrahydrofuran), while low molecular weight oligomers sufficiently differ in their physical properties to be more soluble. However polyglycolide is soluble in highly fluorinated solvents like hexafluoroisopropanol (HFIP) and hexafluoroacetone sesquihydrate, that can be used to prepare solutions of the high molecular weight polymer for melt spinning and film preparation. Fibres of PGA and are particularly stiff (7 GPa) [41].

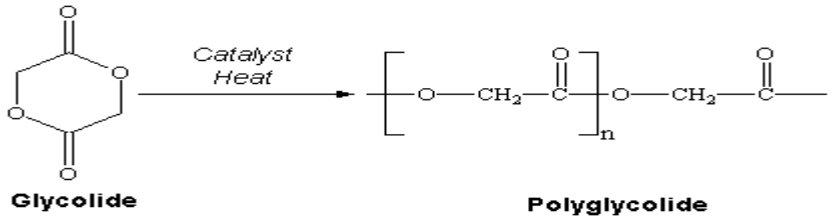


Figure 2.5: Ring open polymerization of glycolide to polyglycolide [66]

D. Poly-Lacti-Glycolyic-Acid (PLGA)

PLGA or poly (lactic-co-glycolic acid) is a Food and Drug Administration (FDA) approved copolymer which is used in a host of therapeutic devices, owing to its biodegradability and biocompatibility. PLGA is synthesized by means of random ring-opening co-polymerization of two different monomers, the cyclic dimers (1, 4-dioxane-2, 5-diones) of glycolic acid and lactic acid. Common catalysts are used in the preparation of this polymer, including Tin (II) 2-Ethylhexanoate, Tin (II) Alkoxides or aluminum isopropoxide. During polymerization, successive monomeric units (of glycolic or lactic acid) are linked together in PLGA by ester linkages, thus yielding a linear, aliphatic polyester as a product [41].

Depending on the ratio of lactide to glycolide used for the polymerization, different forms of PLGA can be obtained: these are usually identified in regard to the monomers' ratio used (e.g. PLGA 75:25 identifies a copolymer whose composition is 75% lactic acid and 25% glycolic acid). All PLGAs are amorphous rather than crystalline and show a glass transition temperature in the range of 40-60 °C. Unlike the homopolymers of lactic acid (polylactide) and glycolic acid (polyglycolide) which show poor solubilities, PLGA can be dissolved by a wide range of common solvents, including chlorinated solvents, tetrahydrofuran, acetone or ethyl acetate [54].

PLGA degrades by hydrolysis of its ester linkages in the presence of water. It has been shown that the time required for degradation of PLGA is related to the monomers' ratio used in production: the higher the content of glycolide units, the lower the time required for degradation. An exception to this rule is its copolymer with 50:50 monomers' ratio which exhibits the faster degradation (about two months) [54].

E. Polyanhydrides

Polyanhydrides are part of the biodegradable polymer class, characterized by anhydride bonds that connect their monomer units to the polymer chain. They are mainly used in medical implants and in the pharmaceutical industry. In vivo they degrade into non – toxic acid monomers, which are after metabolized and eliminated

from the body. Because of their safe degradation products, this polymers class is considered to be a biocompatible one.

Gliadel is one example of a polyanhydride product, often used as a device for the clinical use, in brain cancer treatement. They made of a polyanhydride wafer containing a chemotherapeutic agent. After removal of the cnacerous brain tumor, the wafer is inserted into the brain releasing a chemotherapy agent at a controlled rate proportional to the degradation rate of the polymer. This kind of treatement protects the immune system from high levels of radiaton. Other applications of polyanhydride include the use of unsaturated polyanhydrides in bone replacement and of polyanhydride copolymers as vehicles for vaccine delivery [54].

F. Polyorthoesters

Polyorthoesters are hydrophobic polymers that are mainly used in drug delivery systems. Polyorthoester is fabricated by polycondensation of diketene acetals and diols. This fabrication creates ortho-ester bonds that are stable at neutral pH, but hydrolyse rapidly at phagosomal pH, such as pH 5.5 [50].

G.Polycarbonates

Polycarbonate in its pure form is an amorphous polymer that possesses low moisture absorption, and is not susceptible to microbial attack, which implies non-biodegradability of the polymer. However, the mechanical properties of the polymer attract researchers that are interested in developing polycarbonate-based material that can withstand mechanical loading while the tissue is being regenerated. Since resorbability is the main problem, Bourke et al. [55] investigated a member of the tyrosine-derived polycarbonates that was not only resorbable, but also possessed high strength. Further studies based on polycarbonate’s good biocompatibility and ease of biochemical modification towards cell adhesivity has been undertaken [55].

H.Poly (Gycerol Sebacate)

The constant search for soft and mechanically stable elastomeric materials that could be implanted in dynamic environments led to the investigation of polymers that are analogous with vulcanized rubber, having a crosslinked three-dimensional network in combination with random coil characteristics. Polycondensation of glycerol and sebacic acid renders a polymer having hydrogen bonding interactions through hydroxyl proline hydroxyl groups. The hydrophilic characteristics of the material are a result of the hydroxyl groups attached to its backbone. The material is insoluble but swells in water by approximately 2% [52]. It is totally amorphous at 37 ◦C like vulcanized rubber, a thermoset polymer. However, the uncrosslinked polymer can be melted to a liquid form which is soluble in common organic solvents. It is tougher than hydrogels with tensile strength of less than 0.5MPa and tensile strain more than 300% [52].

I.Polyfumerates

Polyfumarate-based materials have been developed mainly for bone tissue engineering [26–29]. The main advantages of these materials are their injectable and in situ crosslinkable properties. Poly (caprolactone fumarate) (PCLF) and poly (ethylene glycol fumarate) (PEGF) were also investigated as injectable, self-crosslinkable polymers which circumvented the requirement for crosslinking agents that may be toxic. Also, they were shown to harden and self-crosslink when under physiological conditions, and tissue compatibility studies using rat models demonstrated no inflammatory reactions [27].

As the applications of polyfumarate-based materials were first investigated for bone substitutes, mechanical properties are an ultimate concern. Cortical bone has a compressive modulus in the range 17–20GPa, compressive strength of 106–144 MPa, flexural modulus of 15.5GPa, and flexural strength of about 180 MPa [28]. This presents a major challenge for the tissue engineer using polyfumarate-based materials. This unique characteristic of bone is a result of its composite make-up comprising the interaction of inorganic material, i.e. HA, with organic material such as collagen fibres.

J.PEGT/PBT

Polyethylene glycolterephthalate-polybutylene terepthalate (PEGT/PBT) is a thermoplastic polymer resin of the polyester. Engineering resins are made often in combination with glass fiber. Woodfield et al. [56] used a FDM-like technique for producing scaffolds made of polyethylene glycolterephthalate-polybutylene terepthalate (PEGT/PBT) . By varying PEGT/PBT composition, porosity and pore geometry, scaffolds were produced with a range of mechanical properties for engineering of articular cartilage [49].

2.5 Processing Techniques

2.5.1. Conventional Scaffold Fabrication Methods

A.Fiber bonding-non-woven meshes

Some of the first scaffolds used to demonstrate the feasibility of tissue regeneration involved the use of non-woven fibers in the form of tassels and felts consisting in individual fibers placed into a three dimensional pattern. Mikos et al. [46] produced from PGA fibers a porous scaffold intended for liver tissue regeneration .To produce a scaffold the PGA fibers were immersed in a PLLA solution, after evaporation of the solvent, the network of PGA fibers was embedded in a PLLA matrix. The composite is then heated above the melting temperature of PGA (225-230°C) [66]. The PLLA has a lower melting temperature (173-178°C) [64], so it melts first and fills all spaces left by the fibres. This helps to maintain the architecture of fibers so that when the PGA begins to melt, the fiber structure is conserved and fibers at the meeting-points are melting together. The PLLA is then dissoluted with methylene-chloride (PGA is insoluble in it). The porosity of these foams was as high as 81% and pore diameters were in range of 500 μm [46]. Scaffolds with the above described spatial arrangement are suitable for cell implantation and promote cell interaction [46, 56].

Although positive results have been reported using this technique, it must be emphased that this method involves the use of toxic solvents that must be extracted completely. For this reason, the scaffolds must be vacuum dried for several days that inhibits the immediate clinical response, like rejection, inflammation.

B. Solvent casting/particulate leaching

One of the most common and straightforward technique to prepare porous scaffolds is the particulate leaching method, which involves the selective leaching of a mineral, usually NaCl salt or of an organic compound such as sacharose to generate the pores [46]. Solvent casting/particulate leaching involves the casting of a polymer solution and dispersed calibrated porogen particulates in a mold, removal of the polymer solvent, followed by leaching out of the porogen [56]. Scaffolds with more than 70 w% salt exhibited high interconnectivity. Foams fabricated in this manner have been used extensively with various cell types and have shown no adverse effects on new tissue formation, but this technique also involves the use of toxic solvents. Because of the casting and solvent evaporation step, this technique is suitable for thin scaffolds only. One drawback in this technique is the presence of organic solvent, which may be hard to completely remove from the scaffold during the drying process. To circumvent this problem, it was proposed to replace solvent casting by melt-molding resulting in the melt-molding/ particulate leaching method [46].

C.Melt molding/ particulate leaching

The melt molding technique consists in premixing polymer powder and solid porogen particulates and hot-pressing them together. The samples are then subjected to the same solid porogen leaching step as for the solvent-cast samples. In a general manner, the major advantage of combined salt leaching technique is the effective control of porosity and pore size. Materials with porosity levels up to 90% and pore size varying between 100 and 700 µm have been reported by Mikos et al. [46], using the particulate leaching combination technique. The porosity or void volume fraction is given by the amount of leachable particles, whereas the pore size and pore shape of the

porous structure can be modified independently of the porosity by varying the leachable particles characteristics (size and shape). One potential deficiency of the technique, especially for scaffolds requiring lower porosity level is the lack of interconnectivity between the pores. To overcome this inconvenience it has been proposed to partially bond the porogen particles by working in a humid environment in the case of NaCl porogen or by heat treatment in the case of paraffin spheres or sugar particles [46], which lead to a higher interconnectivity from 60% to 70-80%.

D.Gas forming

Gas foaming, also known as gas saturation, eliminates the need for organic solvents during pore-making, and uses gas as a porogen. Initially solid discs of polymer are formed using compression molding. The disks are exposed to high pressure CO_2 (5.5 MPa) for 72 hours at room temperature, than the pressure is rapidly decreased to atmospheric level [46]. This creates thermodynamic instability for the CO_2 dissolved in the polymer disks, and results in the nucleation and growth of gas cells within the polymer matrix. The method resulted in scaffolds with porosities of up to 93% and pore sizes of up to 100 μm. The advantage of this method was that toxic chemicals are not used. But the high temperatures during compression molding still prohibit the incorporation of cells or bioactive molecules during processing. Furthermore, especially on the surface of the compression molded disks the pores were not connected [46].

E.Phase separation/emulsification

Two other methods can be used to prepare scaffolds: emulsification/freeze-drying and liquid-liquid phase separation. With emulsification/freeze-drying, the polymer is dissolved in an organic solvent and then distilled water is added to form an emulsion. The mixture is then cast in a mold and quenched in liquid nitrogen. After that, the scaffolds are freeze-dried at -55°C, to remove the dispersed water and polymer solvents. Scaffolds were reported to contain large porosities (up to 95%), but small pore

sizes (13-35 μm) [46]. Further research into this method is to increase pore size, in order to make the scaffold suitable for cell implantation [46].

Liquid-liquid phase separation uses the advantage of thermodynamic principles to create polymer-rich and polymer-poor phases within a polymer solution; the polymer poor phase is then removed. The polymers are dissolved in naphthalene, phenol or 1, 4-dioxane [46]. These solvents have a low melting point and are easy to sublime. Water can be added to induce phase separation. The polymer solution is then cooled down below the melting point of the solvent and then vacuum dried for several days to promote complete solvent sublimation. Cooling parameters proved to be of critical importance in determining scaffold morphology. Foams of up to 90% porous, with pores of approximately 100 μm, have been reported using this technique [46]

2.5.2 Scaffold Fabrication with Solid Freeform Fabrication Techniques

Control of the microscale polymer scaffold architecture is of fundamental importance in tissue engineering. With solid freeform fabrication (SFF) scaffolds for tissue engineering with different external shape and predefined and reproducible internal morphology can be produced where pore size, porosity and pore distribution can be controlled. SFF it represents a collection of techniques for manufacturing solid objects, by sequential delivery of material to specific points in space. This is done using a computer-aided design (CAD) software that is expressed as a series of cross-sectional layers. Using the data that are introduced in the software, starting from the bottom and building layers up, each newly formed layer adheres to the previous. Basically there are 2 main categories of rapid prototyping techniques for producing scaffolds: the melt–dissolution deposition technique and the particle bonding technique [57-59].

In a typical *melt–dissolution deposition* system, each layer is created by extrusion of a strand of material through an orifice while it moves across the plane of the layer crosssection. The material cools, solidifying and fixing to the previous layer to form a complex 3D solid object [57].

Fused Deposition Modeling (FDM) is a typical example of melt-dissolution deposition technique. In this method, a filament of a suitable material is fed and melted

inside a heated liquefier before being extruded through a moving nozzle having x and y axis control. The model is lowered along the z axis and the procedure is repeated. The system operates in a temperature-controlled environment to maintain sufficient fusion energy between each layer. Although the fiber must also produce external structures to support overhanging or unconnected features that need to be manually removed, the pore sizes in tissue engineering scaffolds are sufficiently small enough for the fiber strand to bridge across without additional support structures [57].

Microsyringe is also used widely in the manufacture of porous scaffolds. The microsyringe expels the dissolved polymer under low and constant pressure to form the desired pattern. The resolution of this method is on a cellular scale, which is remarkably high compared to the other techniques. However, capillaries with a very small diameter require careful handling to avoid any tip breakage. Higher pressure is also needed to expel the material from a small orifice [57].

In *particle-bonding techniques*, particles are selectively bonded in a thin layer of powder material. The thin 2D layers are bonded on top of each other to form a complex 3D solid object. During fabrication, the object is supported by and embedded in unprocessed powder. Therefore, this technique enables the fabrication of through channels and overhanging features and porous structures with controllable porosity. After completion of all layers, the object is removed from the bed of unbounded powder.

The space between the individual granules of powder is responsible for the porosity; by manipulating the region of bonding pore architecture can be controlled; however, the pore size is limited by the powder size of the stock material. To generate larger pores, porogens can be mixed into the powder. The powder-based materials provide a rough surface to the scaffold. Therefore, scaffolds fabricated via a particle-bonding technique might be more advantageous in the context of cell attachment. Typical systems in this category include 3-DP and SLS [57-59].

Three Dimensional Printing (3DP) incorporates conventional ink jet printing technology. The ink-jet print head moves in accordance to the CAD cross-sectional data along the x and y axis and eject a stream of adhesive droplets onto a polymer powder surface to selectively bond a thin layer of powder particles to form a solid

phase. The piston chamber is lowered (z-axis control) and refilled with another layer of powder and the process is repeated. The unbounded powder supports overhanging or unconnected features and needs to be removed after component completion. The resolution achieved is 300 μm. 3DP can be employed by using a particulate leaching technique to create porous scaffolds using polylactic-co-glycolic acid (PLGA) mix with salt particles and a suitable organic solvent [57].

Selective Laser Sintering (SLS) uses a deflected laser beam selectively to scan over the powder surface following the cross-sectional profiles carried by the slice data. It constructs scaffolds by sequentially fusing regions in a powder bed, layer by layer, via the computer controlled scanning laser beam. The interaction of the laser beam with the powder elevates the powder temperature to reach the glass-transition temperature, causing surfaces in contact to deform and fuse together [57] involves selective polymerization of a liquid photo-curable resin by an ultraviolet laser beam. The UV beam is guided (x- and y-axis control) onto the liquid resin surface in accordance to the CAD cross-sectional data, and it solidifies the model's cross-section while leaving the remaining areas in liquid form. After the first layer is built, the elevator holding the model is lowered along the z axis to allow the liquid photopolymer to cover the surface. A 'wiper arm' is then displaced over the liquid to flatten the surface. The procedure is repeated until the model is completed. This system requires support structures to be added to the model, to prevent any overhanging or unconnected features from falling to the bottom of the liquid-filled vat [57].

Table 2.2. Scaffold properties produced by various conventional techniques [57]

Methods	Porosity (%)	Advantages	Disadvantages	Pore size (μm)
Fiber Bonding (Unwoven mesh)	81	Highly porous scaffolds with interconnected pores	Use solvents which are poisonous to cells immersed in them for a long time	500
Solvent casting/Particulate leaching	87	Structure has high strength or electrical conductivity	Organic solvents used contaminate polymer Very long time required.	100
Gas foaming	93	biocompatible	Organic solvents used contaminate polymer	100
Phase separation/emuls-ification	95	Pore size and porosity easily changed	Solvents used are poisonous.	13 - 35

2.5.3. Biomimetic methods for bone substitutes' synthesis

In recent years biomimetic methods to obtain tissue substitutes have received much attention, due to a more natural approach of creating tissue. One biomimetic method for bone substitues' analysis is the substitution in apatite components, while maintaining apatite's basic structure. Ca^{2+} can be substituted by various cations (monovalent- Na^{+}, K^{+}, bivalent-Mg^{2+},Sr^{2+},Ba^{2+},Pb^{2+}, trivalent-Y^{3+}). It is known that the bone regeneration rate depends on several factors, such as: porosity, composition, solubility and the presence of certain elements that, when released during the resorbtion of the ceramic material, facilitate the bone regeneration carried out by osteoblasts. Therefore, small amounts of strontium, zinc or silicates are believed to stimulate the action of these osteoblasts and in consequence, the new bone formation [8]. Carbonate and strontium favour the dissolution, and therefore the dissolution of the implant. Silicates, however are formed to increase the mechanical strength, a very important factor in particular for porous ceramics, and also accelerate the bioactivity of apatite. In recent years, substitution of magnesium (Mg) in the apatite structure has been studied due to its impending role on bone metabolism, reducing cardiovascular disease, promoting catalytic reaction and controlling biological functions [9].

Using a polymeric matrix for the formation of bone like components is another biomimetic approach to bone formation. The polymeric matrix can be embedded into a solution called Simulated body fluid (SBF), an aqueous solution with a concentration that resembles the human body plasma ions one. In time on the polymeric matrix an apatite layer forms, which is an essential condition for biomaterials to bond to living bone or for the bone to start forming.

2.6. Techniques and materials used in the present research

The materials and techniques presented in the next chapter, were chosen in order to combine in the best way the aim of the research (that is producing a scaffold that would substitute hard tissue), managing materials costs and availability of the techniques and equipment. The present research is part of the NOVELSCAFF project,

funded by Marie Curie FP6 Framework, which combines 4 different techniques for producing hard tissue substitutes and one for soft tissue substitutes.

Polycaprolactone was the main material used in the project due to its reduced biocompatibility, costs and availability. Hydroxyapatite is the main component of the natural bone and is a bioactive material. Section3.2 underlines the other benefits of using these specific materials.

Conventional techniques were chosen for this particular research because they are simple and quick with reduced costs. In the same time Plasma Spraying, SLS and 3DPrinting were the other techniques used in the same project.

What this research brings new are the different combinations of the conventional technique, combinations that may simplify even more some of the existing steps and form structures with new morphological features and properties.

CHAPTER 3. EXPERIMENTAL EQUIPMENT, MATERIALS AND PROCEDURES

3.1 Introduction

This chapter outlines the equipment, the materials and the experimental procedures used in the current research. The research investigated the use of bioactive, biodegradable powdered biomaterials using different techniques in order to produce functional gradient scaffolds for bone tissue engineering applications. This means that the structures will mimic the natural tissue by design and internal structural changes, in order to replicate as much as possible the extracellular matrix of the chosen hard tissue. The flow chart in Figure 3.1 shows the steps taken in order to produce and characterize the scaffolds. First the polymer is dissolved in the solvent and the ceramic phase is incorporated, resulting a composite viscous solution which is used in all the techniques applied. If required by the technique, porogen agents are used. The second step includes characterization of the composite samples obtained either by gas forming, phase separation, salt leaching or solvent evaporation and analyzing all those obtained. The best samples with regard to hydrophylicity, morphology, mechanical properties are tested in phase 3, using in vitro tests (simulated body fluid immersion and cell line tests)

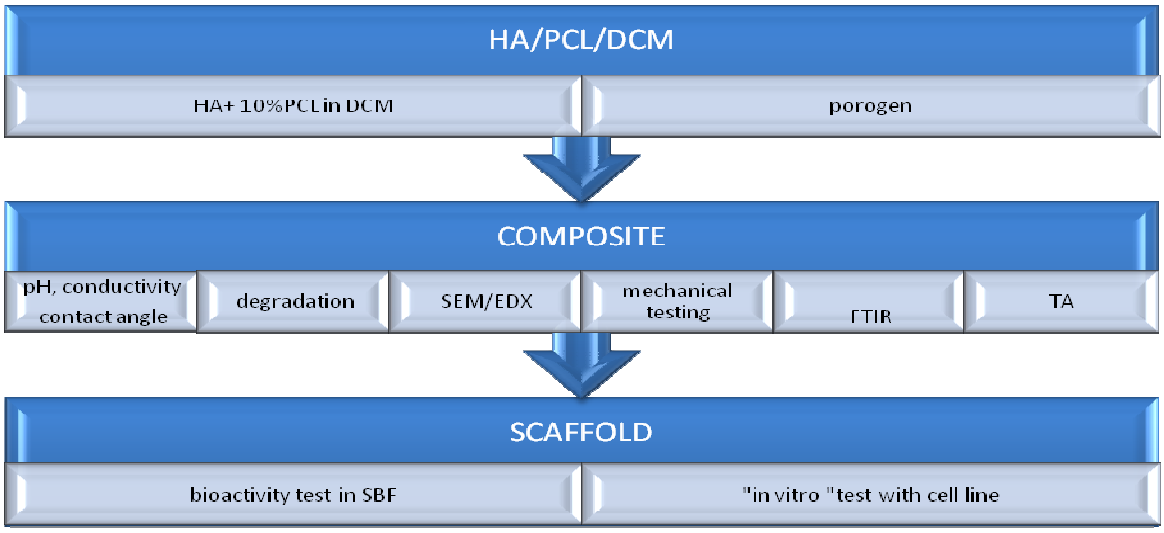


Figure 3.1 The research scheme

Due to the fact that the final product is intended for hard tissue engineering use, the materials to be chosen should combine enough strength to support load, adequate flexibility to withstand shocks and appropriate rate of degradation in order to offer the cells time to attach, proliferate and divide. In addition to these requirements the chosen materials and techniques should be cost effective.

Combinations of conventional techniques were used to induce pore formation, followed by optimization and characterization. Also, a novel technique was developed for producing micropores. The material characterization equipment used in the current investigation is described in Section 3.3. Two methods of investigation to evaluate the *in vitro* behavior of the produced composites that are intended for bone tissue engineering were also conducted, through apatite formation in Simulated Body Fluid (SBF) and cell studies, such as cell proliferation, cell viability and cell morphology. A description of these procedures is outlined in Section 3.4.

3.2 Materials

Characterization of the composite sample was conducted throughout various stages of the scaffold production. The main materials to be used in the construction of the final scaffolds were ε- polycaprolactone (molecular weight 65000Da and 80000Da) and hydroxyapatite, as the ceramic filler of the polymer. Additional materials were used for the preparation of simulated body fluid (SBF) for in vitro bioactivity tests and as porogen, gas forming agents and solvents. Table 3.1 shows these materials and their role in the research work.

Table 3.1 Materials and suppliers used for the research work

No.	Substance name	Used for	Supplier
1	Hydroxyapatite ($Ca5(PO)_3(OH)$)	Composite filler	Plasma Biotal Limited
2	ε- Polycaprolactone	Composite matrix	Sigma Aldrich
3	Dichloromethane (CH_2Cl_2)	Solvent	Fluka
4	Glacial acetic acid (CH_3COOH)	Solvent	Sigma Aldrich
5	Sodium hydroxide (NaOH)	Gas forming agent	Sigma Aldrich
6	Sodium bicarbonate ($NaHCO_3$)	Gas forming agent	Sigma Aldrich
7	Sodium chloride ($NaCl_2$)	Porogen	Sigma Aldrich
8	Potassium chloride (KCl)	SBF	Sigma Aldrich
9	Potassium phosphate dibasis trihydrate ($K_2HPO_4 *3H_2O$)	SBF	Sigma Aldrich
10	Hydrochloric acid (HCl)	SBF	Sigma Aldrich
11	Calcium chloride ($CaCl_2$)	SBF	Sigma Aldrich
12	Sodium sulphate (Na_2SO_4)	SBF	Sigma Aldrich

Polycaprolactone is a member of the alyphatic polyester family. Its degradation products are non toxic and are ultimately metabolized, which has resulted in it becoming FDA approved material. Its degradation involves two stages, comprising of simple mechanisms: random hydrolytic ester cleavage and weight loss through the diffusion of oligomeric species from the bulk. It was chosen for this research due to its low degradation rate, a property which allows cells to attach, proliferate and differentiate. Other advantages are its low melting point (~60°C) and the capacity of dissolving in organic solvents, which can be removed by vacuum drying. Consequently polycaprolactone is not bioactive, and its mechanical properties must be improved in order to be used in load bearing applications.

The polymer and ceramic particles were characterized using scanning electron microscopy with X-ray microanalysis (SEM/EDX) and thermal analysis (DTA/TGA). The composite structures were characterized using SEM/EDX, FTIR, pH, roughness, contact angle measurements, and investigating the degradation rate. In vitro tests were performed in order to determine the bioactive features and biocompatible properties of the produces structures. In order to investigate these features the samples were immersed in simulated body fluid (SBF), prepared in the laboratory, according to Kokubo's formulation [69]. In vitro cell tests were also conducted to evaluate the in vitro behavior of the samples, cell proliferation, cell viability and cell morphology.

Hydroxyapatite is a bioactive material and the main constituent of the human bone. It was used in this research as a filler material, in order to improve the mechanical properties of the polymer. The bending, compressive and tensile strength values of HA lie in the range 38-250, 120-150 and 38-300 MPa [82-84]. Weibull's modulus of dense HA lies in the range of 5-18, characteristic of brittle material and nonstoichiometric HA has the hexagonal space group structure [83].

In order to improve the pore size two different particle sizes (45 µm, 90µm) of the ceramic material were studied and for comparison reasons, glacial acetic acid was also used to dissolve the polycaprolactone, in order to check the viability of the produced scaffolds.

3.3 Scaffold Preparation

As shown in Figure 3.1, the first step was dissolving the polymer in thesolvent and obtaining a viscous polymeric solution. A screening step was implemented in order to find the best concentration of polycaprolactone dissolved in dichloromethane. The dissolved polymer should properly blend with the hydroxyapatite particles, in order to offer the new composite the best combination of mechanical and biological properties. At the same time it should possess an adequate viscosity in order to prevent the hydroxyapatite particles from migrating to the bottom of the composite solution, rather allowing them to suspend (described later).

The screening step revealed that at this stage of the research, 10% polycaprolactone in dichloromethane was the appropriate concentration for the purpose outlined. Into the 10% PCL/DCM solution different hydroxyalapatite quantities (4-70%

HA) were introduced and vigorously mixed, using a magnetic stirrer (Bibby, HB502, Sterilin UK). The magnetic stirrer speed was kept the same for all mixing step (250rpm). After 1 hour of mixing, the solution was left to rest for 24 hours. The next day, the mixing was repeated for 30 minutes and the solution was poured into Petri dishes. Figure 3.2 shows a schematic of the solvent evaporation technique, where 20ml of composite solution was poured into the Petri dish and left to air dry in the fume hood. Generally this technique is used in combination with other conventional techniques, as shown in the next figures 3.3, 3.4 and 3.5, this research work managed to produce porous matrix just by using the solvent evaporation step only.

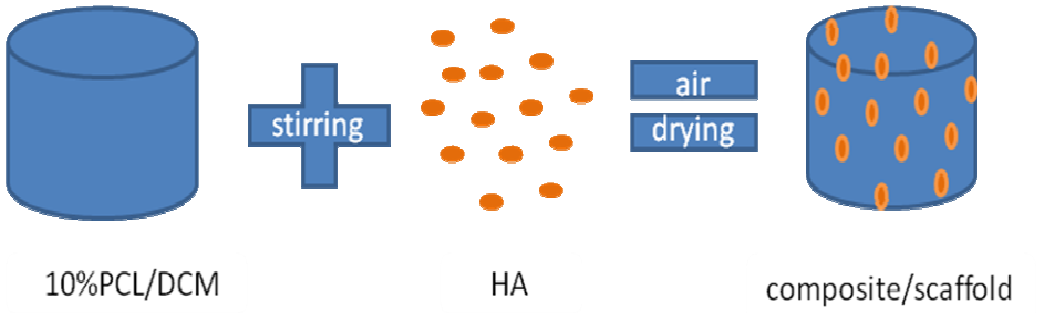


Figure 3.2: Solvent evaporation technique (SE)

The next stage of the process involves creating the pores. For this, various combinations of conventional techniques, specifically salt leaching, gas forming, solvent evaporation and phase separation were used. Separate research schemes for each technique are shown in the following figures (3.3, 3.4 and 3.5). The salt leaching technique involved the use of a porogen agent (salt/glucose particles), which dispersed in the polymer/solvent solution and cast within a glass dish. The solvent was then removed by evaporation and the remaining residual by vacuum drying. In order to remove the salt particle and obtain a porous structure, the dried structure was immersed in water for a convenient period of time, during which time the water was changed several times, to speed up the leaching process. Figure 3.3 shows this technique.

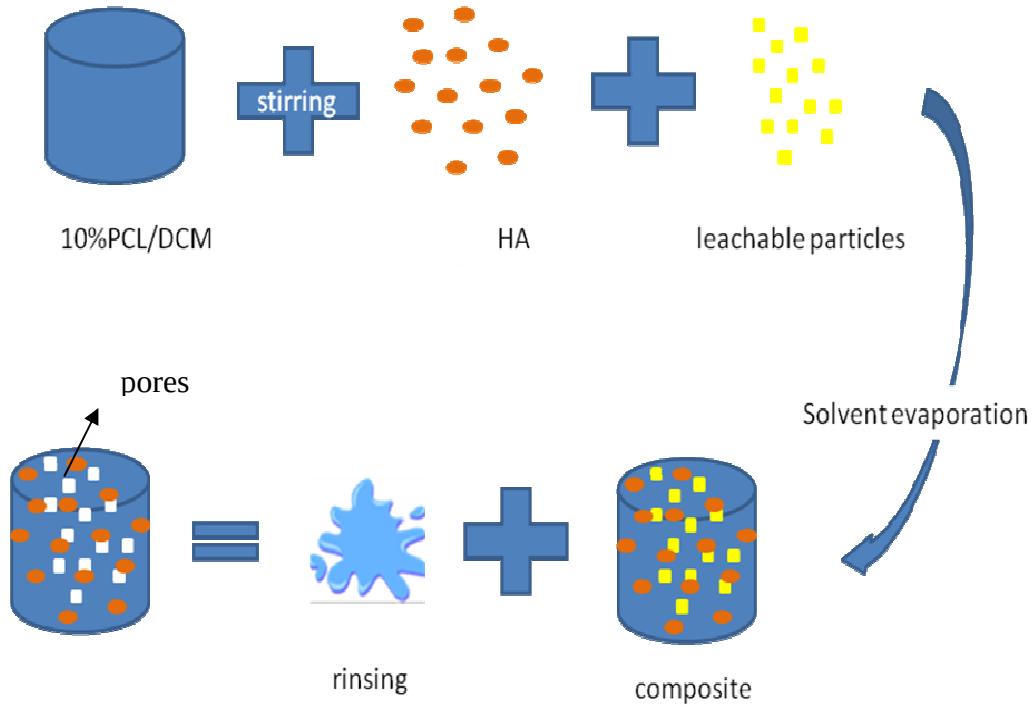


Figure 3.3: Salt leaching technique

In the present research work salt particles of 150 - 425 µm in size, were mixed into the composite solution, poured into a Petri dish, left to dry and, in the end over rinsed for 48 hours in distilled water (the water was changed every 4 hours).

The gas forming technique uses gas forming agents, or even CO_2 gas as the pore forming agent, as shown in the schematic in Figure 3.4. The reason pores form is that the sieved salt particles of ammonium bicarbonate which were dispersed within the polymer-solvent mixture generate ammonia and carbon dioxide gases upon contact with hot water while the matrix solidifies and finally produce highly porous structures. On the other hand when CO_2 gas is used, pressure changes determine the nucleation of pores in the polymer matrix. The salt leaching technique was also used in combination with gas forming, to create an open, interconnected pore structure of the polymer matrix [57].

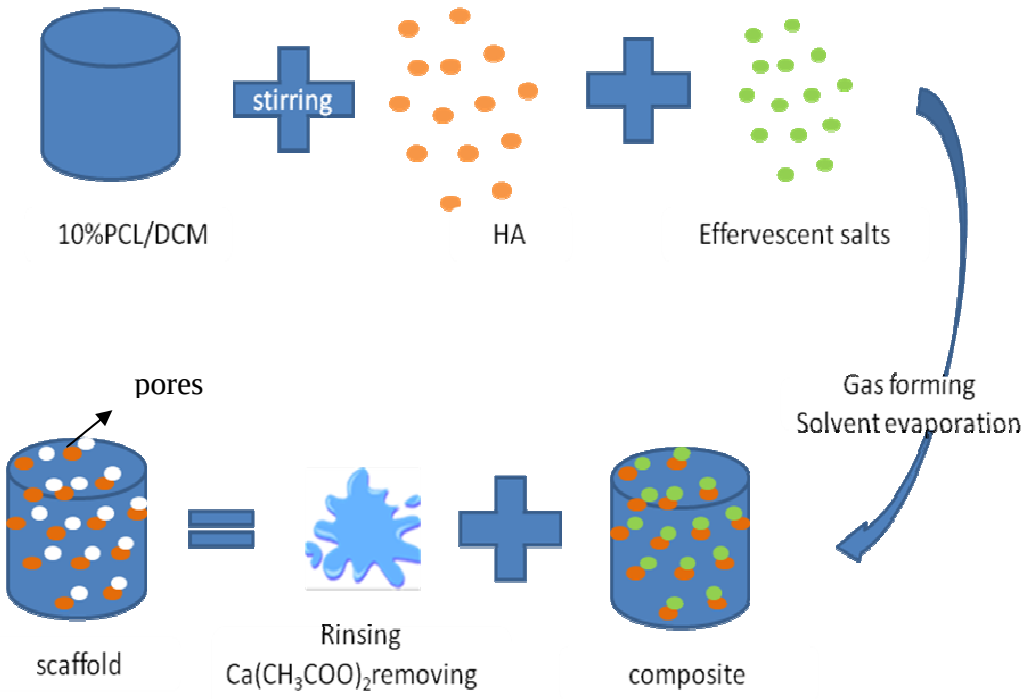


Figure 3.4 Gas forming technique

In this study, the gas forming/ salt leaching method was improved further by either immersing the semisolidified polymer/salt mixture into an aqueous solution of citric acid to yield gas-forming process at room temperature [57] or by combining effervescent salts in the PCL/DCM/HA solution directly or leaving these to react.

The other technique implemented in the research was phase separation, as indicated in Figure 3.5. After dissolving the polymer, water was added and vigourously mixed in a glass container. Immediately the solution was frozen and vacuum dried. To lower the vacuum drying temperature and speed, the samples were placed in between two metal plates. This technique can be implemented when there are multiphasic mixtures.

All the samples were further tested and characterized.

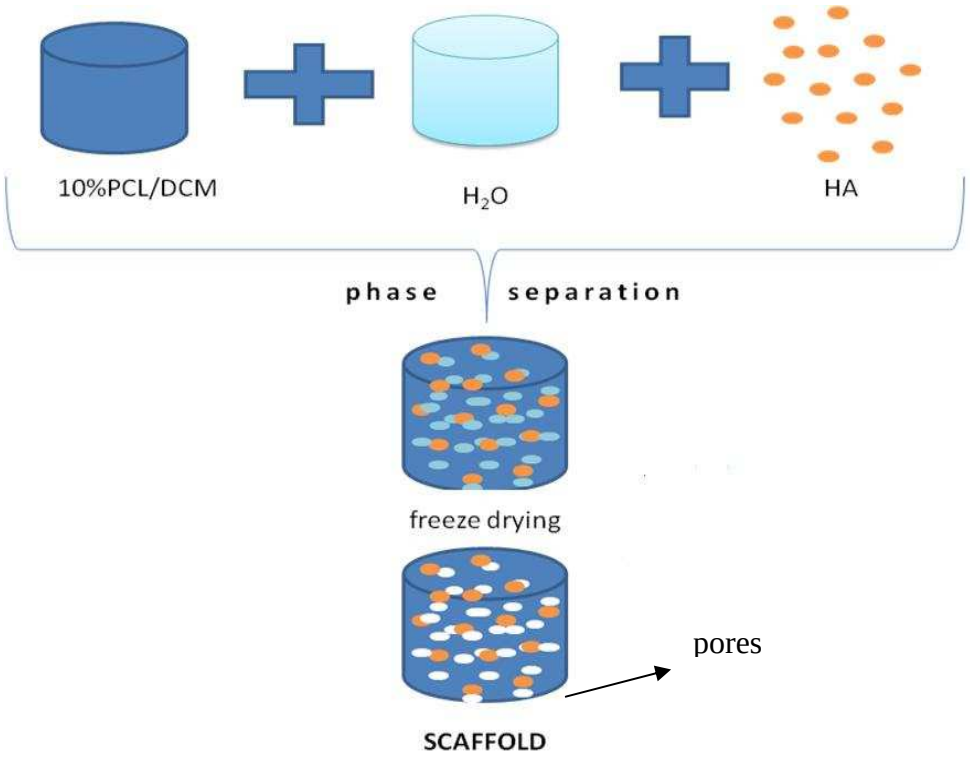


Figure 3.5: Phase separation technique/freeze drying

3.4. Characterization Equipment

The materials and the composite samples were characterized using in house (Materials Processing Research Centre) equipment.

Initially thermo gravimetric and particle size analysis were conducted, using the differential and thermal gravimetric analyzer (DTA/TGA, PL Thermal Sciences Ltd., UK). After the composite samples were prepared, characterization involved the use of SEM/EDX, FTIR, pH/conductivity meter, contact angle and roughness measurements.

3.4.1. Differential and Thermal Gravimetric Analysis (DTA/TGA)

In the DTA, the material to be analyzed and an inert reference material underwent identical thermal cycles, and measurements were conducted to identify any temperature difference between the sample and the reference. The results were plotted

against time, or against temperature in what is called a"DTA curve" or thermogram. Either exothermic or endothermic changes can be detected relative to the inert reference. Thus, a DTA curve provides data on the transformations that have occurred, such as glass transitions, crystallization, melting and sublimation. The area under a DTA peak shows the enthalpy change and is not affected by the heat capacity of the sample.

TGA measures changes in weight in relation to changes in temperature. It is a type of analysis that requires a high degree of precision. TGA is commonly used in research to determine characteristics of materials, to determine degradation temperatures, absorbed moisture content of materials, the level of organic/inorganic components in materials, solvent residues.

Differential Thermal Analysis and Thermo-Gravimetric Analysis (DTA/TGA) were performed on the individual polymeric and ceramic materials, prior to any processing technique, in order to help understand any interaction between the two materials seen subsequently.

3.4.2. Particle Size Analyzer

The method used to determine the grain size distribution is called particle size analysis and the apparatus used for this was called the Malvern particle size analyzer. The particle size distribution is defined in terms of discrete size ranges. The particle size distribution is important in understanding the physical and chemical properties of a material. It affects the reactivity of solids participating in chemical reactions, and needs to be tightly controlled [67-68]. For this research HA powder with particle size of 45 µm and 90 µm was used. The particle size was specified by the supplier and no additional testing was performed.

3.4.3. Scanning Electron Microscopy/ Energy Disperse X-Ray Spectroscopy

Scanning electron microscope (SEM) is a type of microscope (electron microscope) that uses a high-energy beam of electrons. The principle of this technique is that the electrons interact with the atoms that make up the sample and produce signals that contain the information about the topography of the sample's surface, composition

and other properties (e.g. electrical conductivity). The image is an artificial map of the surface because there are no direct ray paths linking the specimen to the projected image, like in the case of optical and transmission microscopes. The area under observation is irradiated with a finely focused electron beam (10- 40 kV) [69]. The scattered electron (SE), including the back scattered electron (BSE) signals, are monitored by a detector and the brightness of the spot on the cathode ray tube (CRT) is controlled by an amplified version of the detected signal. The angle of incidence will vary due to the variation of material's roughness, leading to the development of contrast, which relates to the physical nature of the specimen. The surface facing the light source appears bright while that facing away varies from grey to black. In the end the surfaces that face the detector provide strong enough signals to obtain a useful image, while black are indicative of a tilted surface.

The topography of the composite surfaces was evaluated using Scanning Electron Microscopy (SEM, Zeiss EVO LS15) with image analysis and qualitative EDX capabilities.

The samples were first coated in gold, in order to increase the electrical conductivity of the specimen sample, and also provide a clear analysis of the surface topography and morphological properties. For this purpose an Edward Pirani 501 Scancoat six sputter coater was used to apply a layer of gold onto the samples which took 80 seconds.

3.4.4. Degradation Tests, Contact Angle, Roughness, pH and Conductivity Measurements

Degradation tests: It is desirable for the scaffold matrix produced, to degrade at a rate that will slowly transfer load to the healing bone. Figure 3.4 shows the correlation between the degradation time of the matrix and the built up of the tissue (bone).

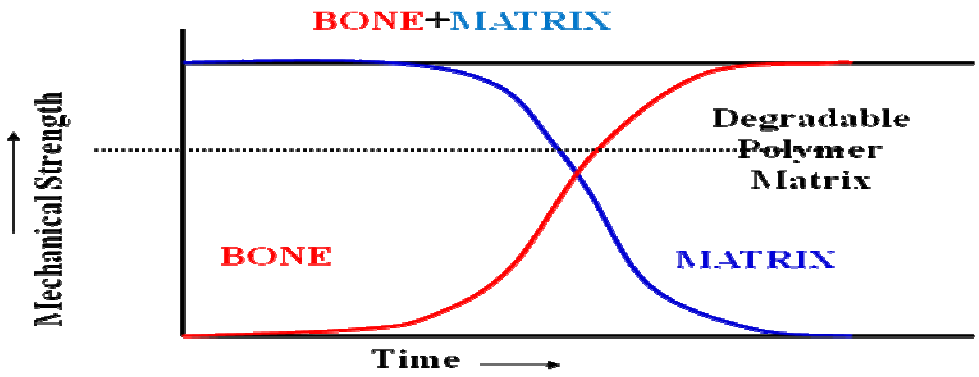


Figure 3.6: Idealised equilibrium between degradation rate of the scaffold and bone regeneration (adapted from [77])

The *degradation* process takes place in 4 steps: water sorption, reduction of mechanical properties (strength and modulus), reduction of molar mass and weight loss. There are two types of degradation: bulk erosion and surface erosion. As defined by Gopferich, "polymer degradation is defined as the chemical reaction resulting in a cleavage of main-chain bonds producing shorter oligomers, monom ers, and/or other low molecular degradation products" [77]. Both *in vivo* and *in vitro* degradation occur in the same rate that shows no significant enzymatic contribution initialy [78]. This also can be explained as several studies have by the fact, that the main mode of degradation for high-molecular-weight aliphatic polyesters is hydrolytic random scission, and biodegradation is supposed to involved just low-molecular weight by-products (M_n< 5000) or sub-micron sized particles that are recognized and ingested by phagocytes.

According to the criteria used to choose the materials and the tissue engineering purpose of the produced samples, degradation tests were conducted in order to investigate if the ceramic particles of HA will change the degradation rate and dynamics. Samples of the same weight and dimensions ($1cm^{2,}$ 50g) were immersed in 50ml deionised water each and kept for 3 months. Every 30 days one sample was removed from the plastic jar, dried and weighted. The results were then recorded for analysis. For biological research the solution that is usually used for degradation tests is phosphate buffer solution (PBS). This is a solution resembling the osmolarity and ion strength of the human body, which helps maintaining a constant pH. In this research

deionised water was used instead of PBS, in order to follow the pH changes as indicator of degradation steps taking place.

Contact angle: A hydrophile material is a material that can transiently bond to water through hydrogen bonding. This property makes the material dissolve more readily in water than in oil or other hydrophobic solvents. The degradation profiles of polymers are related to many factors, in which hydrophilicity plays an important role. Hydrophobicity is also linked with surface energy. Whereas surface energy describes interactions with a range of materials, surface hydrophobicity describes these interactions with water only. Because water has a huge capacity for bonding, a material with high surface energy (high bonding potential) can enter into more interactions with water and consequently will be more hydrophilic. This means that hydropobicity generally decreases as surface energy increases. A simple method that it is used to measure the surface energy and tension is the *contact angle* measurement. This technique is surface sensitive, with the ability to detect properties on monolayers. If a liquid with well – known properties is used, the resulting interfacial tension can be used to identify the nature of the solid. When a droplet of liquid rests on the surface of a solid, the shape of the droplet is determined by the balance of the interfacial liquid/vapour/solid forces. When a droplet of high surface tension liquid is placed on a solid of low surface energy, the liquid surface tension will determine the droplet to form a spherical shape (lowest energy shape). The measurement provides information regarding the bonding energy of the solid surface and surface tension of the droplet. Due to the fact that this method is a very simple one, it has been accepted for material surface analysis related to wetting, adhesion and absorption.

The method used for our tests was the sessile drop technique, which is shown in Figure 3.7, using ArtCAM 130 MI BW monochrome camera, and FTA200 contact angle analyzer software. Contact angle evolution was analyzed after 1, 2 and 3 seconds. Before conducting the measurements the flat samples were cut and cleaned with an air pistol, in order to remove any loose particles.

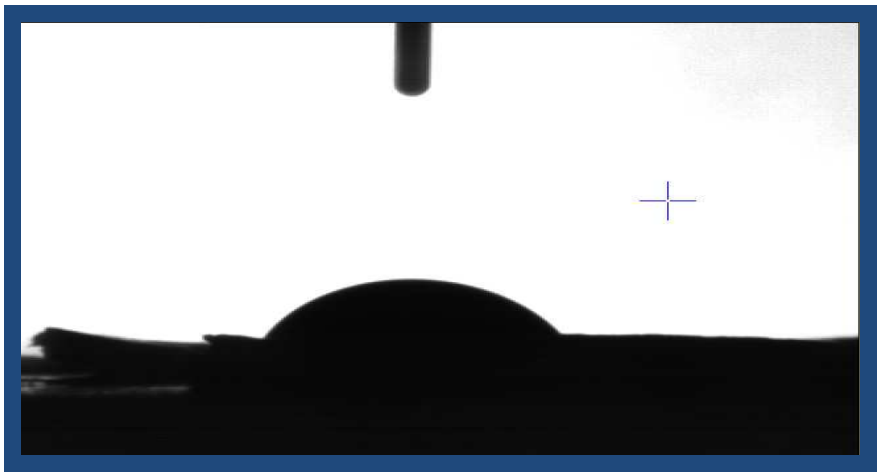


Figure 3.7: Contact angle measurement, using sessile drop method

Roughness : is a measure of the texture of the surface. It plays an important role in determing how a material interacts with its surrounding environment. Rough surfaces will wear more than smooth surfaces and have higher friction coefficients. Also having information on roughness can indicate the mechanical performances of the material, since surface irregularities may form nucleation sites for cracks and corrosion. Roughness can be measured using contact or non- contact methods. Non-contact methods include interferometry, confocal microscopy, electrical capacitance and electron microscopy. For the presented research, a surface roughness tester (Mitutoyo Surftest 402) was used to determine the average and maximum roughness parameters [70].

Conductivity measurement: For normal cell formation pH is a very important factor, due to the fact that cells are very sensitive to it. They are suited to a neutral pH level of 7.3-7.4. Even the slightest change in a pH level can cause serious consequences such as the destruction of the cell or even death of the organism. Every organism takes part in various chemical reactions that give or use up H^+. For this level to stay constant a buffer comes into play to maintain the cells normal pH level. It does this by accepting or releasing H^+. This mechanism of maintaining a proper balance between acids and bases is called acid-base homeostasis. Outside the range of pH that is compatible with life, proteins are denatured and digested, enzymes lose their ability to function, and the body is unable to sustain itself.The conductivity of a solution is its ability to conduct an electrical current, is the reciprocal of its electrical resistance. It is an indicator of the

ionic strength of a solution and it is determined in the same time with the pH determination.

Conductivity and pH measurements were recorded via a Hanna HI 9813 Handheld pH, EC and TCS Meter with Probe (Hanna Instruments, Inc.) with accuracy of pH: ± 0.2 pH at ambient temperature, in order to monitor the degradation process [71].

3.4.5. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is a chemically-specific analysis technique that can be used to identify chemical compounds and substituent groups, measuring the infrared intensity versus wavelength (wavenumber) of light. Based upon the wavenumber, infrared light can be categorized as far infrared (4 ~ 400cm^{-1}), mid infrared (400 ~ 4,000cm^{-1}) and near infrared (4,000 ~ 14,000cm^{1}). It is an absorbance technique. Infrared absorbance only takes place when infrared radiation interacts with a molecule undergoing a change in dipole and when the incoming infrared photon has sufficient energy for the transition to the next allowed vibrational energy state.

A Fourier Transform Infrared (FTIR) spectrometer obtains infrared spectra by first collecting an interferogram of a sample signal with an interferometer, which measures all of infrared frequencies simultaneously. An FTIR spectrometer acquires and digitizes the interferogram, performs the Fourier transform function, and outputs the spectrum.

The spectrums of the samples before and after immersion in simulated body fluid (SBF) were analyzed using a Pekin Elmer Spectrum GX FT-IR system with HATR (Horizontal Attenuated Total Reflectance) accessory (Spectrum V3.01 software). Also EDX results were used to identify the apatite formation on the surface of the sample, as an indicator of bioactivity feature of the scaffold.

3.4.6. *Mechanical Testing*

Experience shows that polymeric materials display a wide range of mechanical behavior, from brittle solids to rubber to plastic to strong fibers. Also it is widely known

that the mechanical characteristics of a polymer alters with changes in temperature as small as a few degrees, and also if it is used to form a composite material.

For the mechanical characterization flat samples (20 mm× 1mm) were manufactured, tensile strength and elastic modulus of the microporous samples were determined, using a Zwick/Roell Z500 N universal testing machine equipped with a 500N load cell (Zwick GmbH, Ulm, Germany). The results were plotted with Test Xpert II (Zwick GmbH, Ulm, Germany).

3.5 "In Vitro" Tests and Biological Cell Responses

3.5.1. Bioactivity Test in Simulated Body Fluid (SBF)

Simulated Body Fluid it is an accellular solution that has inorganic ion concentrations similar to those of human extracellular fluid. It is often used to reproduce the formation of apatite on bioactive materials in vitro. It was first developed by Kokubo and his colleagues [72] and is since known as SBF or Kokubo solution.

SBF has demonstrated its effectiveness via the surface modification of various materials [72]. Initially it was applied as a test to bio-ceramics which were part of bone implants.There are ceramics that bond to the bone through a bone-like apatite layer which forms on the ceramics or modified polymers surfaces. Currently the formation of the apatite-layer is not fully understood. It is known that the biomaterial surface must express OH groups, in order to attract the positive ions from the solution and create nucleation sites. On the surface of organic polymer the apatite layer formation takes place in a two-step biomimetic process [73].

Square samples of $10mm^2$ and 50g were cut from the prepared composite membranes, at least 4 for each HA concentration. The SBF was prepared in the laboratory following Kokubo's recipe [71]. Table 3.2 shows the concentrations of body plasma and SBF [74].

Table 3.2 Ion concentrations (mM) of SBF and human blood plasma [74]

Ion	Simulate Body Fluid	Blood plasma
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	148.8	103.0
HCO^{3-}	4.2	27.0
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5

The samples were immersed in SBF, within plastic containers kept in a water bath (Clifton, Nickel Electro LTD, UK) at 37°C, for 4 weeks. Every week a sample for each HA concentration was taken out, dried and analyzed using SME/EDX and FTIR.

3.5.2. Biological Response of Composite Scaffolds

a. Osteoblasts cells

MC3T3-E1 osteoblast-like cells represent a suitable model for studying osteogenic development in vitro. The osteoblastic cell line MC3T3- has been established from C57BL/6 mouse calvaria. Cells have the capacity to differentiate into osteoblasts and osteocytes and have been demonstrated to form calcified bone tissue in vitro. Mineral deposits have been identified as hydroxyapatite.

b. Cell culture conditions

For determination of cell attachement, MC3T3 Mouse calvarial osteoblast cells were cultured under standard tissue culture conditions (37°C, 5% CO_2) in alpha-MEM medium (Gibco, USA) supplemented with 10% Fetal Bovine serum and 1% Penicillin/Streptomycin. All experiments were conducted with cell between passages 4-8. Samples were sterilized by immersion into 70% Ethyl alcohol for 2 hours and

following washing steps with sterile PBS, stored in culture medium in a CO2 incubator overnight to to promote protein adsorbtion. The cells were trypsinized and the cell number was quantified by tryphan blue and then seeded onto samples, at a concentration 1×105 cells/cm2 in 20µL of medium. After an initial attachement period, the medium was completed to 500µL.

c. Fluorescence Microscopy

To assess cell attachement, proliferation and morphology, samples were stained with DAPI (a DNA binding dye, to stain nuclei) and FTIC –labelled Phaloidin (a fungal toxin with specific affinity to f-actin fibrils, to visualize cell cytoskeleton), samples were fixed with 3.7% formaldehyde for 5 minutes and then rinsed with PBS.Afterwards celles were permealized by treatement with 0/1%Triton X solution. After removal of Triton-X, samples were incubated in 1% PBS-BSA solution at 37°C for 30 minutes to decrease non-specific absorbtion of the dyes. Afterwards 1:1000 dilution of DAPI and 1:200 dilution of FTIC-Phalloidin were applied to the samples and the samples were incubated in the dark for 15 minutes. After washing with copious amount of PBS, the samples were observed under and epifluorescence microscope under single or multiple fluorescence modes (Olympus, Japan).

d. Alamar Blue Cell proliferation assay

Cell attachment and proliferation was quantified by Alamar Blue Cell proliferation assay (AbBiotech, USA) at 1 day and 7 days and 14 days post seeding. Alamar Blue solution (10% in serum free alpha MEM medium) was applied onto the samples and absorbance of the dye at 562 and 595 nm, was determined after one hour of incubation in culture. Absorbance readings were converted to dye reduction % as per instructions of the provider. Dye reduction (%) is indicative of cellular metabolic activity, i.e. higher reduction signifies higher cell number.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Introduction

Several studies have been conducted on building scaffolds for bone tissue engineering, using different types of polymers, or composite materials. There are several factors that influence how the scaffold morphology should behave, what properties it should exhibit. A scaffold intended for bone tissue engineering can be used either outside of the body (*in vitro*) for cell culturing, or implanted in the body (*in vivo*), offering a temporary replacement for the bone, until the seeded cells produce new bone. Research studies have shown that for good scaffold performance, essential properties are required, independent of its place of use, which include: high porosity, appropriate mechanical strength, biocompatibility and bioresorbability. There is no perfect combination of all of these features covering all the applications, since bone itself varies depending on its age and location in the skeletal system. Therefore before a scaffold is fabricated, one needs to define its role.

The present research work aims to produce porous composite scaffolds for bone tissue engineering. Scaffolds characterization will include: porosity, mechanical tests bioactivity and biocompatibility tests. A polymer with an adequate degradation period (PCL) will be used to construct the matrix and a ceramic part to add strength and improve its biofeatures. Ideally it will mimic the natural structure of the extracellular matrix, offering the cells a surface to adhere on, proliferate and differentiate. The preliminary studies were particle size, powder morphology and thermal gravimetric analysis of the materials (Chapter 4.1 and 4.2). The next step included creating the composite membranes of different ceramic concentration and their characterization. In order to produce the composite membranes, improved combinations of conventional techniques were used (salt leaching/ freeze drying/phase separation/ gas forming/solvent evaporation).

The preliminary investigation of this study involved characterization of the HA/PCL composite, with different HA content (1-70%). SEM analysis were performed to observe the morphology of the prepared samples (section 4.2.3) and the viable samples were tested mechanically (section 4.2.4). The next step was the degradation,

contact angle and roughness measurements section 4.2.5). Comparing the results obtained after characterizing all the samples produced, those performing adequately towards the aim were tested for bioactivity features. Samples were immersed in simulated body fluid (SBF) (section 4.2.6); a fluid resembling the human body plasma, and apatite formation was monitored. Finally scaffolds were seeded with cells and cell attachment, proliferation and differentiation was evaluated (section 4.2.7).

4.2. Preliminary Characterization of the Powder Materials

4.2.1 HA Morphology and Particles Size

Figure 4.1 shows the morphology of the initial HA powders. For both types of powders the particle size was specified (45 and 90µm). HA 60 (45µm) is a spherical powder produced by spray drying and the HA 90 (60 µm) was produced by tray drying, giving it an irregular shape. Research has shown that the shape, size and morphology of a particle are factors that influence interfacial bonding [74], which also influence the impact behaviour, important in the case of cranium regeneration. Even though the morphologies of the two powders were different due to the different methods of fabrication, the same porous pattern was obtained, for composites of 1-10% HA in PCL. Thus the results show that following the same technique protocol, the same porous pattern can be obtained independent of powder morphology.

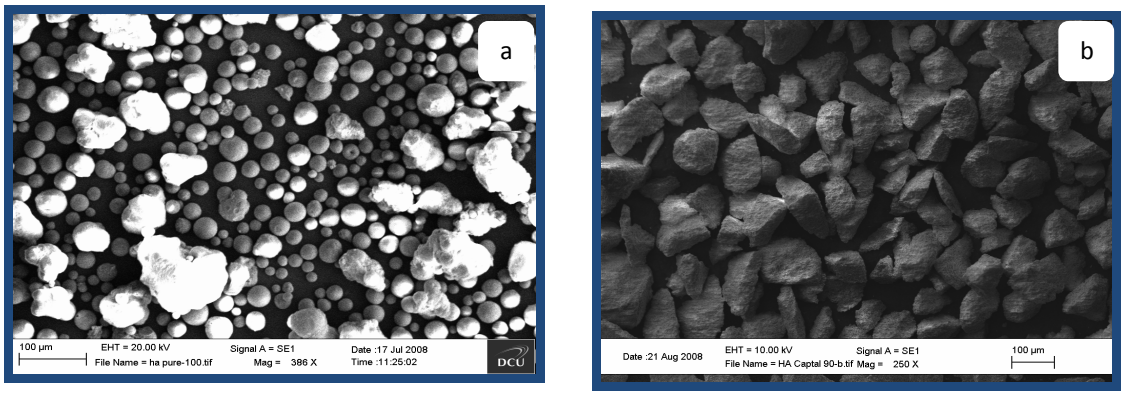


Figure 4.1 Morphology of (a) HA 60 (B) HA 90

4.2.2 Thermal gravimetric analysis

Figure 4.2 shows the DTA/TGA curve for the commercial polymer, PCL, which can be considered its "fingerprint". The results show a melting temperature of 60°C, indicated as a peak on the DTA curve and at 360°C the start of the degradation, indicated on the TGA curve, when the signal begins to drop. For the chosen manufacturing techniques, the polymer does not require melting, so it will not reach 60°C. In the case of sterilization methods like ethylene oxide could be used, which is a gas commonly used to sterilize objects sensitive to temperatures greater than 60°C.

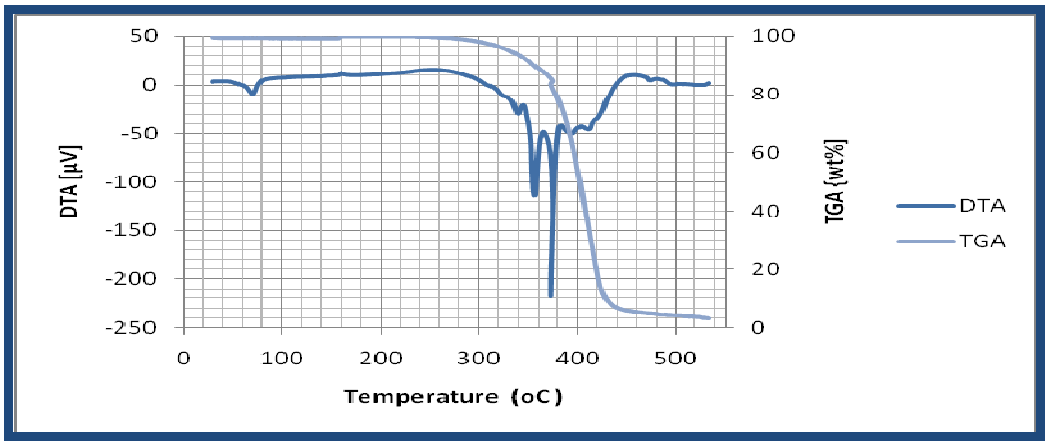


Figure 4.2 DTA/TGA curves for PCL

4.2.3 SEM/EDX Composite Results

Solvent Evaporation Technique

SEM images revealed that the ceramic phase (1-10% HA) was fully covered by the polymeric one. The first sample obtained did not exhibit any porous structure, as shown in figure 4.3, which is not an advantage for cell proliferation. Equally, due to the lack of porosity the hydroxyapatite did not interact with the cells, which would normally enable its bioactive features.

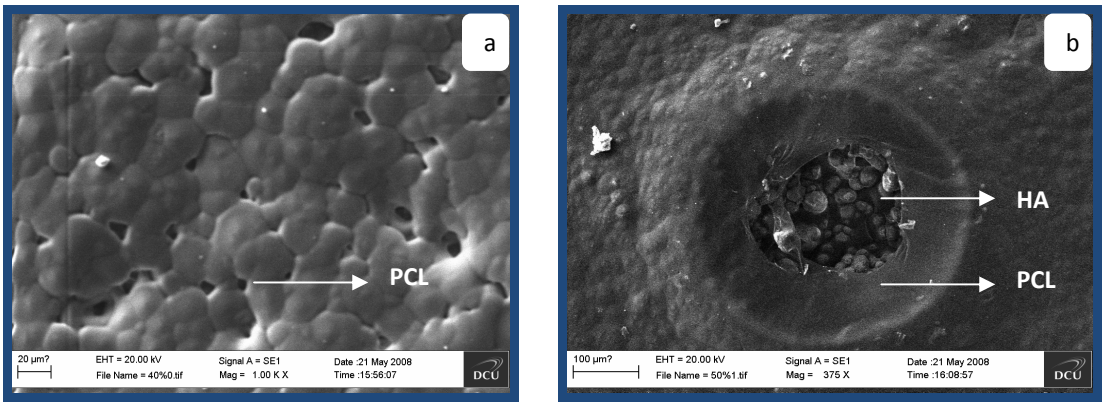


Figure 4.3 a, b. The PCL fully covers the HA particles.
Sample of 4% HA: 96%PCL

Porous composite structures, as shown in figure 4.4, were created by varying the mixing technique. The polymer placed in the solvent solution was vigorously mixed. After the polymer was completely dissolved, the HA particles were added and the complete solution was vigorously shaken for 5 minutes and left to settle for 24 hours. Finally the solution was poured into glass Petri dishes and left to dry. Pores of different sizes were visible on the x-y surface of the sample, as shown in figure 4.4, as opposed to the structure evidenced in figure 4.3. The pore size was found to increase with increased HA particle size. Using HA of 45 µm particle size, the mean pore size was between 6-10 µm, and for HA of 90 µm particle size, the mean pore size increased to between 15-25 µm. A possible explanation for the formation of the pores was formulated: the PCL molecule has one end polar, and another non-polar. The polar end of the PCL reacts with the hydroxyl groups on the surface of the hydroxyapatite. Due to violent mixing (magnetic stirrer) the PCL molecules are torn away from the surface of the HA particles, and the water forms small droplets in the DCM solvent. The charged polar ends of the PCL polymer then stabilize by forming a layer around each droplet of water, causing each droplet to be negatively charged. These charged PCL molecules behave like a surface active agent in the water. So when the water droplets stabilize, this prevents them from coalescence, and they distribute evenly throughout the DCM solvent. During slow evaporation, both the DCM and the water were removed, leaving a system of pores in the PCL matrix, with the pores being equi-distant from each other.

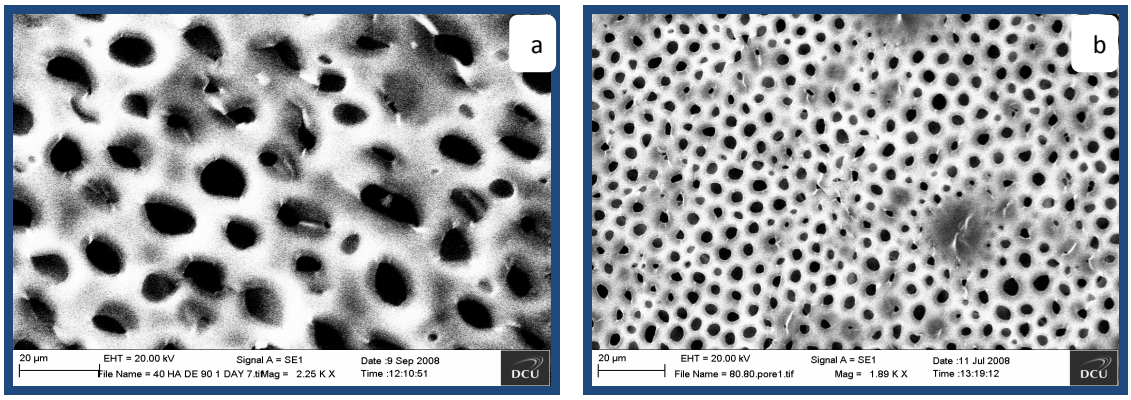


Figure 4.4 HA/PCL/DCM sample with HA particle size of 90 µm (a) and 45 µm (b)

Different morphologies were found using the SEM, for the samples left uncovered to dry in air and the ones covered by a glass plate, as shown in figure 4.5. "Exposed to air" samples "a" dried very quickly by solvent evaporation, where over the 6 hours this allowed the polymer to shrink and separate from the ceramic phase. This behaviour was observed for all samples "exposed to air", independent of the HA concentration. This aspect was improved by lowering the solvent evaporation speed. This was done by covering the Petri dish with another glass plate. The drying time was then 48 hours, where the air surface top aspect of the sample improved. Figure 4.5b shows a sample that was dried for 48 hours (covered with a glass plate). The hypothesis is that the solvent evaporated at a lower rate when the samples were covered and this offered time for the composite blend to spread through and dry slowly.

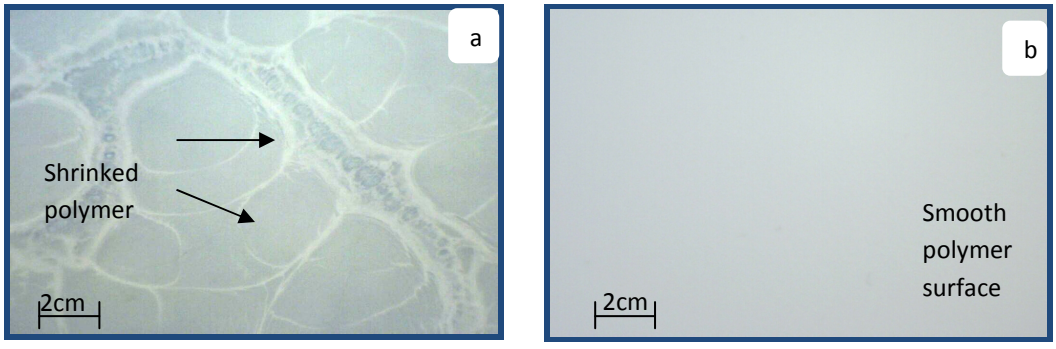


Figure 4.5 Dried composite sample (a- uncovered / b- covered)

EDX analysis showed no trace of HA on the air surface side (top) of the samples, but HA was found on the glass surface side (bottom) of the sample, for the both types of dried samples. The dried composite sample revealed the formation of two different layers of material: a ceramic and a polymeric one. This two layer composite

was the result of the higher density of the ceramic phase (ρ_{HA} = 3.156g/cm3, $\rho_{PCL+DCM}$ ~ 2.5g/cm^3). Figure 4.6 shows the two layers of the composite sample obtained by solvent evaporation technique. EDX analysis confirmed also that the HA was not present on the upper surface of the sample, and that the porous structure had a vertical interconnective orientation.

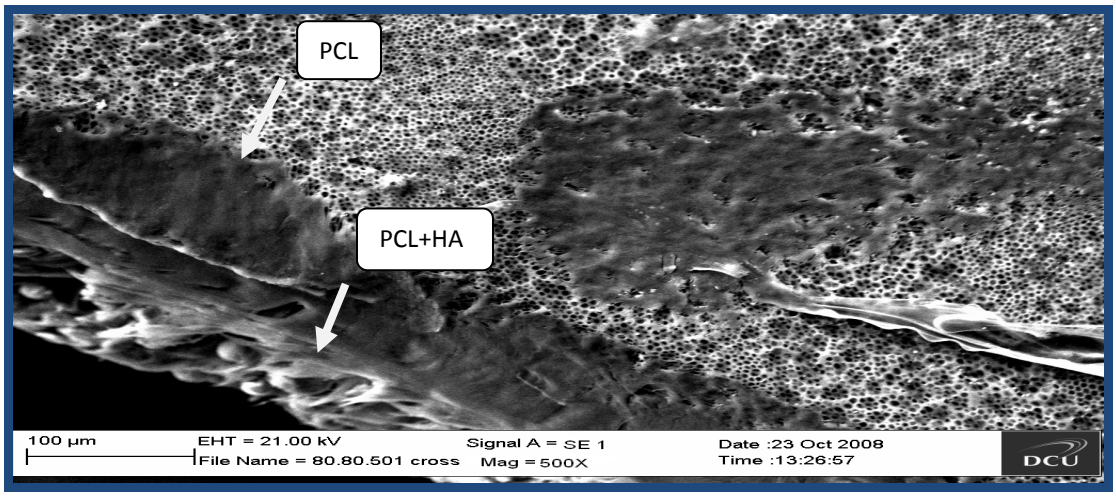


Figure 4.6 The two layers of the composite sample HA/ PCL. Upper level is composed of PCL and the lower is composed of HA+PCL

Salt Leaching Technique

For the leaching technique, salt with particle size varying from 250- 425 µm was added to the composite solution. Three different concentrations of salt, that is 10%, 15% and 20%, added to 2, 4 and 6% HA in PCL were tested and results are shown in figure 4.7.

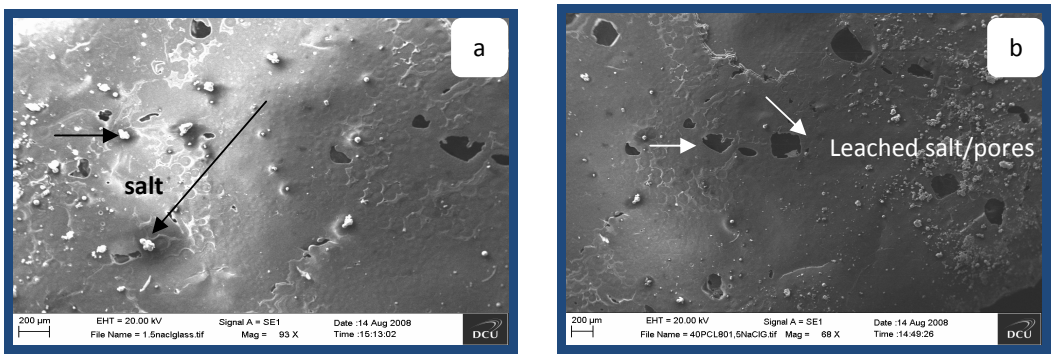


Figure 4.7 Salt leaching technique samples (a- 10% salt, b- 15% salt)

After obtaining the composite solution, the desired amount of salt was weighted and mixed in the solution, and then this solution was poured into the Petri glass dishes. After air drying, covered for 24 hours, under the fume hood the samples were immersed in distilled water (the leaching process). The water was changed every 4 hours, for the first 24 hours, and every 8 hours for the next 2 days, to speed up the leaching time, as indicated by the literature information [30]. After salt leaching, the samples were vacuum dried for another 48 hours and then analyzed. The obtained pores were not interconnected, but randomly distributed throughout the surface. It was visible that some porogen particles were trapped in the dried membrane and in order to remove these particles, the membrane would have to be removed. Due to the fact that the composite scaffold was too thin, cutting away this membrane layer without destroying the sample proved almost impossible.

Different morphology of the sample was produced combining the salt leaching technique with sonication. The viscous composite solution was introduced in the ultrasonic bath (Branson 5210) for 10 seconds and 30 seconds. No heating was added. The ultrasounds agitated the ceramic particles, disperse them in the polymer matrix and provided sufficient energy for chemical reactions to take place. After sonication the solution was rapidly poured into the Petri dish and left to dry in the fume hood. Using ultra sonic the period of time for the molecular re-arrangement of the PCL-HA molecules increased, allowing no time for round pores to form, instead small and irregular shaped ones were created. Figure 4.8 show the sample obtained using ultra sonic for 10 seconds.

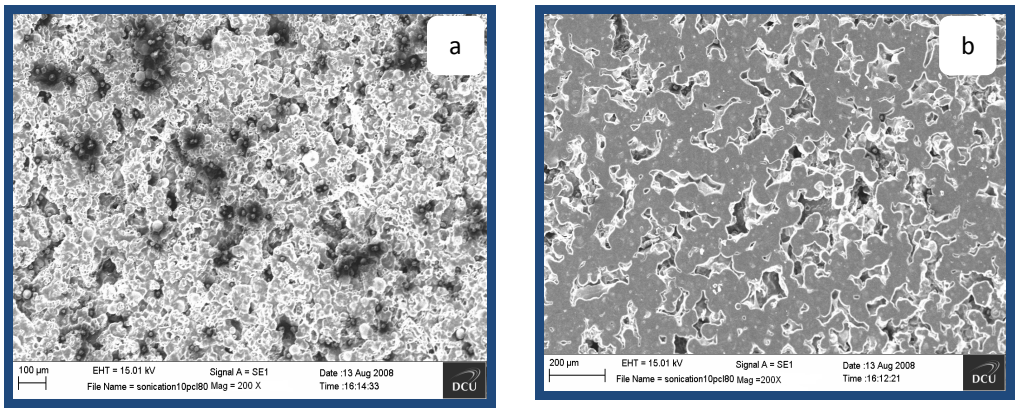


Figure 4.8 Samples obtained with sonication

a.surface exposed to air (AS) b.surface exposed to the glass part of the dish(AG)

The samples prepared using the sonicator for a period of 30 seconds did not behave as expected. The salt and HA particles gathered together around the bottom of the dish, and the polymer was found separately after precipitating, also on the bottom of the dish. This method, if it were to be considered for further studies, should be improved by finding the optimum sonication exposure time.

Gas Forming Technique/Freeze Drying

Another method used to produce porous scaffolds for hard tissue regeneration was the gas forming technique. For this research study, the conventional technique was improved by either immersing the semisolidified polymer/ceramic mixture into an aqueous solution of citric acid at room temperature [57] or by combining effervescent salts in the PCL/DCM/HA solution directly and then leaving these to react. Figure 4.9 shows some samples obtained using this technique in this research.

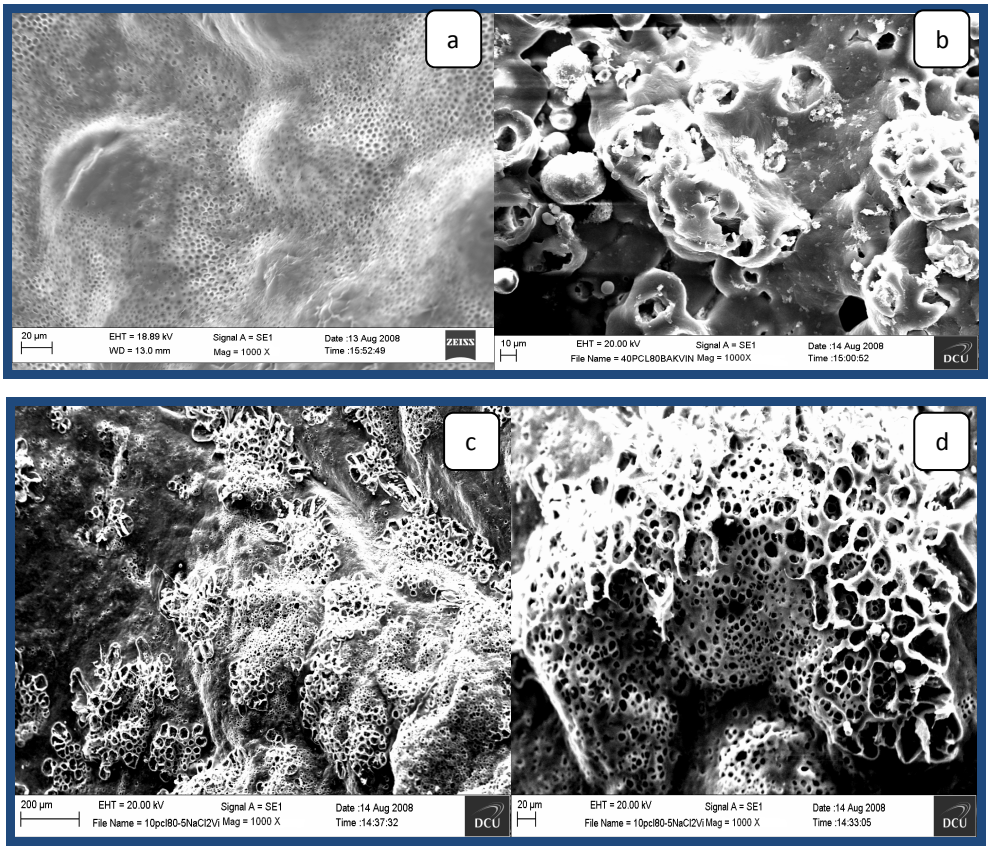


Figure 4.9 Porous samples obtained by gas forming technique/freeze drying (a, b) and without freeze drying (c, d) (effervescent salts were used as gas forming agents)

SEM analysis revealed formed pores of 15-80 µm in size for the gas formed/freeze dried samples. The pores were distributed across the whole surface of the sample. EDX identified HA across the top surface of the sample, which was different to the results found in the samples obtained by solvent evaporation technique. Samples a – b (Figure 4.9) with gas forming onlyrevealed smaller pore sizes than those from c-d, with freeze dried and gas formed, with sizes varying between 15-40 µm. The freeze drying process had the effect of lowering the speed of gas forming and at the same time freezing the pores once the sample froze over.

The gas forming agents used were: baking powder and malt vinegar (5%) (combination 1) and (combination 2): citric acid, baking powder and ethanol. Table 4.2 shows these combinations. After dissolving the PCL in DCM, the HA particles were added and stirred for another 10 minutes at 25rpm in order to increase the viscosity. Then effervescent salts were introduced and immediately the viscous liquid was poured into the glass dishes and frozen, while the components reacted. As discussed previously the gas forming process ceases upon freezing drying. Finally the samples were vacuum dried.

The porosity could be controlled by the amount of bicarbonate incorporated in the polymer and, as literature showed [25, 46], it was possible to make various scaffolds with different geometries and sizes using containers with different shapes, to dry the material. The type of porosity was controlled by the speed of gas forming, which depended on the amount of effervescent salts and container's shape.

Table 4.1: Variation of effervescent salts for gas forming technique at room temperature

4%HA 10%PCL_{80000} (1)	4%HA 10%PCL_{80000} (2)
1g sodium bicarbonate ($NaHCO_3$) **10ml malt vinegar**	**3g citric acid** **1g sodium bicarbonate ($NaHCO_3$)** **20ml ethanol (C_2H_5OH)**

Both combinations of techniques were optimized using data from literature [26]. For the composition (2) in table 4.2, micro particles were formed, but these could not be used for the purpose of scaffolds, since scaffolds must be produced in compact blocks. This also could be a future research recommendation for micro particles production, intended for tissue engineering and drug delivery systems. Micro particles could be carriers for drug delivery or act like micro fillers for bone defects and drug delivery systems at the same time.

Figure 4.10 presents the final samples that were obtained after optimization of the parameters and improvement of the whole technique (phase separation/freeze drying). The conventional technique was simplified, by removing the vacuum drying stage. After mixing all the ingredients of the composite mixture, the solution was left in the freezer for 10 days, during which the solvent and water evaporated (after reacting), leaving behind a porous and thick (1cm) structure.

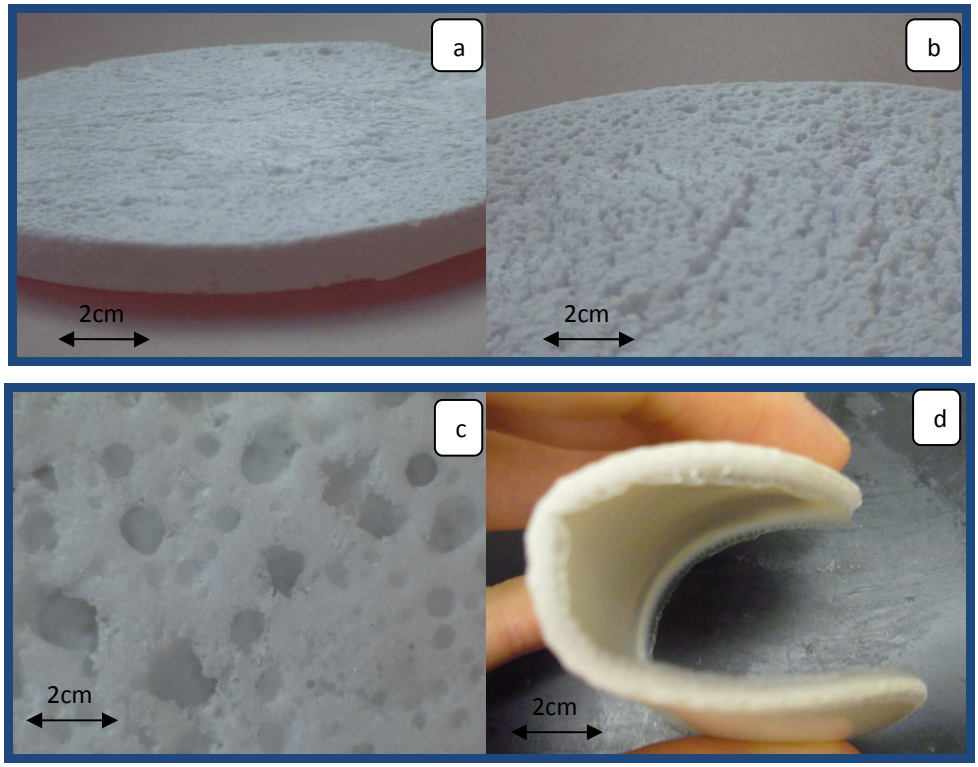


Figure 4.10 Porous scaffolds obtained by phase separation and freeze drying (a and b) 1 cm thick sample (c) porous surface (d) thin sample

4.2.4. Mechanical Testing

Solvent Evaporation Technique

Tensile tests were conducted for the samples that have micropores of at least 30µm and bigger (65- 80 µm), pore sizes that would facilitate tissue infiltration [75]. Figure 4.11 shows the results for samples obtained by the solvent evaporation technique. Standard flat samples of 1cm × 1cm were used for all the tests.

For comparison reasons PCL was dissolved in glacial acetic acid (AcOH), maintaining all the other parameters of the preparation process (rpm, temperature, and concentration). As shown in Figure 4.11 the samples with PCL dissolved in DCM exhibited higher yield strength, of approximately 14MPa, while the ones prepared with AcOH did not exceed 7MPa. In both cases the tensile strength increased with increasing HA content (1-10%), with a sudden decrease for the samples containing greater than 10% HA. In the case of samples prepared using glacial acetic acid, the glacial acetic acid did not entirely break down the bonds which form the PCL at room temperature (22°C), even after 7 days. For the prepared samples the solvent (AcOH) took a long time to evaporate and during its evaporation samples shrunk, making them hard to test, according to Figure 4.12. The samples obtained by gas forming technique were too brittle to be tested mechanically and the ones fabricated by phase separation exhibited strength values of 5-7MPa.

Comparing all the values, one can conclude that the solvent evaporation technique produced membranes with the highest tensile strength. Data from literature [13,30] and this research identified that DCM is a strong solvent that can dissolve the entire polymer mass at room temperature, in a short time and it in combination with a ceramic phase can produce pores of different sizes.

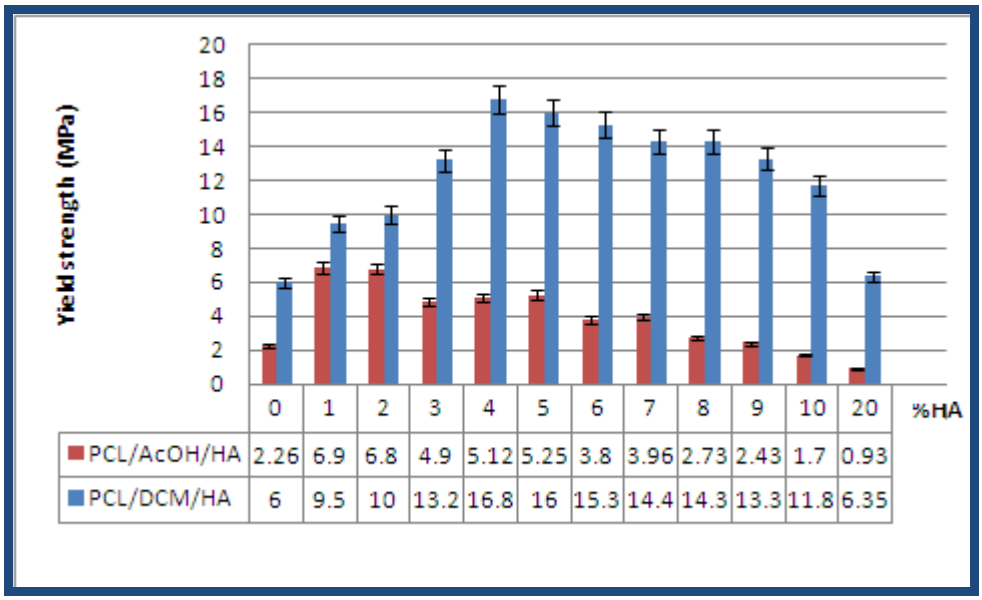


Figure 4.11 Tensile tests for composite samples, where polymer was dissolved in two different solvents (red- AcOH, blue- DCM)

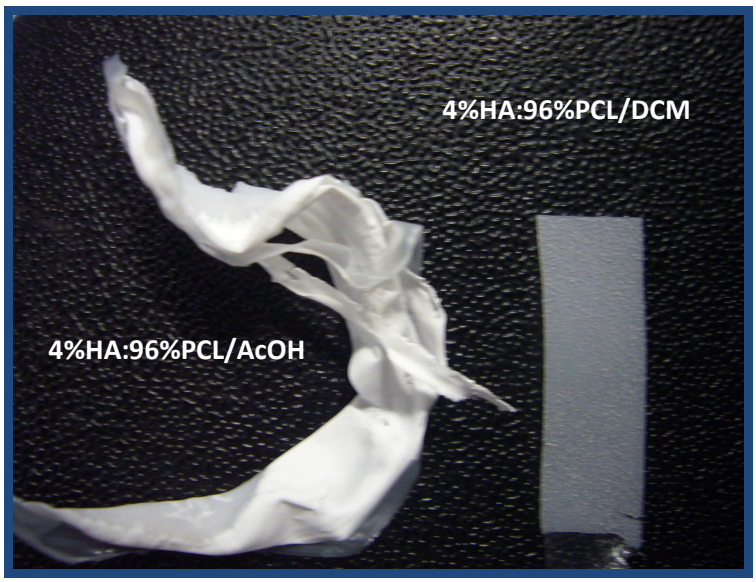


Figure 4.12 Difference between samples prepared with DCM and glacial acetic acid (AcOH), 4%HA: 96%PCL

4.2.5 pH , Degradation Tests, Contact Angle and Roughness Measurements

Figure 4.13 shows the pH and degradation results. Samples produced by solvent evaporation technique, with different HA content were weighted and each one was immersed in 50ml deionised water for 3 months. Every day the pH was measured and

every 30 days one sample (1-10% HA) was taken out from the water, vacuum dried and weighted again. The degraded samples morphology was investigated under SEM, as showed in Figure 4.14.

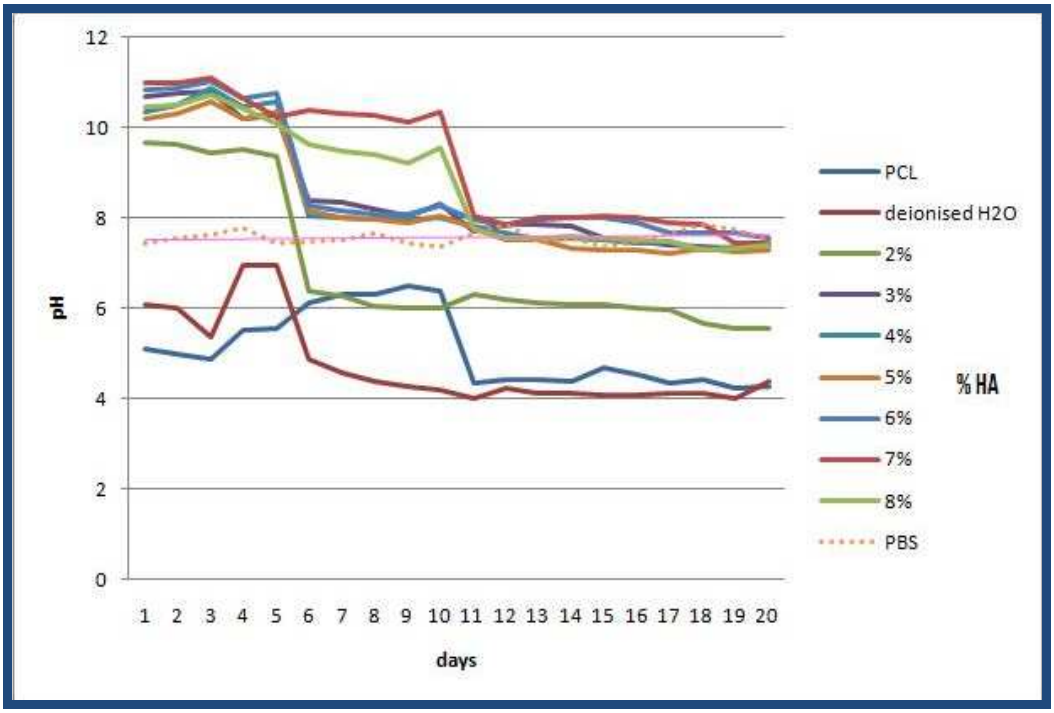


Figure 4.13 Variation of the pH for the first 30 days, in deionized water, for samples of PCL/DCM/HA

As shown in Figure 4.13 the addition of the ceramic phase caused that the pH to became alkaline (pH = 8-10), compared to the pure PCL with an indicated pH = 6 (acidic). After the first 4 to 10 days, the pH values of the composites approached more neutral values, depending on the HA content (the higher the ceramic content was, the longer the period for the pH to approach neutral values), which may indicate that acidic products were released from the composite. Over the following 10 days (days 10 to 20), the pH values remained almost in the range of 7-8 for samples with 3-8%HA, where pure PCL, 2% HA and deionised water produced acidic values.

Compared to the studies made for accelerated degradation, samples that were treated with NaOH, showed slow degradation. To verify if the PBS produced a constant neutral pH value, a second degradation test in PBS was conducted for 1 month (pH was remained between 7.37-7.87, see figure 4.13- dashed orange line).

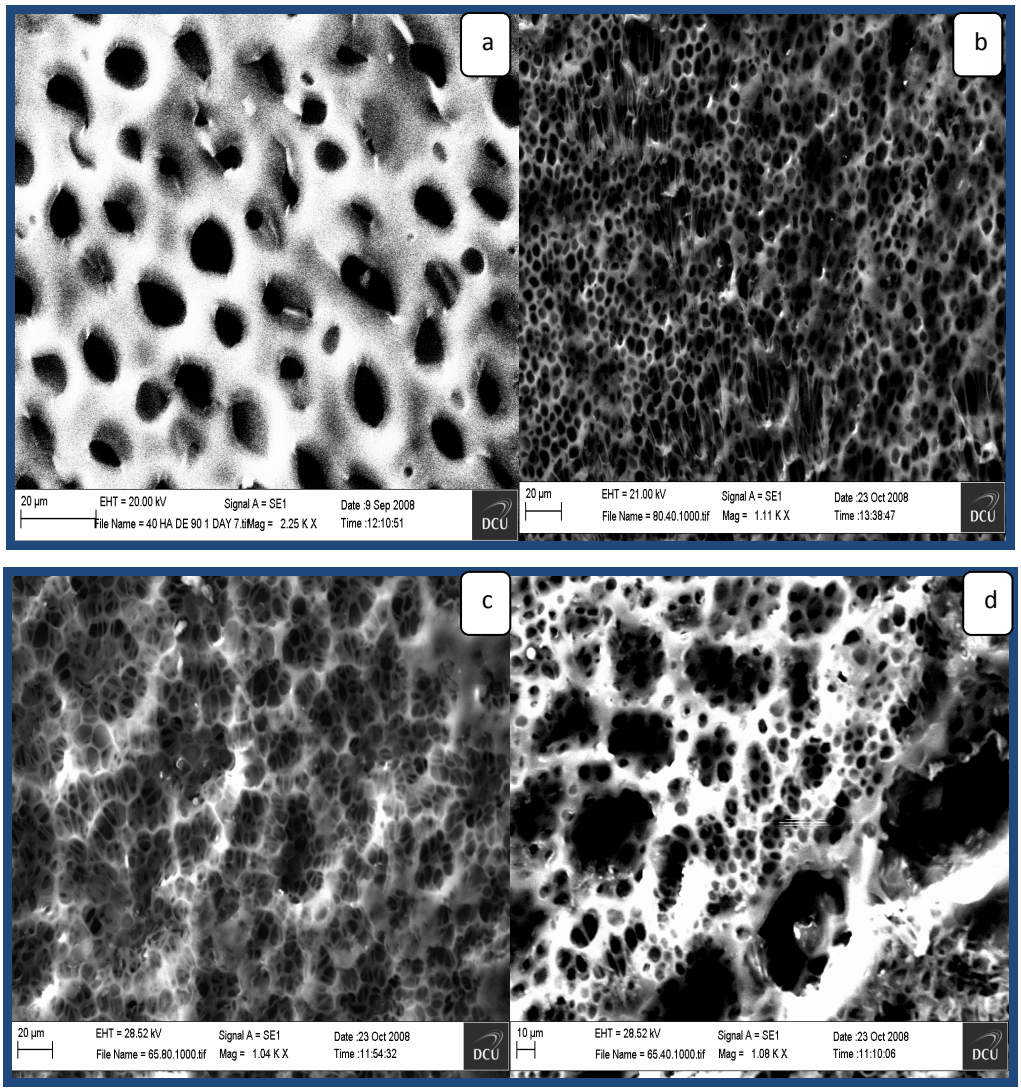
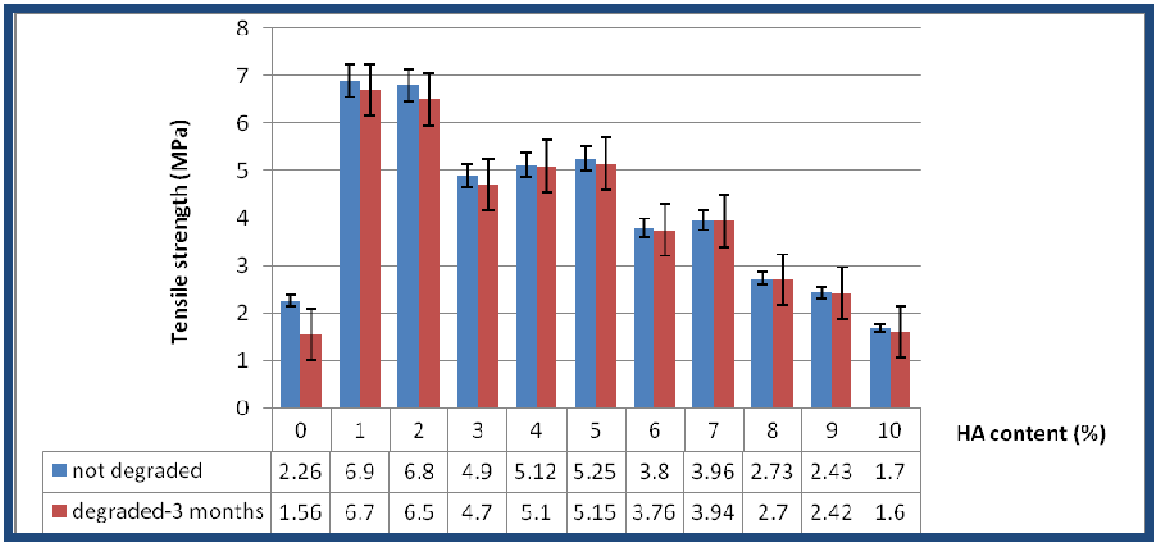


Figure 4.14 shows the SEM images of the degraded samples,
after 1(b), 2(c) and 3(d) weeks compared to the prepared ones (a)

The samples degraded in Figure 4.14 (b-d), physically had a jelly-like aspect, with larger pores and thinner pore walls. Even when the pore walls became thinner, they remained fused and attached together. There was no trace of HA powder dislodged from the degraded and dried samples. It appeared like the scaffolds degraded via a surface erosion pathway homogeneously, throughout the scaffold structure, through the thinning of the pore walls. The tensile yield strength of the degraded samples did not decrease significantly after 3 months either. Figure 4.15 shows that all the samples behaved in a similar manner compared to the non degraded ones. With only 2% weight loss after 3

months, it was concluded that the polymer based structures degraded at a slow rate, as expected [25].



	0	1	2	3	4	5	6	7	8	9	10
not degraded	2.26	6.9	6.8	4.9	5.12	5.25	3.8	3.96	2.73	2.43	1.7
degraded-3 months	1.56	6.7	6.5	4.7	5.1	5.15	3.76	3.94	2.7	2.42	1.6

Figure 4.15 Tensile strength for degraded and non degraded composite samples

Contact Angle

Contact angle measurements showed that the samples produced by solvent evaporation technique were hydrophilic, with an average contact angle of 60-70°. Figure 4.16 describes the results for samples with a ceramic HA content of 1- 10%, for the top surface of the samples. Wettability is a very important property of biomaterials because cells adhesion and proliferation rate are higher on a hydrophilic surface [37]. Interesting results were obtained for the samples with 6 % HA and 7% HA, which exhibited contact angle values of 81.6° and 84.6°, showing the material to be less hydrophobic, hence would improve the cell adhesion. The 8% HA result was similar to the other ones (76.3%). Increased irregularities on the sample surface may have contributed to changes in the contact angle results. At the same time it was observed that an increase in the ceramic phase caused a decrease in hydrophilicity, which was in contradiction with the hydrophilic features of the hydroxyapatite. This could be explained by an increase in roughness. However roughness and surface features may be improved by future research.

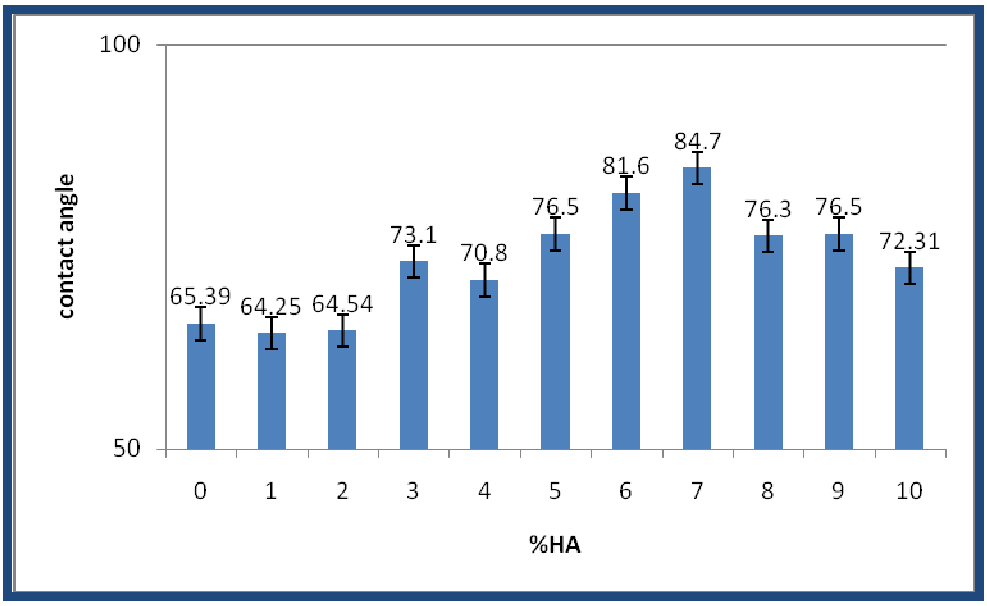


Figure 4.16 Contact angle measurements for samples containing 0 -10% HA(top surface)

Roughness

The roughness measurements results are shown in Figure 4.17. Roughness is a measurement of the texture of the material, with direct implications on how the material will interact with the environment, such as cells in this case. Samples prepared by salt leaching showed a rougher surface, due to the presence of larger pores. Samples obtained by gas forming and phase separation techniques were more rough than those obtained by simple solvent evaporation technique. Samples obtained by solvent evaporation showed that as the HA content increased (1-10%) the roughness increased. Also when the HA content was found to lie between 10 -27%, the roughness decreased, and beyond 27% HA samples became too brittle to be used.

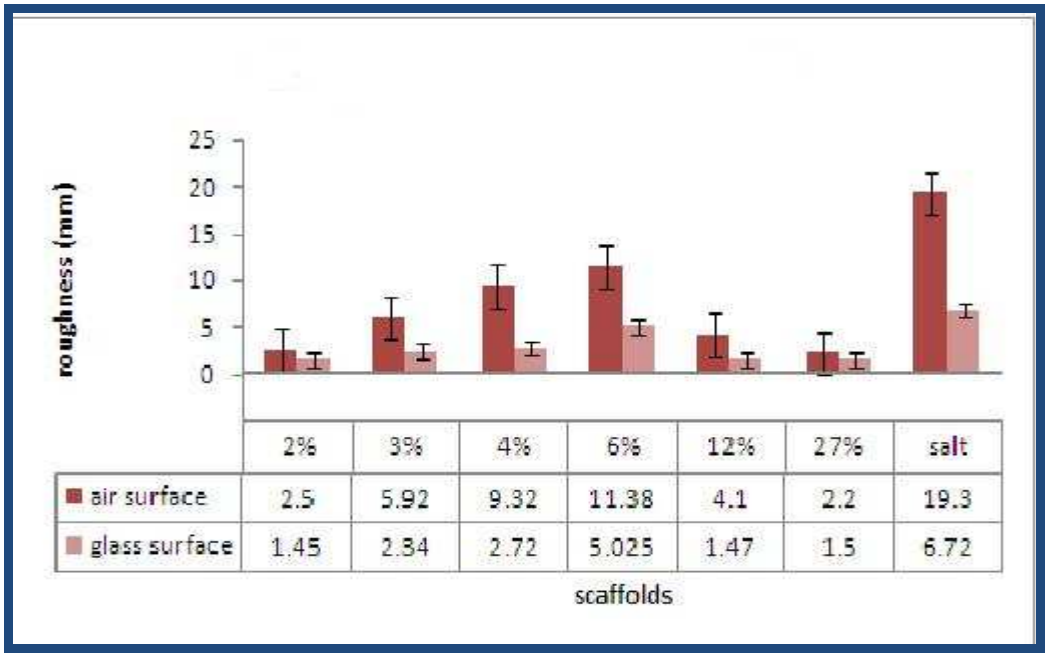


Figure 4.17 Roughness measurement for a selection of composite samples

A possible explanation for this behaviour can be described in correlation with the SEM images (Figure 4.4, 4.6). At a low HA content, the porosity was lower. With an increase in HA content, there was an increase of the porosity and therefore roughness. This behaviour is valid for HA content of 1- 10%. For more than 10% HA, HA particles agglomerated and the porosity decreased, no micropores were formed and therefore the roughness results decreased.

4.2.6 In Vitro Bioactivity Test. Simulated Body Fluid and FTIR Spectrums

Simulated Body Fluid was created in house, using Kokubo' recipe [71]. Square samples were immersed in SBF and left for 28 days, at 37°C. Every 7 days one sample was taken out, dried and analyzed. Results from SEM/ EDX and FTIR were correlated, to reveal if any apatite was formed on the surface of the samples, which would have seen as an indicator of bioactivity features.

Figure 4.18 show SEM/EDX images of the samples after 2 and 4 weeks of immersion in SBF. A delicate layer of needle like apatite appeared after 2 weeks, and they became thicker after 4 weeks, as figure 4.18 (b) shows. FTIR analysis confirmed the structures formed were apatites, comparing them to the spectrums of the samples

before immersion. Figure 4.19 shows the spectrums obtained after 7, 14 and 21 days in SBF.

There were no obvious change on the surface of pure PCL samples, which indicated that pure PCL is a non-active material in SBF and no hydroxy- carbonate apatite (HCA) was identified. HCA is a component normally found when HA interacts with SBF [79-81] and the lack of HCA on the surface of the material indicates that the material has no bioactive features. In the same time, according to literature [79,80] the fact that no HCA are formed on the surface of the material does not show that the material will not bind *in vivo*. Instead there were changes observed on the surface of the composite samples, as the days progressed. Thin needle like structure formed after 14 days, and agglomeration of the hydroxy-carbonate apatite was observed after 21 and 28 days.

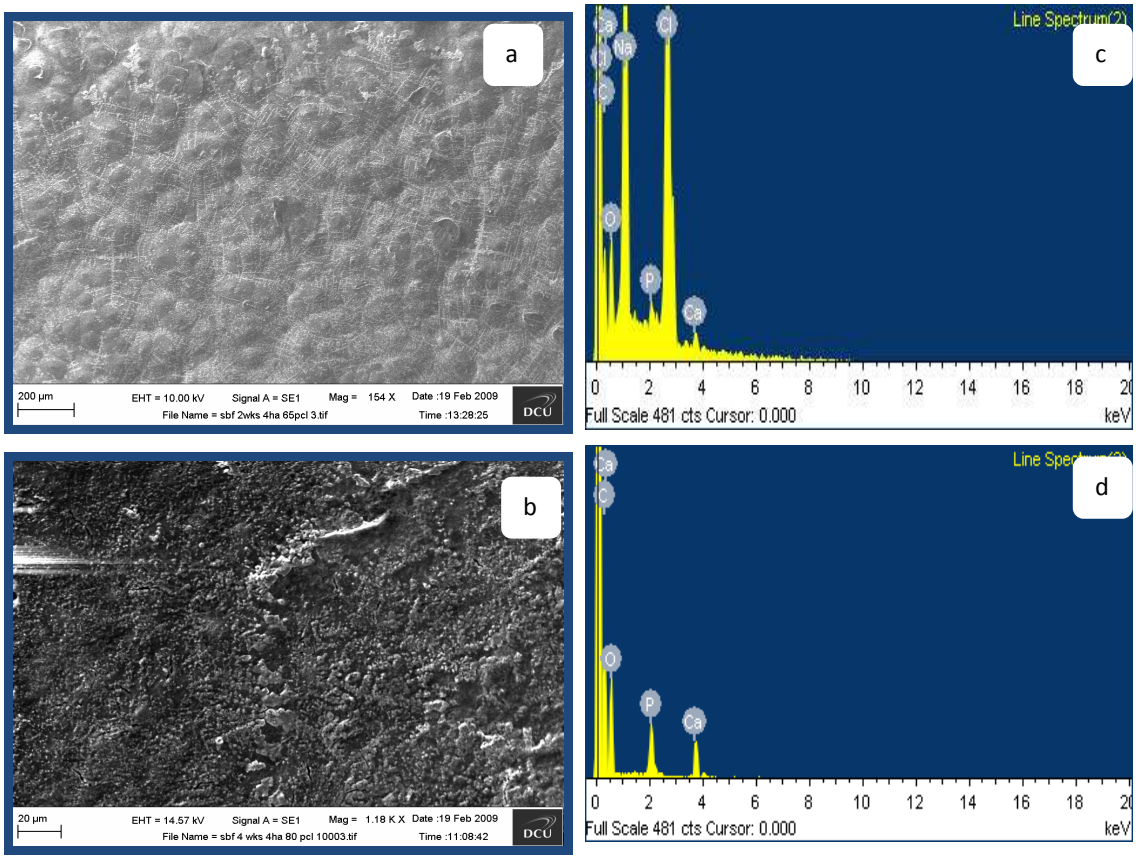


Figure 4.18 SEM/EDS results for samples after 14 (a, c) and 28 days (b, d) in SBF.

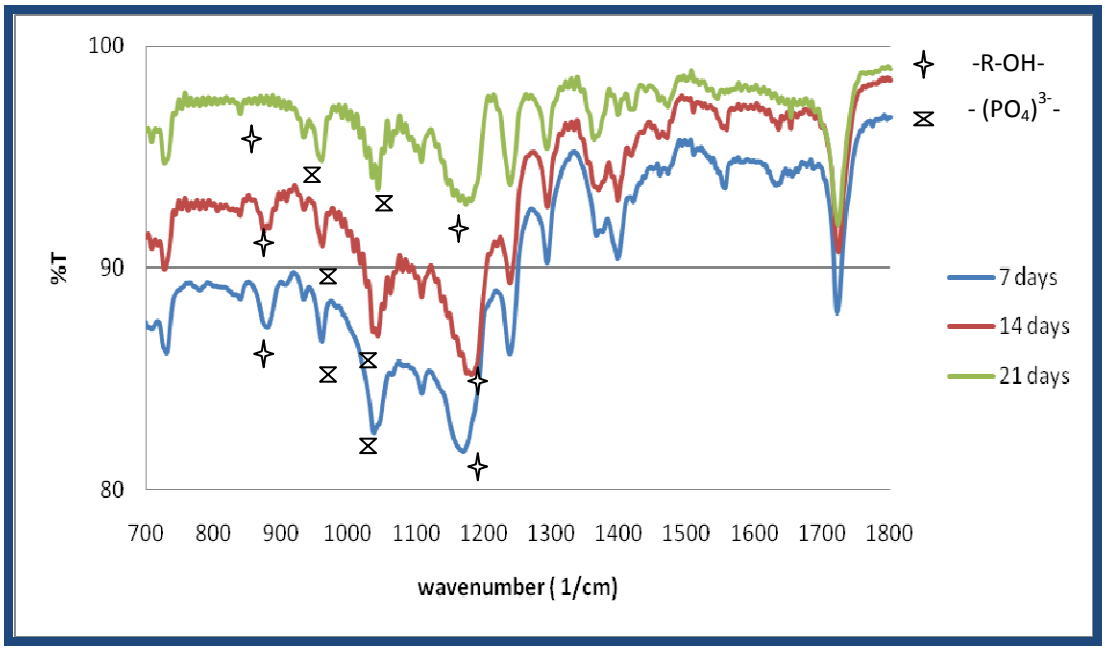


Figure 4.19 FTIR spectrums after 7,14 and 21 days in SBF immersion

Observation of all the samples of HA/PCL/DCM showed that there was a gradual formation of an apatite layer, spread across the entire samples surface. Comparing samples immersed in SBF and those not soaked, there was a large distribution of apatite crystals after 14 days, on the surface of the sample. By day 21, larger crystals appeared covering the first formed a layer, and by day 28 a thick crystals layer was observed under SEM. EDX revealed Ca and P ions on all the samples immersed in SBF, initiating on day 7.

FTIR also indicated the presence of apatite layer, as Figure 4.18 shows. Peaks that corresponded to HA were located at 1169 and 843 cm^{-1} for the hydroxyl compound and peaks at 1040 and 962 cm^{-1} belong to the phosphate compound.The apatite crystals as previously mentioned contained phosphate and hydroxyl compounds. Peaks at 1632 and 1554 cm^{-1} belonged to the C=O band of the PCL and this can be an indicator that the PCL matrix has undergone a chemical reaction in the SBF environment from day 7 onwards.

4.2.7 Cell Biocompatibility

Figure 4.20 shows cells proliferation rate on four different types of composite scaffolds, the composition of which is described as follows:

1. 4% HA in 10% PCL solution in DCM, mixed with water and produced by phase separation /freeze drying technique

2. 4% HA in 15%PCL solution in DCM, produced by gas forming using effervescent salts in combination of 3:1 (baking soda: vinegar)

3. 4% HA in 10% PCL solution in DCM, produced by simple solvent evaporation technique

4. 4% HA in 10% PCL solution in glacial acetic acid (AcOH), produced by simple solvent evaporation technique

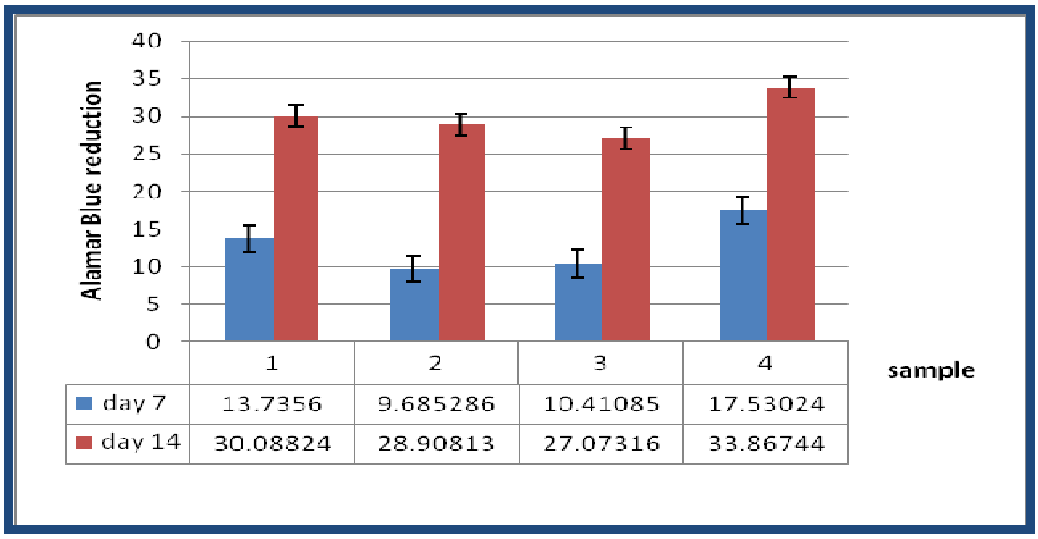


Figure 4.20 Cell proliferation for 4 sample types

Between day 7 and day 14 the cell number doubled for all of the 4 types of scaffolds. It was also observed that a linear growth in cell proliferation for all the samples. Samples exhibiting larger pore sizes allowed cells to penetrate through the pores, as showed in Figure 4.22. Comparing the results for the samples obtained by solvent evaporation technique, the cells interaction with the polymeric matrix prepared with glacial acetic acid was improved, however the mechanical results were not as

encouraging (4%HA:96%PCL/AcOH exhibited a tensile yield strength of 3MPa, compared to 5 MPa for 4%HA:96%PCL/DCM). The cells attached and grew across the entire composite matrix. Available spaces were filled by cells endings where possible. Where the pore size was less than 20µm, the cells did not penetrate the scaffold and for this reason surface attachment was observed, as shown by Figure 4.21 and Figure 4.22 a, b.

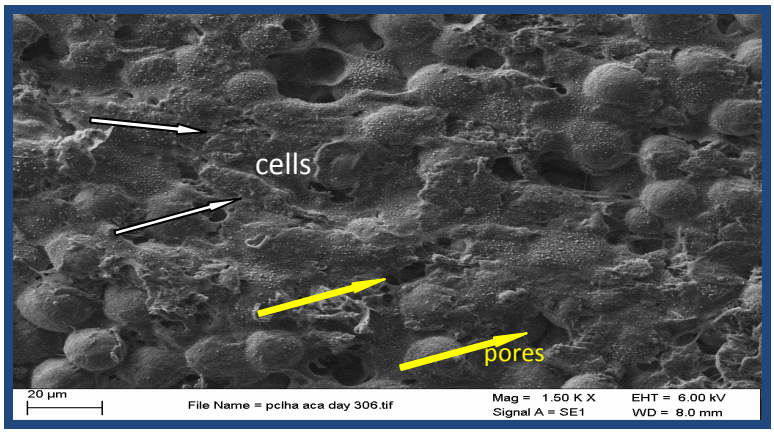


Figure 4.21 SEM images of bone cells attached on a PCL/DCM/HA surface

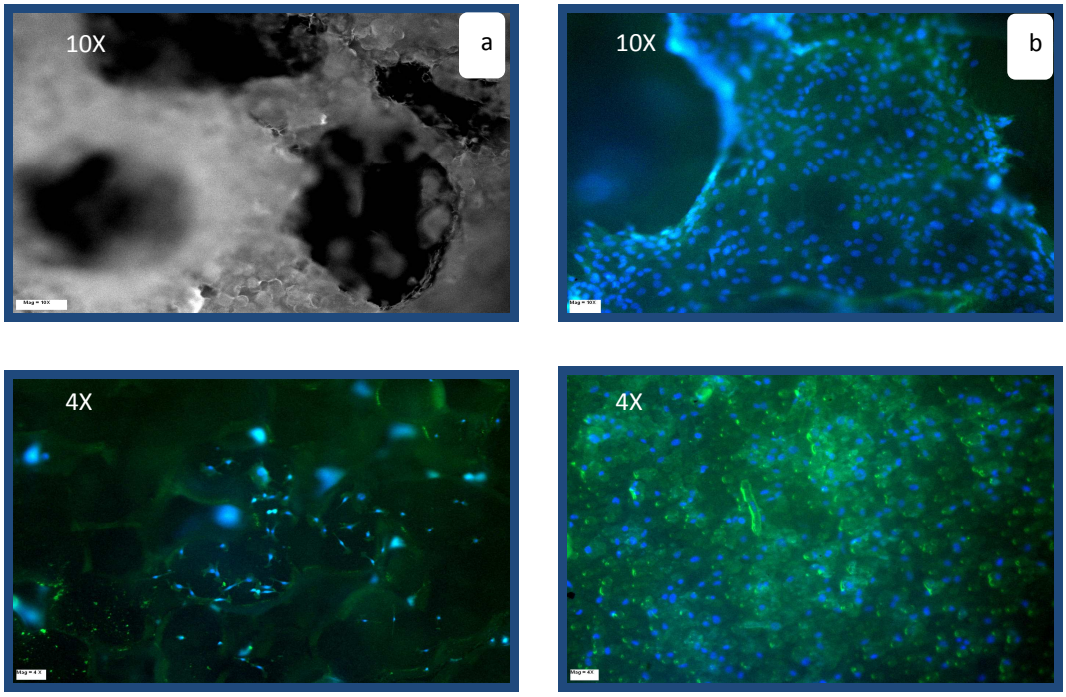


Figure 4.22 Bone cells attached on porous scaffolds composite of (a) PCL/HA/DCM and (b) PCL/HA/AcOH

Summary

All sets of experiments showed that biocompatible and biodegradable structures can be manufactured using different combinations of conventional techniques, having PCL and HA as the main materials. Samples of 4-6 %HA in PCL/DCM, fabricated using solvent evaporation and phase separation techniques exhibited the best mechanical and morphological results. Composites were porous and flexible, but at the same time evidenced a tensile yield strength of 6-8MPa. Cell culturing *in vitro* tests showed all the samples were biocompatible, and Simulated Body Fluid tests revealed their bioactive features as well. Thus the aim to produce functional bio-scaffolds was achieved and these kind of composites could be used for skull regeneration or low bearing application, or for cell culturing also. Table 4.2 shows the mechanical/biological characteristics of the scaffolds produced and tested, with a tick rating for the presence or absence of the porous structure, apatite formed on the surface of the sample and cell attachment and proliferation and 1-3 quantifying the rate of cell attachment/proliferation

Table 4.2 Mechanical/Biological characteristics of the scaffolds produced

4%HA: 96%PCL	Thickness	Porous pattern	Yield tensile strength (MPa) (5mm thickness)	Apatite	Cell attachement /proliferation
Solvent evaporation	5mm- 10mm	✓	16-17	✓	3
Salt leaching	5mm	•	-	•	1
Phase separation	≤ 10mm	✓	14-16	✓	3
Gas forming (effervescent salts)	≤ 10mm	✓	9	✓	2

✓ - apatite /porous pattern/ cells attachment and proliferation present

• - apatite /porous pattern/ cells attachment and proliferation absent

Level 1-3: 1=low, 2= medium, 3=high

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

5.1 Conclusions

This research work shows that different combinations of conventional techniques can be used to produce porous structures for bone substitution. Polycaprolactone and hydroxyapatite were the main materials to be used for this work, to produce properties like elasticity, biocompatibility, slow degradation rate and bioactivity and construct a rigid porous scaffold. A new method (self assembly during solvent evaporation) of producing micropores in the composite structure was developed. Other conventional techniques, like salt leaching, phase separation, freeze drying and gas forming, were adapted to achieve the construction of a viable and biocompatible scaffold. The structures produced (4-6%HA in PCL/DCM, formed by phase separation/freeze drying) resulted in 75-80% porosity, which is one of the main requirements when building a scaffold for bone regeneration. Scaffolds with different porosity and mechanical properties were produced using solvent evaporation, gas forming and phase separation techniques. The inconvenience of the salt leaching technique was the formation of a “skin layer” on the bottom surface of the sample (layer that does not exhibit any pores) and this research tried to overcome this disadvantage, building thicker scaffolds from which this layer could be cut off. Samples were produced either as thin samples or thicker blocks, with various shapes. Slow mass loss and thickening of the structure were observed during degradation tests. At the same time the degradation speed can be influenced by the ceramic and polymeric phase and the technique used. The manufactured samples were easy to handle and cut, giving the possibility to create scaffolds of different shapes. Tensile yield strength was about 6-8MPa, making these scaffolds more appropriate for low bearing bone application, like facial and skull reconstruction. Cell culture tests confirmed that all samples were biocompatible. Cells attached and penetrated the structures that exhibited pores larger than 50µm.

5.2 Recommendations

Further investigations should include other calcium phosphate, ceramic material combinations, as tri-calcium phosphate (TCP), TiO_2, Ytrium Stabilized Zirconia (YSZ) in order to improve the mechanical properties and maintaining the open porosity. At the same time optimization of phase separation parameters would lead to improvement in morphology and mechanical properties. Another possible direction for this research area could be the development of a multiphase and gradient porous structure, using the same components, structure that could be intended also for cartilage regeneration.

PUBLICATIONS ARRISING FROM THIS RESEARCH

- E. I. Paşcu, T. Prescott, J. Stokes, *Biocomapibility and Bioactivity Studies on Polycarpolactone/Hydroxyapatite Gradient Scaffolds* The 2nd Marie Curie Training: Fabrication and Characterization of Tissue Engineering Scaffolds, 3B's Research Group - Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine Avepark, Guimarães, Portugal, 7-18th of September 2009

- E. I. Paşcu, T. Prescott, J. Stokes, *Study on Polycaprolactone/Hydroxyapatite Gradient Scaffolds for Tissue Engineering,* Proceedings of the 35th Biomedical Engineering Conference, MIT-Division, Boston, USA, pp.48, 3-5thof April 2009

- E. I. Paşcu, J. Podporska, T. Prescott, J. Stokes, *Preliminary study on hydroxyapatite/polycaprolactone gradient materials for tissue engineering,* Proceedings of the 9th Advanced Summer Course in Cell-Materials Interactions, Porto, Portugal , 16-20th of June 2008

- E. I. Paşcu, J. Podporska, T. Prescott, J.Stokes, *Preliminary study on hydroxyapatite/polycaprolactone gradient materials for tissue engineering,* Book of Abstracts, Biomedical Research Symposium , Dublin City University, Ireland, 10th of June, 2008

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