Development and Characterisation of Completely Degradable Composite Tissue Engineering Scaffolds

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Chapter 1. Introduction

The objective of this PhD thesis is to develop and optimise scaffolds for tissue engineering. The structure of the Introduction is meant to guide the reader through the main aspects of the background, the materials and the processing of these scaffolds. The first section is a review of the development of tissue engineering and its main principles. Special attention is paid to bone tissue and bone tissue engineering. The following section describes biomaterials for tissue engineering applications and describes the materials used in this PhD thesis in detail: polylactic acid polymer and a calcium phosphate soluble glass. Finally, a description of scaffold design principles and the main scaffold processing techniques is detailed in the third section.

Tissue Engineering: history, definitions and applications

The field of tissue engineering developed as a response to the problems associated with the replacement of tissues lost to disease or trauma. Currently, tissue replacements must overcome important challenges such as rejection, chronic inflammation and severe organ donor shortages[1]. In fact, thousands of patients die every year in waiting lists for organ transplantation[2]. The driving force behind tissue engineering is the desire to avoid these problems by creating biological substitutes capable of replacing the damaged tissue.

Nowadays, damaged tissue can be replaced by xenografts, allografts or autografts. A xenograft is a graft of tissue proceeding from another species. Xenografts offer the advantage of availability in a variety of shapes and sizes, but they also imply a non-negligible risk of immunological reactions and infections. Allografts are grafts made of tissue from a human donor, usually post-mortem. This tissue must be thoroughly sterilised in order to avoid immunological reactions in the receiver and infections. Their limitations include donor shortages and risks of infections as mentioned above. Autografts are grafts made of tissue obtained from the patient who receives the graft: a self-transplant of tissue in other words. Autografts are in some way a gold standard

because they avoid most problems related to transfection and rejection. They do involve significant donor site morbidity and chronic donor shortages however. For example, in the case of bone replacement with tissue from the iliac crest, patients often complain of more pain in the hip area (iliac crest) than at the implantation site.

The idea behind tissue engineering is to create or engineer autografts, either by expanding autologous cells *in vitro* guided by a scaffold, or by implanting an acellular scaffold *in vivo* and allowing the patient's cells to repair the tissue guided by the scaffold. In both cases, the scaffold should degrade in time with tissue regeneration, so that once the tissue has matured the scaffold no longer exists as such and the newly created tissue can perform the function of the lost tissue[3]. This approach avoids some of the drawbacks of the grafting techniques discussed above. Namely, small number of cells are harvested from the patient, thus avoiding the problems of tissue shortage and donor-site morbidity. The cells are seeded into a scaffold which will eventually degrade completely, thus eliminating the presence of a foreign body at the implantation site and its consequent chronic inflammation. Finally, the use of autologous cells avoids problems of rejection and transfection (Figure 1.1).

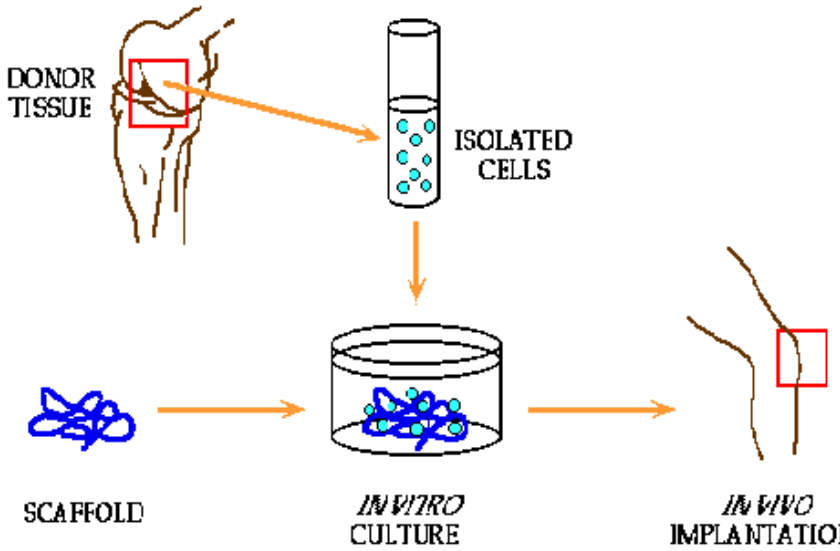

Figure 1.1: Schematic diagram of the different phases in Tissue Engineering, from scaffold fabrication and cell isolation to *in vivo* implantation

The term **tissue** refers to: an aggregate of cells usually of a particular kind together with their intercellular substance that form one of the structural materials of a

plant or an animal. This intercellular substance, or extracellular matrix, is a crucial part of a tissue and acts both as a structural framework and as a regulator of cell behaviour. The word **engineering** is defined as: a) to contrive or plan out usually with more or less subtle skill or craft, and b) to guide the course of. In effect, Tissue Engineering, uses multidisciplinary tools to produce a surrogate extracellular matrix meant to guide cells into creating new tissue[4].

One of the classical definitions of Tissue Engineering was postulated by Langer and Vacanti in 1993 as:

- "…an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore maintain, or improve the tissue function.[5]"

Many other, more or less similar definitions of tissue engineering can be found in the literature. Being a relatively new field, tissue engineering, is not always clearly defined, and may span from decellularised extracellular matrices, to exclusively cellular implants or non-biodegradable biomaterial scaffolds. Some examples of these definitions are:

- "... The process of creating living, physiological, three-dimensional tissues and organs utilizing specific combinations of cells, cell scaffolds, and cell signals, both chemical and mechanical.[6]"
- "...some combination of cells, scaffold material, and bioactive peptides used to guide the repair or formation of tissue.[7]"
- "…the three-dimensional assembly over time of vital tissues/organs by a process involving cells, signals and extracellular matrix.[4]"
- "The field of tissue engineering exploits living cells in a variety of ways to restore, maintain, or enhance tissues and organs[8]"
- "…products or processes that (1) combine living cells with biomaterials, (2) utilize living cells as therapeutic or diagnostic reagents, (3) generate tissues *in vitro* for therapeutic implantation, and (4) provide materials or technology to enable any of these approaches.[9]"

Already, one can infer two of the basic building blocks of tissue engineering: a) cells, and b) scaffolds. The third building block is signalling; biochemical and biomechanical signals which will coax the cells into creating tissue. Alternatively, these concepts can be interpreted as: a) biological and b) engineering challenges[8], bearing in mind that engineering challenges span both cell, scaffold, and signal treatment and vice versa. Thus the field of tissue engineering must combine the knowledge and practices of life scientists and engineers in order to create viable tissues.

Cells are one of the basic components of tissue and are critical in all tissue engineering applications[4;8;10]. Whether cells are directly implanted into the body or are cultured in vitro before implantation, their source and type must be chosen carefully. Furthermore, the harvesting, expansion and differentiation of cells imply many challenges which have retarded the implementation of cellular grafts. Skin tissue engineering grafts such as Apligraf® and Dermagraft® are the exception, partly due to the relative simplicity of the structure of skin as an organ, and partly due to the ease with which skin cells can be cultured and expanded in vitro maintaining the appropriate phenotype[8].

If one considers the cellular approach, the first issue is the cell source: autologous, allogenic, or xenogenic, with the advantages and disadvantages discussed above. Due to the problems associated with the expansion and maintenance of the phenotypes of cells, cell type is also critical. Cells can be adult or embryonic stem cells (pluripotent, totipotent, …) capable of self-renewal and differentiation into various cell lineages. They can also be adult cells at different stages of maturation and differentiation. Cells can also be generated by nuclear transplantation or manipulation ex-vivo[4]. Though stem cells hold enormous promise for this application, stem cell technology is still rather recent and must solve numerous engineering and ethical shortcomings. The chosen cell source and type should also guarantee sufficient supply and be free of pathogens and contamination.

Once cells are harvested they must be kept alive and expanded for a certain time *in vitro.* During this phase, cells must retain the desired phenotype be it undifferentiated or differentiated. Finally, the cells must be seeded onto a scaffold and should retain their function within the construct. Thus, the construct must also provide the mechanical and chemical cues the cells require.

The signals the cells receive from their environment (in this case the scaffold) will in fact determine whether the scaffold turns into integrated tissue. First of all the right cell types must adhere to the outer surface of the scaffold and be able to migrate into it. This is achieved if the scaffold has cell-adhesion sites distributed with the appropriate density to promote cell migration[11].

Once the cells have colonised the scaffold, they should begin proliferation or differentiation in order to produce the tissue which is being replaced. Cells receive the cues for proliferation or differentiation via the integrins with which they anchor onto the extracellular matrix (ECM) or scaffold, and via growth factors and cytokines. The mechanical stimuli they receive also induce mechanotransduction which allows them to behave and thus remodel tissue in function of the mechanical environment. The integrin-mediated signalling pathway is indeed complex. Cells attach onto proteins of the ECM via integrins and apply traction forces on them, thus stretching the ECM which in turn extends proteins revealing hidden binding sites on the protein structure. The ECM is thus an active environment that interacts with the cells very differently than the relatively passive artificial scaffolds[12-16] (Figure 1.2).

The addition of growth factors in scaffolds may solve the challenges of inducing cell proliferation and differentiation. The dosage and distribution of the growth factors within the scaffolds, however, is not straightforward. In any case, signalling and cellular mechanotransduction are critical issues in tissue engineering. They determine cell phenotype, proliferation and differentiation[3;4].

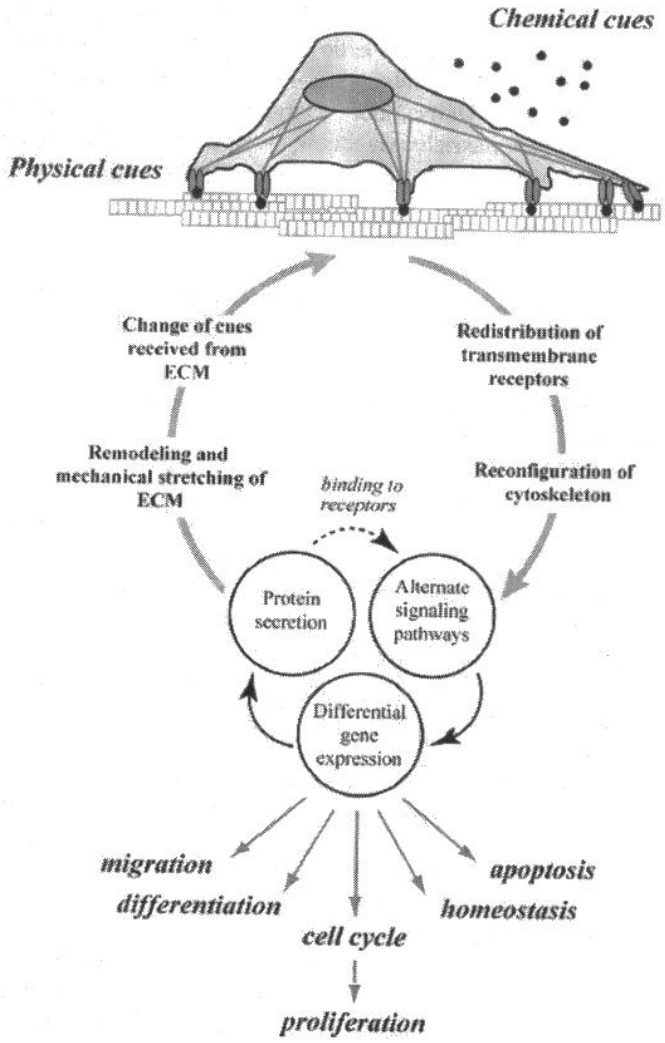

Figure 1.2: Schematic image of the dynamic reciprocity between cells and their extracellular matrix. From [11]

Scaffolds are the other major component of the tissue engineering approach; the choice of scaffold includes its constitutive material, its design and the surface or molecular treatment it may carry. First of all, the biomaterials the scaffolds are made of must be biocompatible. In addition to its biocompatibility, the material's chemical and physical configuration must be adequate for the application, this includes its degradability. The degradation of the biomaterial should be in phase with and of course should not harm tissue regeneration. Degradation by-products should not be toxic and should be easily and rapidly removed or diluted at the implantation site. The ability to eliminate the degradation by-products will largely depend on scaffold design. The design of the scaffold is another crucial issue. This design will determine its structure,

porosity and interface with the cells and surrounding materials. The design must also be adapted to the application creating scaffolds with cubic or tubular pore shapes, for example.

Furthermore, the biomaterial must be processable, a major challenge in the case of brittle ceramic biomaterials for example. Ease of processability, such as easy conformation or injectability in the case of plastics can often determine the choice of a certain biomaterial. The combination of biomaterial and scaffold design will in turn determine the ease and cost of manufacture, manipulation and sterilisation of the construct.

Sterilisation is an absolutely crucial step in scaffold production, both at a laboratory and at an industrial level. The optimisation of sterilisation protocols adapted to each application is, nowadays, an active field of research. Sterilisation of medical materials has typically been endeavoured by autoclave, UV, gamma or other irradiation, or ethylene oxide treatment. These treatments are often poorly adapted to polymeric scaffolds due to their low melting points, complex geometries and hydrolytic degradation mechanisms. Furthermore, certain sterilisation methods provoke undesirable polymer chain scission or reticulation which alter final properties. The effect of the sterilisation procedure on the final chemical, mechanical and surface properties of the scaffold must be accounted for in the scaffold design process. During the development of this thesis, various reports describing the noxious effects of ethylene oxide were published. Indeed, traces of ethylene oxide remain entrapped within the structure of complex geometries after sterilisation. An enhanced evacuation protocol applying high vacuum for several days must be applied in order to reduce the risks of this sterilisation method. Due to the growing concern on the effects of ethylene oxide, sterilisation by radiation is now considered more appropriate for the sterilisation of complex geometries. [17-19].

The mechanical properties of the scaffold, another decisive aspect, will be largely determined by the choice of materials and design. Ideally, the mechanical properties should be adapted to the implantation site, and allow the patient to lead as “normal” a life as possible.

Issues such as storage, transport, scale-up to industrial dimensions and legal approval must be addressed. The scaffolds must retain their shape and properties during

transport and storage in order to begin successful commercialisation. Industrial scale-up is often complicated due to the difference between a laboratory environment and an industrial production site. The scaffold must be both sterile and clean, thus the production must take place in clean conditions, or a cleaning protocol must be implemented before packaging. Finally, legal approval and the necessary certifications are essential in order to commercialise a product meant for medical applications. In order to be sold in Spain, the scaffolds must comply with the standards described in the Real Decreto 414/96 which regulates all sanitary products. In Europe the standards are set by the European Union in the Medical Device Directive MDD 93/42CE. Scaffolds fall into the III category of medical devices, which are medical devices meant for permanent use which are not directly in contact with the blood stream nor the central nervous system, but do exert a biological effect or are absorbed totally or partially [9;20].

From an engineering and biological point of view, tissue engineering holds many other challenges which span beyond the strict definition of cells and scaffolds. Perhaps the most critical issue of all is to understand and to define the native tissue which is meant to be replaced; this may seem trivial at first, but it holds the key to most issues in tissue engineering. In fact, the function of the tissue must be completely understood, biologically and biomechanically, in order to replace it optimally. The mechanical characterisation of biological tissue is, however, often complex due to testing conditions, the tissues' inherent anisotropy, and limited sample life-cycle (a bone sample can only be tested for a certain number of days before it begins to degrade). Furthermore, the mechanical signals regulating tissues must also be ascertained.

Another important issue is the comparison between scaffolds or constructs. The variety of biomaterials, fabrication techniques and cell types used makes it difficult to understand which factor has determined better cell attachment, proliferation or differentiation in each study. Certain construct parameters, such as pore interconnectivity, are often difficult to quantify and thus to compare. Finally, the design and implementation of bioreactors to culture tissues *in vitro* are an inherently engineering responsibility, as well as cell storage and cell shipping.[21]

Bone and Bone Tissue Engineering

Bone and connective tissue are the main building blocks of the human skeletal system. Bone is made up of organic and inorganic or mineral matter. The organic matter is concentrated in the bone matrix, which consists mainly of 90% collagen fibres and other noncollageneous proteins. Collagen is the most abundant protein in the body. The collagen subunit, tropocollagen, is a rod about 300 nm long and 1.5 nm in diameter, made up of three polypeptide strands, each of which is a left-handed helix. There is a regular arrangement of amino acids within each polypeptide strand, it follows the sequence: Glycine-X-Y. Where X represents various amino acids residues and Y is almost always Proline or Hydroxyproline. Collagen assembles in an organised pattern within the bone microstructure and modulates bone calcification sites (Figure 1.3). The noncollageneous proteins in bone play an important role in bone remodelling and in osteogenesis and include: growth factors, cytokines, osteonectin, osteopontin, osteocalcin, bone sialoprotein, hyaluronan, thrombospondin, proteoglycans, phospholipids, and phosphoproteins [22-24].

Non collageneous protein	**Functions**
Alkaline Phosphatase	A phosphotransferase; potential Ca^{2+} carrier; hydrolyses inhibitors of mineral deposition such as pyrophosphates
Osteonectin	May mediate deposition of hydroxyapatite; binds to growth factors; may influence cell-cycle antiadhesive protein
Hyaluronan	May capture spaces destined to be bone
Osteocalcin	May regulate activity of osteoclasts and their precursors; may mark turning point between bone resorption and formation, regulate mineral maturation
Thrombospondin	Cell attachment, binds to heparin, platelets, type I and C collagen, thrombin, fibrogen, laminin, plasminogen, and plasminogen activator inhibitor
Fibronectin	Binds to cells, fibrin, heparin, gelatine, collagen. Organises

	the intracellular cytoskeleton by means of receptors. Helps create a cross-linked network in the ECM by binding to other ECM components.
Vitronectin	Cell attachment protein due to binding sites for integrins, binds to collagen, plasminogin and plasminogen activator inhibitor, and to heparin
Osteopontin	Binds to cells, inhibits mineralisation and nitric oxide synthase; may regulate proliferation, tissue repair, and initiate mineralisation
Bone sialoprotein	Binds to cells, binds Ca^{2+}; may initiate mineralisation
Albumin	Transports proteins; inhibits hydroxyapatite crystal growth

Table 1. 1: Noncollageneous proteins found in bone and their functions. Adapted from [25].

The mineral matter of bone is a calcium phosphate called hydroxyapatite (HA): $Ca_{10}(PO_4)_6(OH)_2$. The HA crystals are thought to occupy the spaces between the collagen fibrils, although their exact shape is under discussion. The mineral phase of bone acts as an ion reservoir and largely determines the mechanical properties of bone. In fact, the mechanical properties of bone result from the impregnation of the soft organic matrix with the very hard and brittle HA crystals[26-28].

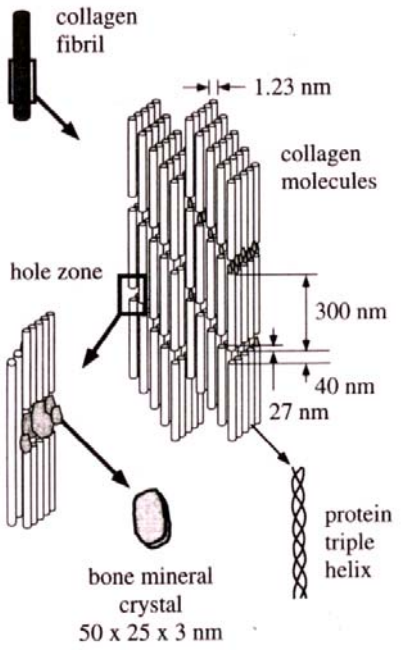

Figure 1.3: Arrangement of collagen fibres and hydroxyapatite crystals within bone microstructure. From [22].

Bone's function is both biomechanical and metabolic. Biomechanically, bone acts to: a) maintain the shape of the skeleton, b) protect soft tissues in the cranial, thoracic and pelvic cavities, c) transmit the forces of muscular contraction during movement, and d) supply a framework for bone marrow. Metabolically, bone a) serves as a reservoir for ions, especially calcium ions, and b) contributes to the regulation of the extracellular matrix composition.

Macroscopically, bone is made up of cortical and cancellous bone. Cortical, or compact bone is very dense and contains only microscopic channels. It forms the outer wall of bones and bears most of the supportive and protective function of the skeleton. Cortical bone represents 80% of the total bone mass in the human body. Cancellous bone makes up the remaining 20% of bone mass in the body. It consists of trabeculae which form an interconnected lattice (Figure 1.4). Cancellous bone can be found in vertebrae, fracture joints, ends of long bones and in foetuses.

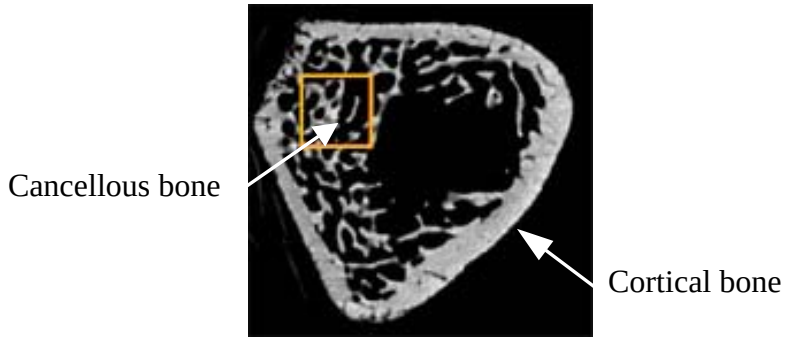

Figure 1.4: Three-dimensional reconstruction of a cross-section of a long bone showing the cortical and cancellous regions. Adapted from [29].

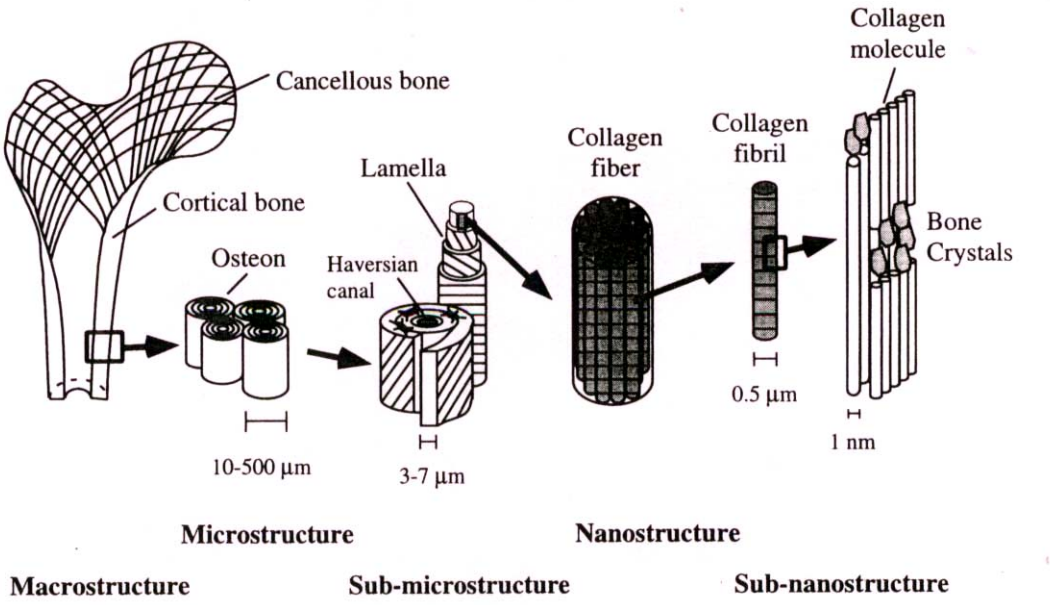

Figure 1.5: The hierarchical structure of bone from macrostructure to sub-nanostructure. From [22].

Mature bone is lamellar bone. New bone, whether it is formed after an injury, at the ends of long bones or in the embryonic stage, is woven bone. Woven bone consists of a matrix of coarse interwoven collagen fibres arranged randomly and thus has low strength. It is temporary and is eventually replaced by lamellar bone. Lamellar bone is built up of layers or lamellae made of collagen fibres than run parallel to each other. Both woven and lamellar bones contain small cavities called lacunae which are connected to each other by means of tubular canals called canaliculi. Specific bone cells

called osteocytes remain entrapped within these lacunae and receive and transmit nutrients and stimuli from and to the bone through the canaliculi.

The lamellae in adult cortical bone are arranged in an organised pattern called osteons or haversian systems. The osteon consists of a central canal called the osteonic (haversian) canal, which is surrounded by concentric rings (lamellae) of matrix. Between the rings of matrix, the osteocytes are located in the lacunae. The osteonic canals contain blood vessels that are parallel to the long axis of the bone. These blood vessels interconnect, by way of the canaliculi , with vessels on the surface of the bone.

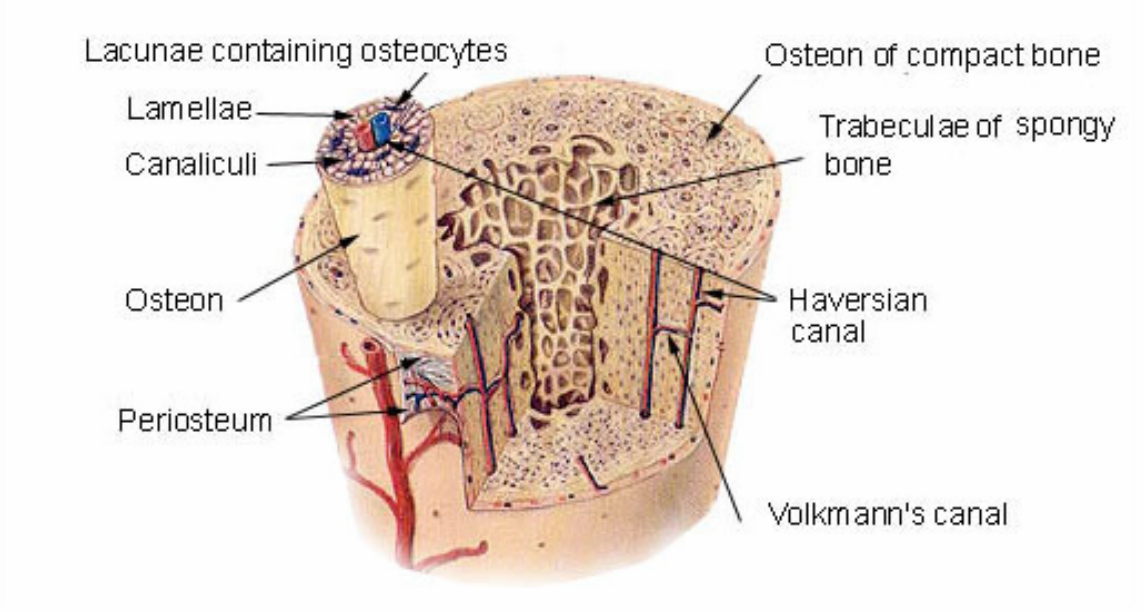

Figure 1.6: The microscopic structure of cortical and cancellous bone. From [30].

Bone is a self-repairing structural material; it is capable of adapting its mass, shape and properties to the changes in mechanical and physiological requirements. This capacity stems from the fact that bone is in fact alive, and contains cells which work continuously to regenerate and repair it. There are three specific bone cells: osteoblasts, osteocytes and osteoclasts.

Osteoblasts

Osteoblasts are mononuclear cells responsible for bone formation. Osteoblasts create the osteoid or organic matrix, and then mineral salts are precipitated within the matrix from the ions in the extracellular fluid. Thus, the osteoid is a template for the

organisation of the bone structure. The morphology of osteoblasts is typical of their function; they have a round shape and an organelle-rich cytoplasm. Osteoblast cells have a polar appearance probably due to the asymmetric environment they dwell in. In fact, osteoblasts exist at the interface between the newly forming bone surface and other tissues such as bone marrow or the periosteum.

Osteoblasts secrete a large variety of macromolecules such as: collagens, alkaline phosphatase, osteonectin, fibronectin, vitronectin, osteopontin and bone sialoproteins. Many of these bone proteins contain specific motifs, namely the RGD sequence, which are recognised by cellular receptors. The secretion of these proteins varies in space and time. Thus fibronectin and osteonectin are expressed early in osteoblastic cultures, whereas osteocalcin is expressed upon matrix mineralisation.

Osteoblasts are thought to influence matrix organisation even after protein secretion, although the exact mechanism is not fully understood. The mineralisation of the matrix is regulated by osteoblasts by the alkaline phosphate enzyme secretion. This enzyme is expressed at very high levels early in osteoblastic differentiation.

Osteocytes

Osteocytes are mature osteoblasts which have become entrapped in lacunae within the bone matrix. They have low synthetic activity but are believed to control the remodelling of their local environment. Osteocytes' most remarkable morphological feature is the network of cellular processes that run through the bone canaliculi and link them to each other and to cells on the bone surface.

Osteocytes monitor mechanical load and tissue damage in bone, and trigger the adaptive responses such as bone generation or resorption. Ostecytes are believed to sense mechanical loads through fluid movements through the canalicular system and shear stresses in the matrix. They also sense tissue damage and initiate the tissue reparation mechanism. Programmed cell death or apoptosis of osteocytes may be the trigger for bone turnover to repair damaged sites.

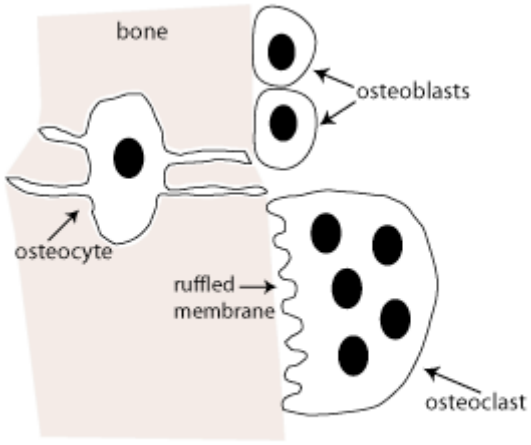

Figure 1.7: Schema describing the different kinds of cells present in bone: Osteoblasts, Osteoclasts and Osteocytes. From [31].

Osteoclasts

Osteoclasts are very large multinucleated cells specialised in bone resorption. Osteoclasts resorb bone by dissolving the mineral phase and enzymatically digesting the organic macromolecules. Morphologically, they have a ruffled bordered plasma membrane at their resorbing surface, a zone where attachment to bone takes place, and numerous lysosomes.

Osteoclasts resorb the mineral phase of bone by secreting H^+ ions, which lower the pH of their fluid environment. This leads to the release of Ca^{2+}, HPO_4^{2-} and H_2PO^{4-} from the hydroxyapatite. The organic phase of bone is degraded by means of a series of proteases and glycosidases including matrix metalloproteinases and several lysosomal cysteine proteases. [25;28;32]

Mechanical properties of bone

The mechanical properties of bone can be measured by testing whole anatomical units or specimens prepared to isolate particular structural components. The mechanical properties of cortical bone have been well documented. They can be measured via traditional testing techniques such as: uniaxial compressive or tensile testing, or three or four-point bending. They can also be tested using ultrasound techniques or micro and nanoindentation. Cortical bone exhibits a high degree of anisotropy and values of mechanical properties vary between animal species, bone location and testing

conditions, age and disease. Testing conditions, for example, may vary between testing dry samples, testing wet samples at 37°C and embedding them or not.

Cortical Bone	**Strength (MPa ± s.d.)**	**Elastic Modulus range (GPa)**
Compression	200 ± 36	18.6 ± 28.8
Tensile Test	141 ± 28	7.1 - 28.2
Torsional Test	65 ± 9	/
Cancellous Bone	**Strength range (MPa)**	**Elastic Modulus range (MPa)**
Compression	1.5 - 38	10-1570

Table 1. 2: Mechanical properties of human cortical bone. From [22;33;34].

Measuring the properties of cancellous bone is far more complex than in the case or cortical bone. The complexity is due to the small dimensions of the individual trabeculae. It is speculated that differences in moduli between cortical and cancellous bone are entirely due to the bone density. Thus, as can be seen in Table 1. 2, some authors find value of Elastic Modulus of cancellous bone as high as those for cortical bone [27;33;35].

Bone Tissue Engineering

In sum, bone is a complex tissue with multiple cell phenotypes, distinct tissue types, high vascularisation and which plays a very demanding mechanical role. Furthermore, bone's structure is highly anisotropic and it remodels itself along local stress fields lines in order to optimise its properties[36]. Given this scenario, tissue engineering, which would allow the body to generate its own bone tissue, seems a sound approach to repair bone.

Despite the multiple functions bone has in the body, its biomechanical role is the most compromised upon injury. Indeed, the other bones in the body can compensate for the injured bone's metabolic function, but if a bone broken or injured, it can no longer support the load it is meant for, and the body remains handicapped. Bone transplantation's aim is thus to restore the biomechanical function of injured bone. Trabecular bone autografts are the gold standard in bone transplantation. The high porosity of trabecular bone allows the surrounding tissue to vascularise the graft in a

matter of weeks and grows new bone within months. Compact bone autografts offer higher initial strength. Their vascularisation and tissue in-growth, however, can only take place through the osteon canals, and osteoclasts must resorb the bone in the graft before new bone can be generated. Thus, the bone tissue engineering scaffold should ideally resemble trabecular bone's architecture, biochemistry and mechanical properties[37].

Cells for bone tissue engineering should ideally be autologous. The bone marrow is an extraordinary source for bone regenerating cells, and many of the engineering problems associated to their culture and expansion have been solved. The necessary signals or soluble factors include bone morphogenetic proteins and growth factors which promote bone growth.

No single material possesses all the criteria required for successful bone grafting. One approach is to design composite materials that combine the strengths of the parent phases and minimise their drawbacks. The combination of polymeric and ceramic materials could improve the mechanical properties of the material and enhance its biological properties. These concepts will be developed in detail in the following sections[32;38;39].

Biomaterials for Tissue Engineering applications

A biomaterial is a "material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body"[40]. As Hench and Polak describe in their key article[41] published in 2002, biomaterials have evolved during the past 50 years, and can now be considered "third-generation biomaterials". Initially, biomaterials were chosen because of their biological inertness, the goal was to minimise the body's immune response to the foreign material. Though this goal is still valid today, scientists have come to understand that complete biological inertness is synonym to non-recognition by the body. This lack of biological recognition is often accompanied by fibrous tissue encapsulation and chronic inflammation, which in turn compromise the mechanical performance and long-term biocompatibility of the prosthesis. Thus, second-generation biomaterials were developed seeking to tailor or enhance biological recognition in an attempt to improve the biomaterial-body interface.

Second generation biomaterials used bioactive components that could elicit a controlled action and reaction in the physiological environment. Two very typical examples of these components are synthetic hydroxyapatite and Bioglass®. Both were used as porous scaffolds, coatings or powders, and by the mid-80s these new bioactive materials had attained clinical use for various dental and orthopaedic applications. The biomaterial-body interface problem was also addressed by exploiting resorbable materials, thus eliminating the interface all together. Resorbable polymers are the main example of these resorbable materials, namely polylactic and polyglycolic acid which decompose hydrolytically into H_2O and CO_2. They are used as sutures, screws in orthopaedics and in controlled-release drug-delivery systems.

Third-generation biomaterials are being designed at present, expanding the concept of biological recognition to **specific** biological recognition. Thus, third-generation biomaterials aim to stimulate precise cellular responses: interaction with distinct integrins, stimulation of cell differentiation or the activation of certain genes. It is also important to emphasize that these biomaterials are being **designed**. That is, third-generation biomaterials are no longer borrowed from existing materials and adapted to a medical application. Instead, they are being designed prior to their development. In this way, the properties of bioactivity and resorbability are being combined to create materials capable of helping the body repair itself better or faster than it could do on its own.

Typically, biomaterials can be divided into: polymers, metals, ceramics and natural materials. Composite biomaterials are created by combining two or more of these fields. The material used in this thesis is a typical third-generation composite biomaterial. A resorbable polylactic acid polymer has been combined with a biodegradable calcium phosphate glass in order to create a composite material. This composite material has then been shaped and processed into a scaffold for tissue engineering applications. Both materials will be described in detail in the following two sections.

Polymers for Tissue Engineering applications: Polylactic acid

Polymers have found widespread use in biomedical applications for more than fifty years now. Polymers classify as the largest class of biomaterials. They often present the advantages of degradability and easy processability with respect to ceramic or metallic biomaterials [42;43]. Both natural and synthetic polymers are used for medical applications. Natural polymers can be of both plant and animal origin. Some examples of natural polymers derived from plants are cellulose, sodium alginate or natural rubber. Examples of those derived from animals are collagen, or hyaluronic acid. Natural polymers offer the advantage of biological recognition, which reduces problems such as platelet adhesion, and indiscriminate protein adsorption. This makes them ideal candidates for cardiovascular tissue engineering, where these issues are crucial [44]. They often require chemical or physical pre-treatment, however, to enhance their material properties, increase their resistance to enzymatic or chemical degradation, and reduce immunogeneicity. These treatments, cross-linking with glutaraldehyde for instance, may have toxic effects and affect cell growth. Natural polymers may also include pathogenic impurities and in general offer low reproducibility.

Synthetic polymers, on the other hand, offer high reproducibility and the possibility of large-scale production, as well as controlled mechanical and biodegradability properties. They lack, however, biological activity and may be very hydrophobic. Some synthetic polymers include; polyethylene (PE), Polypropylene (PP), poly(ethylene terephtalate), polytetrafluoroethylene (PTFE), the polyhydroxyester family : polylactic acid (PLA) and polyglycolic acid (PGA), polyhydroxybutyrate (PHB), copolymers of PHB and hydroxyvalerate (PHBV), polycaprolactone (PCL), polyethylene oxide (PEO), polyanhydrides, and polyorthoesters [6].

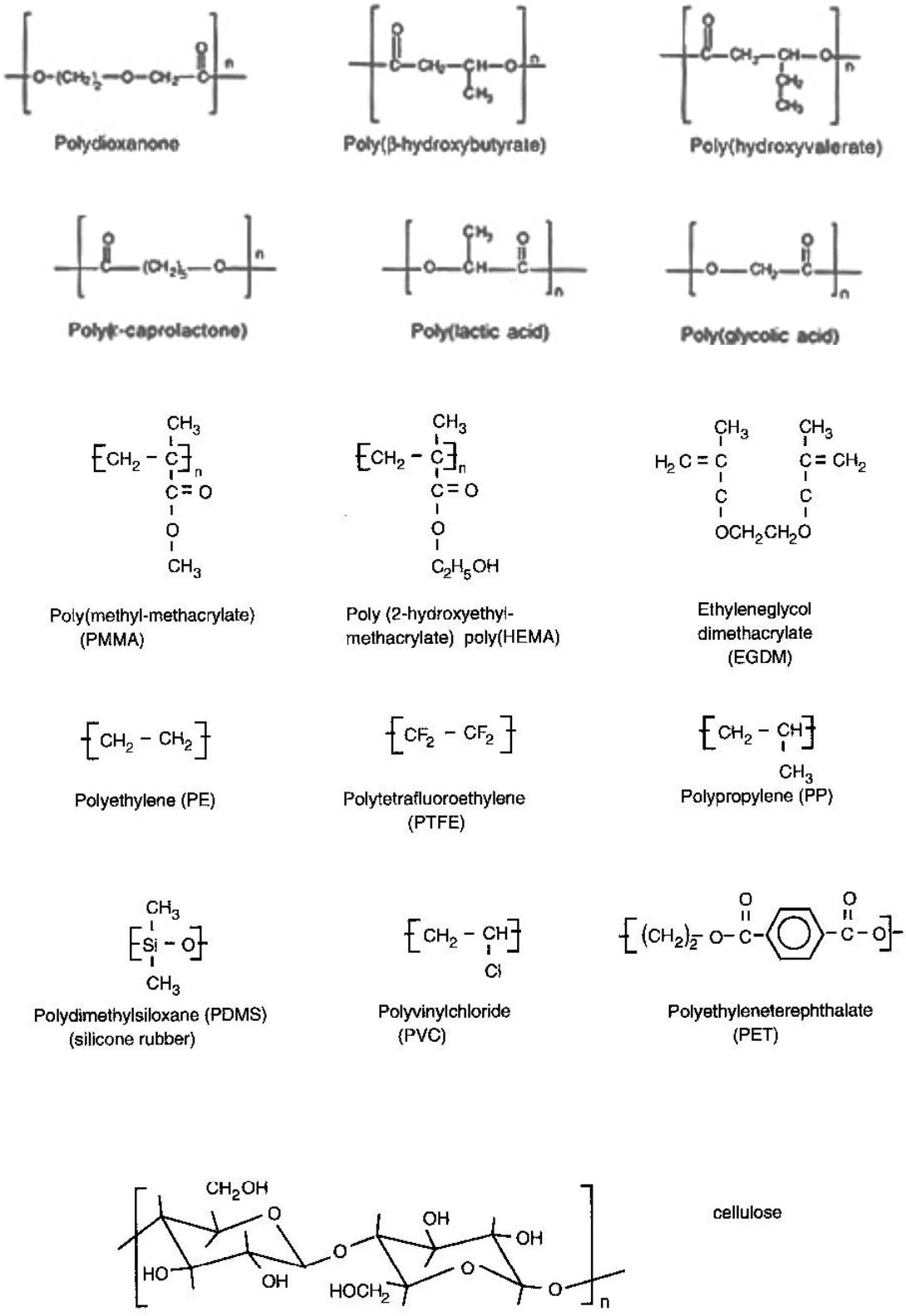

Figure 1.8: Examples of polymers used as biomaterials. Adapted from [43].

Degradable polymer	Current major research application
Synthetic degradable polyesters	
Poly(glycolic acid), poly(lactic acid), and copolymers	Barrier membranes, drug delivery, guided tissue regeneration (in dental applications), orthopaedic applications, stents, staples, sutures, tissue engineering
Polyhydroxybutyrate(PHB), polyhydroxyvalerate (PHV), and copolymers	Long-term drug delivery, orthopaedic applications, stents, sutures
Polycaprolactone	Long-term drug delivery, orthopaedic applications, staples, stents
Polydioxanone	Fracture fixation in non-load-bearing bone, sutures, wound clip
Other synthetic degradable polymers	
Polyanhydrides	Drug delivery
Polycyanoacrylates	Adhesives, drug delivery
Poly(amino acids) and "pseudo"-Poly(amino acids)	Drug delivery, tissue engineering, orthopaedic applications
Poly(ortho ester)	Drug delivery, stents
Polyphophazenes	Blood contacting devices, drug delivery, skeletal reconstruction
Poly(propylene fumarate)	Orthopaedic applications
Some natural resorbable polymers	
Collagen	Artificial skin, coatings to improve cellular adhesion, drug delivery, guided tissue regeneration in dental applications, orthopaedic applications, soft tissue augmentation, tissue engineering, scaffold for reconstruction of blood vessels, wound closure
Fibrinogen and fibrin	Tissue sealant
Gelatin	Capsule coating for oral drug delivery, haemorrhage arrester
Cellulose	Adhesion barrier, haemostat
Various polysaccharides such as chitosan, alginate	Drug delivery, encapsulation of cells, sutures, wound dressings
Starch and amylose	Drug delivery

Table 1. 3: Degradable polymers and their current research applications. From [45] and [46].

Polylactic acid

One of the most widely used synthetic polymeric materials is Polylactic Acid (PLA). PLA is a biocompatible, thermoplastic, resorbable aliphatic polyester, it is FDA approved, and has been used clinically as sutures, bone fracture fixation devices and as drug release systems. PLA is produced by the ring-opening polymerisation of lactide; a cyclic diester. The polymerisation requires heat and a metallic or an organometallic catalyst. Stannous octoate is the most commonly used catalyst because it is FDA approved.

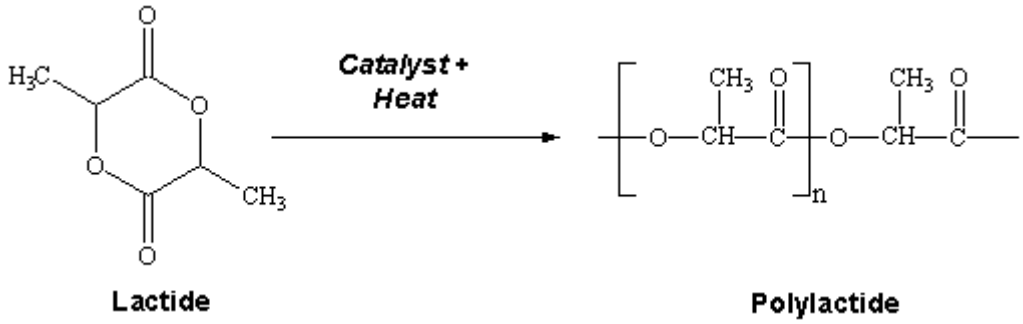

Figure 1.9: Synthesis of Polylactide by ring-opening polymerisation of lactide.

PLA is a chiral molecule, and thus exists as two stereoisomers or enantiomers: L-lactide and D-lactide. PLA can be polymerised into four morphologically distinct forms: P-DD-LA, P-LL-LA, P-DL-LA and a P-meso-LA. (In order to simplify the nomenclature, the P-LL-LA and P-DL-LA will be called PLLA and PDLA respectively from now on in the text). PLLA is a semicrystalline polymer, with a glass transition temperature (Tg) ranging between 50-65°C, and a melting Temperature (Tm) of between 170-190°C. PDLA is an amorphous polymer with a Tg ranging between 50-60°C (Figure 1.10). As with all polymers, crystallinity, Tg and molecular weight modulate PLA's mechanical and degradation properties. PLLA is the most frequently employed polylactic polymer because it yields the L-lactic acid upon hydrolytic degradation which is the naturally occurring stereoisomer of lactic acid (Figure 1.10)[47].

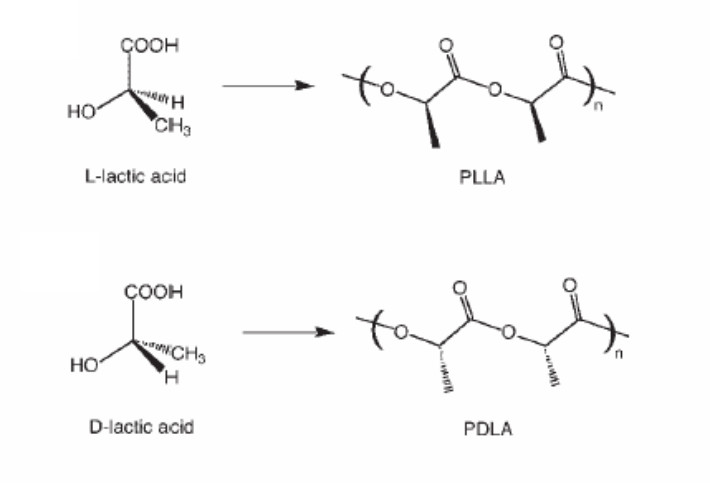

Figure 1.10 : The L-lactide and D-lactide stereoisomers of polylactic acid, and their polymers.

PLA degrades by non enzymatic autocatalytic cleavage of its ester bonds, and is finally eliminated as CO_2 and H_2O[48] (Figure 1. 11). First, polylactic acid degrades into lactic acid which in turn gives pyruvic acid. In the presence of sufficient oxygen, pyruvic acid is converted into carbon dioxide, CO_2, and acetyl-coenzyme A. This coenzyme is the main input to the citric acid or tricarboxylic acid cycle; a series of chemical reactions of central importance to all living cells. Within the citric acid cycle, acetyl-coenzyme A reacts with oxoaloacetate to produce citrate which is the first and the last product of the cycle.

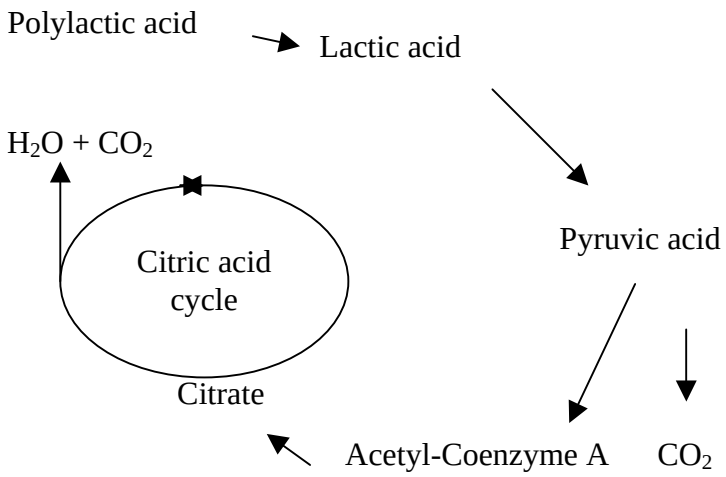

Figure 1. 11: Schematic description of the degradation of polylactic acid within the metabolism

The autocatalytic cleavage of the PLA ester bonds has important effects on the degradation behaviour of PLA implants. The cleavage of the ester bonds in PLA releases lactic acid which tends to acidify the milieu of the PLA implant and may be toxic to the surrounding tissues[49]. This is one of the main drawbacks of PLA implants and its application as a tissue engineering material. This effect may be curbed by ensuring high fluid flow at the site of implantation. The addition of a second phase which could buffer PLA's acidity, in other words implanting a composite material is another possibility, although the buffering effect *in vivo* must be demonstrated. Alternatively, the second phase may enhance mechanical properties, improve the degradation profile or increase bioactivity by creating a bone-bonding apatitic layer on the surface of the material, [42;50]. PLA has been previously combined with particles or fibres of bioactive glass, wollastonite, carbonated fluoroapatite, hydroxyapatite, α-tricalcium phosphate, and calcium carbonate [51-54].

In fact, the use of a composite materials may be the best compromise to attain all tissue engineering requirements. This approach has been used in this thesis; the addition of a biodegradable calcium phosphate glass phase is meant to enhance the mechanical, chemical and biological properties of the PLA.

Ceramics for Tissue Engineering applications: Calcium Phosphate biodegradable glass

Ceramics include a broad range of inorganic and non-metallic compounds. Although their application in tissue engineering is recent, ceramics have been applied to medicine for centuries now as eyeglasses, diagnostic equipment, chemical ware, fibre optics for endoscopies etc. Specifically, as biomaterials, ceramics are used to replace hard connective tissues.

Ceramic biomaterials, or bioceramics, can be classified according to their relative chemical activity (Figure 1.12), which correlates with the rate of formation of an interfacial bond with the body. In fact, implant failures often originate at biomaterial/tissue interfaces due to movement between the two, or in other words: due

to poor interfacial bonding. In order to solve interfacial issues, the implantation sites and fits must be chosen carefully. In the case of nearly inert bioceramics for example, an extremely close fit will minimise fibrous tissue encapsulation and thus guarantee implant success. In the case of porous implants, a biological fixation can be established if the host tissue grows into the pores.

Bioactive ceramics develop a strong interfacial bond which can even sustain significant mechanical forces[55;56]. This group includes the bioactive glasses (namely Bioglass®), glass-ceramics and calcium phosphate compounds such as the apatites. Bioactive ceramics develop a carbonated hydroxyapatite layer on their surface which forms a uniting interface with the surrounding tissue. The most common calcium phosphate implant material is hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, whose composition is similar to the mineral component of bone. Bioactive glasses contain SiO_2, Na_2O, CaO and P_2O_5 and tend to have: 1) less than 60% mol of SiO_2, b) a high Na_2O and CaO content, and a high Ca/P ratio. The Ca/P ratio is important because it largely determines the acidity and solubility of the compound. Stoichiometric hydroxyapatite has a Ca/P ratio of 1.67. Ca/P ratios lower than 1 give extremely high acidities and solubilities.

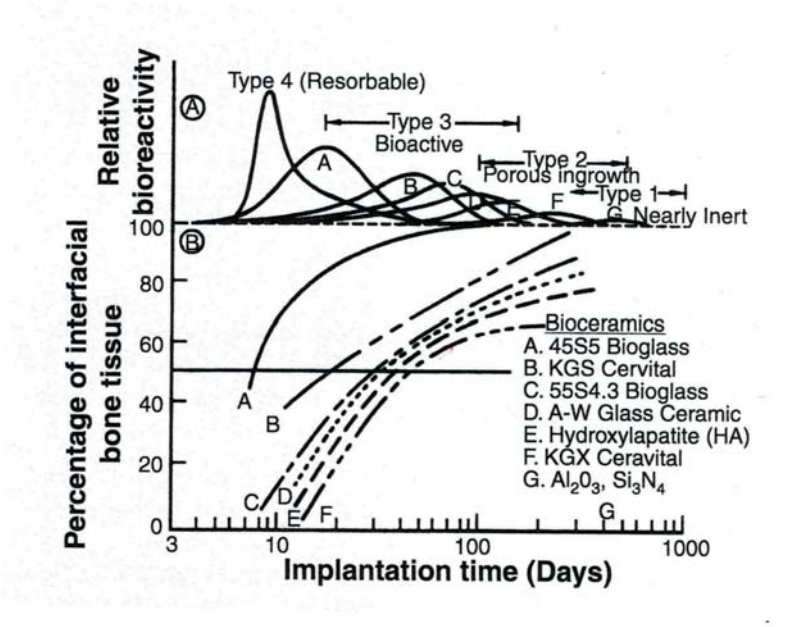

Figure 1.12: Classification of Bioceramics according to their chemical activity. From [57]

Another approach to interfacial issues is to eliminate the interface altogether by using resorbable materials: polymers (described above) or ceramics. Resorbable

bioceramics include tricalcium phosphate (TCP) and soluble biocompatible glasses. As with biodegradable polymers, these bioceramics must maintain their strength and stability during degradation and their degradation rate must match the rate of repair of the body. They are often applied in particulate form in order to enhance their resorbability.

Calcium Phosphate biodegradable glass

Phosphate glasses were developed about 100 years ago for use as achromatic optical elements. They possess very high transparency to UV light and have relatively low dispersion and high refractive indices[58]. These glasses are very soluble in water, and are thus unstable for many applications. This high solubility has however been exploited for biomedical applications by creating biocompatible soluble glasses with a similar composition to that of the mineral phase of bone.

The glassy state is difficult to define. Basically a glass is a non-crystalline solid with the atomic arrangement of a liquid but whose atoms cannot move. The ASTM defines glasses as: “The inorganic product of fusion which has cooled to a rigid condition without crystallising”. Another definition of glass is an: “amorphous material with no long range order presenting a glass transition at its Glass Transition Temperature (T_g). The behaviour of glasses during cooling and heating is in fact one of their distinct characteristics (Figure 1.13). Glasses do not possess a melting point, instead, they can move reversibly from their solid to their liquid state without creating a new phase. The temperature at which glasses pass from a solid to a liquid state is called its T_g. At this temperature glass molecules vitrify and lose their mobility. The T_g is actually a kinetic transition, because glass is in a supercooled liquid state before and after the transition.

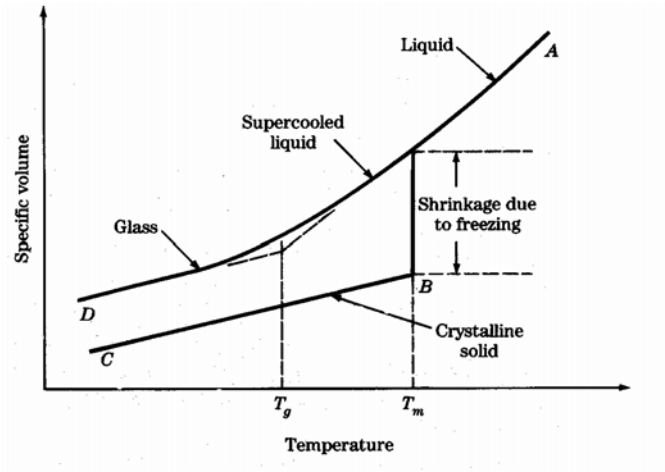

Figure 1.13 : Comparison between the thermal expansion behaviour of crystalline solids and non-crystalline solids (glasses) during cooling

Two main theories describe the structure of glasses: the Zachariesen-Warren[59] or Random Network Theory, and the Van Wazer theory[60]. The theories will be reviewed briefly in order to illustrate the structure of glasses and of phosphate glasses in particular.

Zachariesen-Warren Theory

This theory was developed during the 30's to explain the lattice structure of glasses. It stems from the observation that the mechanical properties of oxide glasses and oxide crystals are comparable, and thus the atoms in both must be linked together by equivalent forces: i.e. covalent bonds. Thus, the atoms in glass form an extended three-dimensional network without periodicity; in other words, the atoms in glass form an infinitely large unit cell with an infinite amount of atoms (Figure 1.14b). Only a few oxides and fluorides can form glasses, and in all cases the oxygen atoms must form tetrahedra or triangles around the cation. The author concludes that an oxide glass can be formed if the following conditions are met:

- The sample contains a high percent of cations surrounded by oxygen tetrahedra or triangles
- The oxygen tetrahedra o triangles share only corners with each other
- Some of the oxygen atoms are linked to only 2 cations

The glass-forming cations are called network formers, and have small ionic radii and large valences. The most common network formers are: B^{3+}, Si^{4+}, P^{3+}, P^{5+}, As^{5+}, and Ge^{4+}. Other cations are usually present in glasses, they are called network modifiers. The network modifiers occupy the holes of the network (Figure 1.14c) and should have large ionic radii and low valences. If, on the contrary, the network modifiers are small and highly charged cations (Ti^{4+} or Mg^{2+}), they tend to devitrify the structure.

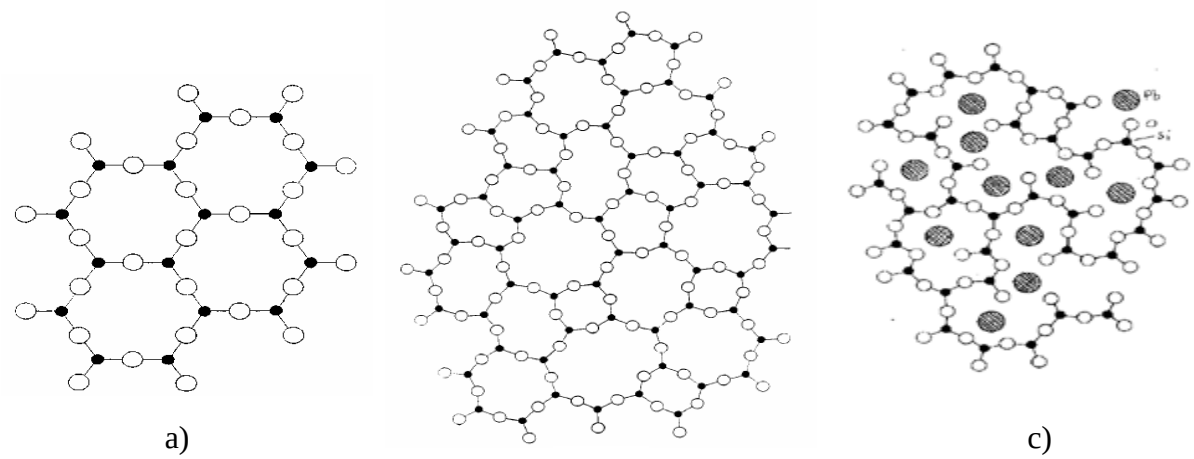

Figure 1.14: Two-dimensional lattice structures of a) a crystalline oxide, b) a vitreous oxide: random network, and c) a vitreous oxide with modifier cations. The small black circles represent glass-forming cations, the white circles represent oxygen atoms, and the large grey circles represent the modifying cations. Adapted from [59] and [61].

Van Wazer Reorganisation Theory

The Zachariesen-Warren and Van Wazer theories are practically equivalent when the number of network modifiers is small. If the number of network modifiers increases, the arrangement of the network forming ions varies adopting chain or ring formations. Van Wazer developed a Reorganisation Theory which describes this arrangement.

Phosphates are defined as those compounds of phosphorus in which each phosphorus atom is surrounded by four oxygen atoms arranged at the corners of a tetrahedron. These PO_4 tetrahedra can be interconnected by forming chains, rings and branched polymers. The PO_4 tetrahedra can be considered “building blocks” of the phosphate structure and can only exist in certain configurations (Figure 1.15).

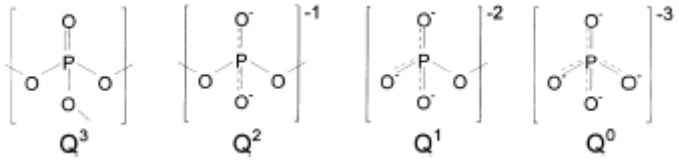

Figure 1.15: Tetrahedral sites that can exist in phosphate compounds. From [58]

Following the schemas in Figure 1.15:

- Q_3 is called a "branching point", each oxygen is shared with neighbouring PO_4 groups.
- Q_2 is called a "middle group", two oxygens are shared with neighbouring groups and there is one negative charge
- Q_1 is called an "end group", one oxygen is shared and there are two negative charged
- Q_0 is called an "orthophosphate" or "monophosphate group" , there are three negative charges

Van Wazer postulates a Reorganisation Theory, based on the ratio (R) between network modifiers and phosphorus atoms, where M stands for a cation network modifier or an organic radical:

$$R = \frac{M_2O}{P_2O_5}$$

He assumes that parts of molecules are continuously exchanging with similar parts of other molecules i.e. reorganising. The M_2O/P_2O_5 ratio imposes the number of oxygens shared between phosphate tetrahedra, or in other words, the number of branching, middle and end phosphate groups in a given composition. Thus, in the extremely ionic case, R=0, there exist only branching units and the three-dimensional network is a Zachariesen-type random network. As R increases, the network becomes more linear until R=4 where very short or unitary chains exist (Figure 1.16).

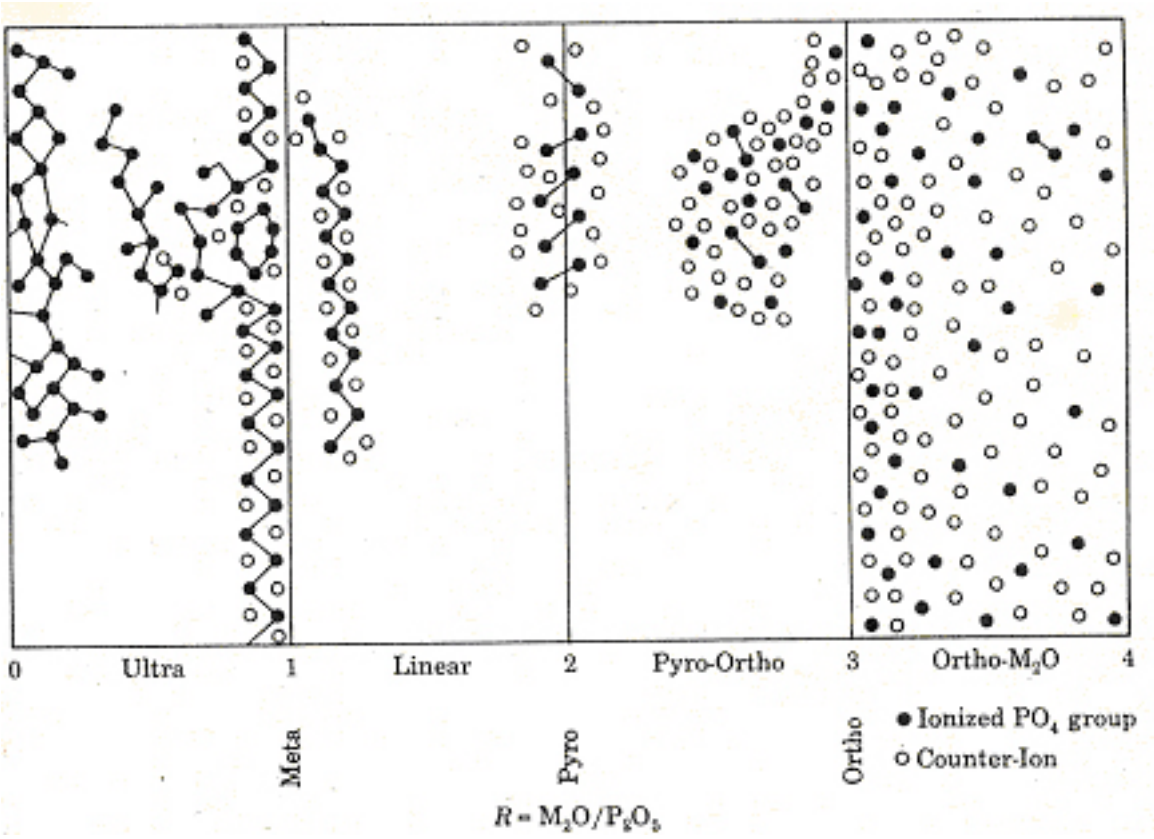

Figure 1.16: Schematic structure of phosphate compounds as a function of the ratio of network modifiers and phosphate ions. From [60]

Phosphate glasses have the same structure as the phosphate compounds described in Figure 1.16, and can be classified by the ratio between network modifiers and phosphorus atoms. The phosphate glass used in this thesis is considered a metaphosphate glass, its composition is listed in Table 1. 4, its R=1.19. Although strictly speaking, a metaphosphate compound has an R=1, the difficulty of obtaining exactly stoichiometric compositions means in reality metaphosphate glasses are accepted as having a molar composition of P_2O_5 between 40% and 50%.

% P_2O_5	% CaO	% Na_2O	% TiO	R=M_xO/P_2O_5
45.4	43.7	5.9	4.8	1.19

Table 1. 4: Molar composition of the phosphate glass used in this thesis**.**

Metaphosphate glasses are less reactive than their ultraphosphate counterparts, and are thus more resistant to water. Spectroscopical techniques have demonstrated that

their structure is formed by long chains of Q_2 middle groups ended by Q_1 end groups forming a linear polymer-like structure[62]. The number of end groups increases as the percentage of network modifiers increases: R increases, thus creating shorter chains. The modifying cations can create ionic bonds with the phosphate chains whose strength depends on the valence and ionic radius of the cation. Thus, varying the content of modifying cations can finely modulate the mechanical and dissolution properties of metaphosphate glasses.

Scaffolds for Tissue Engineering applications

In tissue engineering, as in many other engineering fields, the design of the construct may be as crucial as the material it is made of. As was mentioned earlier, scaffolds, cells, and signals, are the main building blocks of Tissue Engineering. Scaffolds must provide the cells with the appropriate physical support, chemical signals and mechanical signals to allow them to generate tissue. Scaffold design and development is mainly an engineering challenge and is in fact the goal of this thesis.

Tissue engineering scaffolds are meant to be colonised by cells and should transmit the chemical and physical cues necessary to ensure adequate tissue growth. An ideal tissue engineering scaffold should fulfil a series of requirements[63]:

- It should have a reproducible microscopic and macroscopic structure with a high **surface/volume** ratio suitable for cell attachment
- The material it is made of should be **biocompatible**. The scaffold should perform its function with an appropriate response of the host and it should not induce adverse responses [64].
- The scaffold should have an adequate **porosity**; this includes the magnitude of the porosity, the pore size distribution and its interconnectivity
    - This will allow cell in-growth and vascularisation, and
    - Promote metabolite transport
- The scaffolds should have appropriate **mechanical properties and support** to resist physiological forces within the implantation site and similar flexibility to

the surrounding tissue. Ideally it should support the mechanical load on the damaged tissue while it regenerates

- The scaffold material should be **biodegradable.** Its degradation products should not be toxic and should be easily eliminated from the implantation site by the body.
- The scaffold's **degradation** rate should be adjusted to match the rate of tissue regeneration, so that it has disappeared completely once the tissue is repaired.

At a macroscopic level (mm-cm) the shape and composition of the scaffold will determine its cytotoxicity and the ability of the cells to penetrate its structure. At an intermediate level (100 μm), the pore size, orientation, interconnectivity and surface chemistry will determine the cell differentiation and proliferation behaviour as well as the supply of nutrients and the evacuation of waste products. At a microscopic level (1μm), the local surface texture and porosity will affect protein adsorption and cell adhesion.[6] Thus a thorough characterisation of each level of the scaffold and a proper design are crucial for understanding its behaviour as a tissue engineering construct.

The optimum porosity, pore interconnectivity and mechanical properties of tissue engineering scaffolds are yet to be determined. These requirements vary with cell type, implantation site and cell culture conditions[65]. Journal articles on polymeric-based scaffolds for musculoskeletal tissue engineering applications describe porosities ranging from 30% to 98%, and Young Moduli ranging from 100 kPa to 100 MPa [66-74]. Many fabrication methods have been developed in order to attain these requirements, some are: solvent-casting and particulate leaching, thermally induced phase separation, gas-foaming fibre bonding, and three-dimensional printing.[42;75]. These methods are briefly described below.

The solvent casting and particulate leaching method was developed by Mikos et al. [76] amongst others for polylactic and polyglycolic polymers, and several authors have used the method to manufacture composite scaffolds [53;77;78]. It consists of dissolving a polymer in a solvent and then adding particles of a leachable porogen: salt particles, glucose, paraffin spheres, etc. The mixture forms a thick paste which is left to dry in air or under vacuum until the solvent has evaporated completely. The porogen is then leached out and leaves behind a network of interconnected pores (Figure 1.17). In the case of composites, the second phase is added with the porogen and remains within the structure after the porogen is leached out. Additionally, a thermal treatment can be used to modulate the crystallinity of the polymer by melting the polymer and controlling the cooling rate. The advantages of the solvent casting method are that it is a simple and fairly reproducible method which does not require sophisticated apparatus. The disadvantages include thickness limitations intrinsic to the particle leaching process, limited mechanical properties, and some authors question the homogeneity and interconnection of the pores in the scaffolds, as well as the presence of residual porogen and solvent [79].

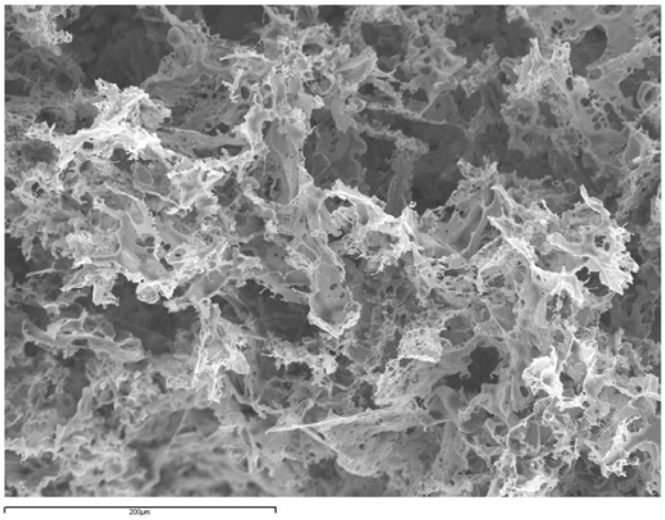

Figure 1.17: Scanning microscopy image of a composite: biodegradable glass/ PLA scaffold made by solvent casting. From [80].

Thermally induced phase separation was first applied to PLA scaffolds by Schugens et al. [81;82], several authors have applied this technique to composite scaffolds [83-86]. It consists of inducing a solid-liquid or liquid-liquid phase separation. This is done by dissolving the polymer in a solvent and quenching the solution at a certain temperature. The quenching induces a phase separation into a polymer-rich phase and a polymer-poor phase. The solvent must then be removed from the phase separated solutions either by freeze-drying, or by solvent extraction. The solvent leaves behind a microstructural foam (Figure 1.18). The main advantage of the phase separation method, is that pore morphology and orientation can be tailored by altering the thermodynamic and kinetic parameters of the processing. Its disadvantages include the use of potentially toxic solvents and a high degree of anisotropy of the porosity. The latter may actually be beneficial for certain biomedical and industrial applications such as nerve regeneration, filtration membranes, mechanically damping materials, packaging etc[71;87].

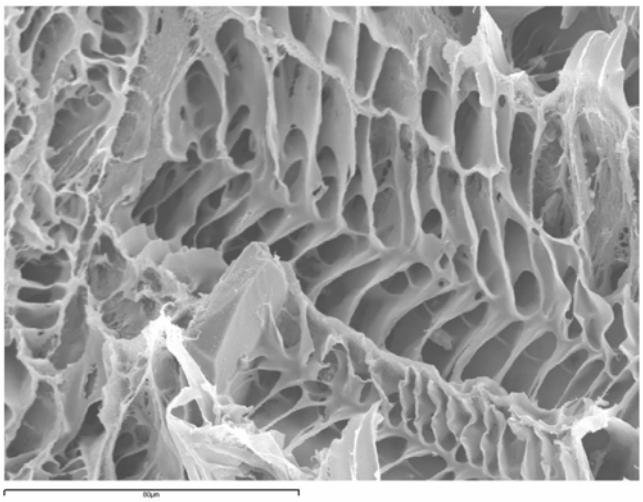

Figure 1.18 : Scanning electron microscopy image of a composite: biodegradable glass/PLA scaffold made by phase separation and freeze drying. A glass particle can be seen to the left of the image. From [80]

The gas foaming process is used to fabricate highly porous foams without the use of organic solvents[88-90]. Organic solvents may leave residues behind which can have toxic effects *in vitro* and may cause inflammation *in vivo*. The process consists of saturating the polymer mix with gas at high temperatures and pressures. Then, a thermodynamic instability is created by quickly decreasing the temperature and pressure which stimulates the nucleation and growth of pores of gas within the polymer (Figure 1.19). Gas-foaming yields high porosities (up to 93%) and varying the temperature, pressure, and rates of parameter reductions can modulate pore sizes. The main disadvantage is due to the poor interconnectivity of the porosity, and the fact that surfaces are mostly nonporous.

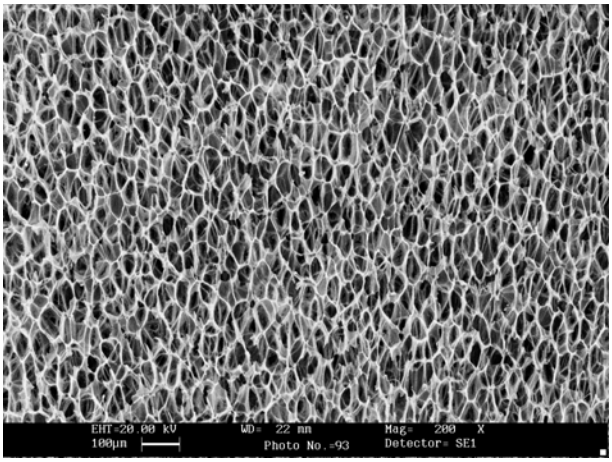

Figure 1.19 : Scanning microscopy image of a composite: biodegradable glass/PLA scaffold made by a gas foaming process. By Salerno,A. and Charles-Harris, M.

The fibre bonding method was first developed by Cima et al. [91] who produced scaffolds made of polyglycolic (PGA) acid polymer. They took advantage of the fact that PGA was available as sutures and thus in the shape of long fibres. Mikos et al. [92] improved the structural stability of the constructs developing a fibre bonding technique in which the PGA fibres are joined at their cross-linking points by “sintering” above their melting point temperature. The main advantage of the fibre bonding technique is the very high surface area/volume ratio which makes them ideal for tissue engineering applications.

The electrospinning process uses an electric field to control the formation and deposition of polymer fibres on a substrate. Sheets and cylindrical shapes can be fabricated with this technique.

Solid free-form techniques allow the production of complex scaffolds from CAD models; this is actually one of the major challenges of conventional scaffold production techniques Their main advantage is the possibility of creating intricate three-dimensional shapes and guaranteeing pore interconnectivity. In these techniques, the manufacturing is realised layer by layer until the complete scaffold is created. One of the most consolidated techniques, and probably the oldest, is 3D printing. In this case, a thin layer of polymer powder is spread over a piston and a liquid binder (usually chloroform) is printed onto the layer. The following layer is created by lowering the piston and applying another layer of polymer powder. Thus, the previous layer is joined to the present layer by the binder creating a three-dimensional shape. The operation parameters can be controlled exactly via a computer-assisted- design program and the process takes place at room temperature. Some other techniques include the following.

- Stereo-lithography: photo-polymerisation by a low-power highly focused UV laser.
- Selective Laser Sintering: laser sintering of polymer powders.
- Fused Deposition Modelling: extrusion of the polymer, the polymers used in industry are usually: ABS (standard and medical grade), elastomer (96 durometer), polycarbonate, polyphenolsulfone.
- Solid Ground Curing: similar to stereolithography: but entire layers are cured at time thanks to the use of photomasks.
- Ink-Jet Printing: 3D binding of polymer powders by a binder fluid; usually needs post process infiltration.

The main limitation of these techniques are their resolution limit (50μm to 300 μm depending on the technique) which makes it difficult to design scaffolds with fine microstructures. [93;94].

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