Faculdade de Engenharia da Universidade do Porto

Preparation and characterization of bioceramic based spheres for bone regeneration

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Abstract

For several decades, there has been a significant increase in bone defects, which are responsible for affecting and limiting a large percentage of the population. The rise in the occurrence of bone fractures is due to the increase in the longevity of the population and the high incidence of trauma, as well as diseases associated with the bone. Thus, with constant scientific evolution, synthetic bone substitutes have been increasingly developed in order to treat bone defects, as an alternative solution for auto and allografts, since they present several limitations, such as limited availability, donor morbidity and disease transmission. Regarding to bone substitutes on the market, they still present some limitations, namely osteoinduction and osteogenesis properties.

This dissertation has as main objective the preparation and characterization of bioceramic microspheres for bone regeneration and subsequent association with drugs. Its function will be to fill injured sites and stimulate the cells responsible for bone production, to accelerate the regeneration process.

For this, it was necessary to study a method of creating open and interconnective porosity, since it is a characteristic very important for bone regeneration. Therefore, it was studied two approaches of producing porous microspheres, namely the addition of porogenic agents and a syringe foaming method. Microspheres produced by the two approaches were characterized using different techniques. By these analysis it was possible to conclude that syringe foaming method proved to be the best method on producing porous microspheres, since this method allowed the production of open and interconnected pores with pore sizes needed to vascularization.

Furthermore, to ensure a more efficient bone regeneration, it was intended to associate drugs to the hollow microspheres. Hence, it was performed a release study of an antibiotic, namely gentamicin sulfate, which is responsible for combating bone infections, caused by bacteria. From a preliminary study, it was obtained an interesting profile that lasted approximately 7 days.

Key words: bioceramics, bone regeneration, drug delivery systems, hollow microspheres, porous microspheres, drugs.

Resumo

Durante várias décadas, tem-se verificado um aumento significativo de defeitos ósseos, responsáveis por afetarem e limitarem uma grande percentagem da população. O aumento da ocorrência de fraturas ósseas deve-se ao aumento da longevidade da população e à elevada incidência de traumas, bem como doenças associadas ao osso. Desta forma, com a constante evolução científica, os substitutos ósseos sintéticos têm sido alvo de desenvolvimento para tratar defeitos ósseos, como uma alternativa para auto e aloenxertos, uma vez que apresentam várias limitações, como disponibilidade limitada, morbidade do dador e transmissão de doenças. Relativamente aos substitutos ósseos existentes no mercado, estes apresentam ainda algumas limitações, nomeadamente ao nível da osteoindução e osteogénese.

Esta dissertação tem como principal objetivo a preparação e caraterização de microesferas biocerâmicas, que funcionam como um substituto ósseo capaz de associar fármacos, de forma a promover a regeneração óssea. A sua função será preencher locais lesados, de forma a combater as infeções e estimular as células responsáveis pela produção óssea, para acelerar o processo de regeneração.

Assim, foi necessário estudar um método de criação de porosidade, que fosse interconectiva, uma vez que é uma característica muito importante para a regeneração óssea. Portanto, estudaram-se duas abordagens de produção de microesferas porosas, nomeadamente a adição de agentes porogénicos e o método "*syringe-foaming*". As microesferas produzidas pelas duas abordagens foram caracterizadas utilizando diferentes técnicas. Dessa análise, foi possível concluir que o método "*syringe-foaming*" demonstrou ser o melhor método para a produção de microesferas porosas, uma vez que possibilitou a produção de poros abertos e interconectivos e tamanho de poros necessários para a vascularização.

Por conseguinte, para garantir uma regeneração óssea mais eficiente, é importante a realização de estudos relativos à impregnação e libertação de fármacos em microesferas ocas. Por isso, foi realizado um estudo de libertação de um antibiótico, nomeadamente a gentamicina, que é responsável pelo combate de infecções ósseas, causadas por bactérias. Deste estudo preliminar, foi possível obter um perfil interessante que durou aproximadamente 7 dias.

Palavras chave: biocerâmicos, fármacos, microesferas ocas, microesferas porosas, regeneração óssea, sistemas de libertação de fármacos.

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Abbreviations and Symbols

List of abbreviations

ASA	Acetylsalicylic acid
BG	Bioactive glass
COX	Cyclooxygenase
DCM	Dichloromethane
DEX	Dexamethasone
ETOH	Ethanol
FTIR	Fourier transform infrared spectroscopy
Hap	Hydroxyapatite
IBU	Ibuprofen
LbL	Layer-by-layer technique
LVR	Linear viscoelastic region
MIP	Mercury intrusion porosimetry
NSAID	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffer saline
PEG	Poly (ethylene glycol)
PS	Polystyrene
PVA	Poly (vinyl alcohol)
SEM	Scanning electron microscopy
TCP	Tricalcium phosphate
TEOS	Tetraethyl ortosilicate
TEP	Triethyl phosphate
XRD	X-Ray diffraction

Introduction

The evolution of scientific knowledge, techniques and materials are revolutionizing the biomedical field, enhancing alternatives for the treatment of bone defects, using different bone substitutes. Biomaterials, such as bone substitutes, are used regularly in regenerative dentistry and also in orthopedics, for bone regeneration. An ideal bone substitute is biocompatible and have osteoinductive or osteoconductive properties. The bone substitutes on the market present some limitations, namely lack off osteoinduction and osteogenesis. Hollow microspheres have some unique properties including high specific surface area and good flow ability, so have potential applications as controlled release carriers for drugs.

Therefore, the main objective of the dissertation is the preparation and characterization of bioceramic based spheres for bone regeneration and subsequent association of drugs.

This work developed under this dissertation is inserted in Stephanie Soares' PhD thesis that is underway. The dissertation is organized in an article structure, containing 4 chapters. For the development of the dissertation, it is important to perceive some theoretical concepts that are scrutinized in the first chapter. The second and third chapters are dedicated to two different approaches of producing porous spheres used for this work, followed by the fourth chapter with the main conclusions and future perspectives.

Chapter 1

1 – State of the art

1.1 – Bone

The skeletal system is composed by bones, cartilages and ligaments, which confer together a suitable framework. This system performs several integral functions in the maintenance of body systems, such as protection of vital internal organs, providing physical support and site of muscle attachment for locomotion, blood cells production, mineral storage (retaining reserve stores of some ions like, calcium and phosphate) and having an important action in the ionic gradient balance and in the homeostasis regulation [1].

Osteogenesis is the process responsible for the formation of the bone, which is the main calcified tissue present in the skeletal system of vertebrates [1].

1.1.1 - Bone Tissue

The bone tissue is composed by the bone matrix (calcified extracellular matrix) and the bone cells. The composition of the bone matrix is responsible for the characteristics of the bone. The bone cells are responsible for the production of the bone matrix, and are cloistered in order to allow replacement by a new matrix, destroying the old one [2,3].

- Bone matrix

The bone matrix is constituted approximately by about 35% of organic material and 65% of inorganic material. The organic material consists essentially of collagen and proteoglycans (large molecules consisting of polysaccharides attached to core proteins). The inorganic material consists essentially of calcium phosphate crystals (hydroxyapatite). Collagen and mineral components are responsible for the main functional characteristics of the bone tissue, so they provide, respectively, flexible matrix resistance and compressive strength (ability to bear weight) [2,3].

- Bone cells

Bone cells are classified in osteoblasts, osteocytes and osteoclasts, which have different functions and origins. These bone cells have functions in the formation, repair and remodeling of bone [2,3].

a) **Osteoblasts:** Cells are responsible for ossification or osteogenesis. Osteoblasts produce the organic material of the bone matrix and they participate in the mineralization of the bone tissue.
b) **Osteocytes:** Cells found inside of the bone matrix, occupying the gaps (lacunae). Osteoblasts are surrounded by bone matrix, becoming a mature cells, which are identified as osteocytes. These cells are able to produce components that are needed to maintain the bone structure and metabolism.
c) **Osteoclasts:** Cells that are giant, multinucleated and extensively branched. These cells are responsible for bone remodeling that occurs along the growth of the bone.

1.1.2 - Bone Physiology

To better understand bone physiology, it is important to recognize the different stages connected with it, such as bone formation and growth, bone remodelling and repair, and bone disorders.

Bone tissue is classified as reticular or lamellae bone, according to the organization of collagen fibers in the bone matrix. So, reticular bone (where osteoblasts intervene for bone formation) is the first to be formed during fetal development, being also considered as the immature bone. In reticular bone, the collagen fibers are randomly oriented and have various directions. Lamellae bone is considered as the mature bone, having collagen fibers that are parallel to one another. Thereby, bone growth occurs by the deposition of new bone lamellae onto existing bone or other connective tissue. As osteoblasts deposit new bone matrix on the surface of bone between the periosteum (resistant connective tissue membrane that completely surrounds the bones, except in the cartilaginous joints) and the existing bone matrix, the bone increases in width, or diameter. This process is called appositional growth. Growth in the length of a bone is the major source of increased height in an individual [1-3].

In terms of bone repair situations, these occur when bone is broken and needs to be repaired. Beyond the breaking bone, the blood vessels in the bone are also damaged, thus the vessels usually result in blood clot formation in that damaged area. So, bone fracture repair involves three stages: inflammation, repair and remodelling. The inflammatory phase is the shortest of the three and consists in a releasing of chemoattractants for phagocytic cells that remove cellular debris from the wound site. After this, osteoblasts proliferate from the periosteum around the fracture begin to cover the blood clot. This phase of the process it is the beginning of bone repair [1-3].

As previously mentioned, the bone tissue is classified as reticular or lamellae bone, whereby reticular bone is the first one to be formed. Once the reticular bone is formed, the osteoclasts degrade the reticular bone and the osteoblasts construct a new matrix that correspond to lamellae bone. This process of replacing old bone with new bone named remodelling. Bone remodelling occurs in all bone and it is the main responsible for changes in bone shape, the adjustment of bone to stress, bone repair and calcium ion regulation in the body fluids [1-3].

1.1.3 - Bone Pathologies

Increasingly, there is a significantly rise in bone diseases that are directly related to problems of growing relevance, such as the aging of the population and the increasing appearance of injuries associated, for instant, with sport.

Some examples of bone diseases:

Osteoporosis is a disease that structural degradation and decreased bone mineral density increase the risk of bone fractures that occurs during aging. This disease causes decreased absorption of minerals and calcium. It is the major cause of fractures and falls in elderly people. The most affected areas are vertebrae, bones of the forearm and hips.

Osteomyelitis is an infection of the bone tissue, preferential caused by bacteria and may also be by fungus. When the bone gets infected, inflammation of the bone marrow occurs.

Paget disease is represented by a disorder of bone remodeling that typically begins with excessive bone reabsorption followed by an increase in bone formation. This disease is a disorder of osteoblasts and osteoclasts, which are responsible for the reconstruction and breakdown of the bone tissue, creating a structurally disorganized mosaic of the bone. This condition causes the bones to become thicker, but also more fragile.

Osteogenesis imperfect is a genetic disease characterized by brittle bones, which break easily. It is caused by a genetic deformation in the production of collagen (protein that is necessary to make the bones strong). It affects the bones of the inner ear, which can cause hearing loss, as well as weak teeth and a curved spine.

1.2 - Biomaterials for bone regeneration

A biomaterial is now defined as a material that interacts with biological systems in order to evaluate, treat and replace tissues, organs or functions of the organism [4].

Certainly, the materials employed in biomedical technology are increasingly being designed to have specific and desirable biological interactions with their surroundings, rather than the older common practice of trying to adapt traditional materials to biomedical applications. In this way, the progress of biomaterials has been dominant, since they represent a range of materials with characteristics that allow to satisfy the desired functionality, being able to replace, repair and improve organs or functions of the organism. Thus, biomaterials have a huge impact in the treatment of injuries or bone diseases [4].

1.2.1 - Classification

In general, biomaterials can be classified according to their chemical composition and biological behavior. Firstly, the biomaterials can be distinguished into metals and metal alloys, ceramics, polymers and composites. The classification according to the biological behavior is based on the response of the host tissue.

When biomaterial is placed on the human organism, it interacts with the host's tissue. So, exist four types of responses between implant and tissue:

- If the material is toxic, the surrounding tissue dies;
- If the material is nontoxic and inactive (almost inert), a fibrous tissue of variable thickness forms;
- If the material is nontoxic and active (bioactive), an interfacial bond forms;
- If the material is nontoxic and dissolves, the surrounding tissue replaces it.

Thereby, based on these different interactions, biomaterials can be classified as bioinerts, bioactive, biotolerant and bioreabsorbable [3,8].

A bioinert material is the one that retains its physical and chemical properties when implanted, presenting a minimal or even none biological interaction with surrounding tissues [3,8]. Examples of this class are alumina and zirconia.

A bioactive material is a material that interacts with the surrounding tissues at the interface, demonstrating an actively interaction with the organism (biological response), without the formation of an interface membrane, through chemical bonds. These biomaterials have in their composition calcium and/or phosphorus ions (in the case of bone substitutes) that will establish a chemical bridge with the surrounding bone. Examples of this class are hydroxyapatite (Hap), bioactive glasses and glass-ceramics [3,5].

A biotolerant material represents the group of materials that are moderately accepted by the organism, causing an organic reaction, through the presence of a fibrous capsule [5,6].

And finally, a bioreabsorbable material is a material that after a variable time implanted, is degraded, solubilized or phagocytosed by the organism. Tricalcium phosphates, bioactive glasses (BG) and gypsum are included in this class [3,5].

1.2.2 – Bioceramics

Taking into account the different classes that the materials can assume, ceramics stand out for this work, essentially the class of the bioceramics. Bioceramics are biocompatible ceramic materials that have been used on dental and orthopedic applications. The biocompatibility of these materials results from their chemical composition, which contains common ions with the constituents of the physiological environment (calcium, potassium, magnesium, sodium) and ions of very limited toxicity to body tissues (aluminum and titanium). Regarding to bioceramics, they include three different types, such as ceramics, glasses and glass-ceramics [6-8].

Ceramics are inorganic compounds that present low electrical and thermal conductivity and are materials more resistant to high temperatures and abrasive environments than polymeric materials. These materials have good dimensional stability, are resistant to wear and tear and are stable in acidic environments. However, they present some limitations as brittleness and susceptible to fractures. Therefore, they are difficult to manufacture and have low mechanical reliability. They are very sensitive to the presence of fissures and other defects, which can lead to fractures and contribute to an early rupture of the material, while they are being used. Due to these factors, the ceramics are little indicated for applications in regions submitted to high tensions and local support [5-8].

Thereby, ceramics, glasses and glass-ceramic materials are all constituted with non-metallic inorganic compounds and consolidated by thermal treatment at high temperature. However, they present differences on microstructure because, ceramics are crystalline solid materials, glasses are non-crystalline amorphous solids and glass-ceramics are constituted by an amorphous phase and one or more crystalline phases [7-9].

Therefore, in terms of applications, bioceramic materials are those specifically designed to be used in the manufacture of surgical implants, prostheses, and artificial organs, such as to fulfill a particular physiological function in the human body. Bioceramics have good biocompatibility and are the materials that best support bone integration (interaction between bone and implant) [5,6].

1.2.2.1 - Calcium Phosphates

Calcium phosphates are a good example of an ideal biomaterial and are widely used in biomedical applications, to repair damaged parts of bone tissue. These materials have a chemical composition that is roughly equivalent to the inorganic matrix of human bone, such as calcium and phosphate ions, providing an ionic balance between the biological fluid and the material. Thus, they have good biocompatibility and bioactive behavior, allowing migration, bone cells attachment and proliferation. So, they promote high levels of osteoconduction [9-11]. An example very well-know is hydroxyapatite (Hap), which is the most commonly used calcium phosphate ceramics for bone replacement [12]. Hap is a stable calcium phosphate that has low solubility in biological fluids, so it has the ability to remain stable in physiological

environment. Besides these, Hap has attracted special attention because of its affinity with biopolymers, proteins and enzymes [13,14]. Although Hap is very similar to the bone mineral phase and it is a good option for bone substitute [15]. They have a slow resorption rates, when are compared to glasses [16].

A convenient way to classify these biomaterials is by their $\frac{\mathrm{Ca}}{\mathrm{P}}$ molar ratio. A high value of this ratio promotes formation of apatite crystals, on the other hand a lower ratio do not promote the formation of bone. Calcium phosphates have a range molar ratio between 0.5 to 2. These phosphates can be transformed into biocompatible and osteoconductive ceramics. Hap has a molar ratio of 1.67 [17].

1.2.2.2 - Bioactive glasses

Bioactive glasses (amorphous solid materials) are not a very conventional ceramics (crystalline solid materials) and represent a group of surface reactive glass-ceramic biomaterials [7].

Silica bioactive glasses are obtained by a combination of silica based material with a biocompatible material, such as calcium phosphate. So, the main components of the silica bioactive glasses are sodium oxide (Na_2O), calcium oxide (CaO), phosphorus pentoxide (P_2O_5) and silicon dioxide (SiO_2). There are different types of bioactive glasses because of the percentage variability of each one of those components in the glass.

Thus, bioactive glasses represent the type of glasses that when they are exposed to human physiologic environment, they tend to form a strong mechanically bond with the living tissues. They develop a carbonate substituted hydroxyapatite-like layer on the glass surface, when they are in contact with the body fluid. So, the strong bond results on the synthesis of apatite crystals that are similar to bone and allows to repair and replace diseased or damaged bone. So, they promote osteointegration [18,19]. These type of glasses are reported to be able to stimulate more bone regeneration than other bioactive ceramics [7,12].

1.2.3 - Bone Grafts Substitutes on the market

The rising standard of living has driven considerable scientific and engineering efforts on the discovery, development and utilization of biomaterials [20]. In order to meet the needs of the biomedical community, a wide range of materials have been researched [21]. These advances allow the development of different forms of treatment for diseases previously seen as untreatable.

Nowadays, the most economically successful clinical segment are orthopedic implants, and the field of biomaterials is growing at a rapid rate.

The requirements of a graft depend on the fracture or bone defect in question. For bone grafts substitutes, there are a few aspects to be taken in consideration, such as, structural support, osteoconduction, osteoinduction and osteogenesis [6,18,22]. However, it is difficult

to produce a bone graft substitute with all these aspects. As table 1.1 shows, there are several examples of bone graft, which are not able to suit all the specifications. For a certain bone graft substitute be considered for marketing purpose, it is essential to demonstrate safety and efficiency of the graft, assured by biocompatible and osteoinductive or osteoconductive [6].

Table 1.1 - Bone graft substitutes [6].

Examples of Bone grafts substitutes	Characteristics
Calcium phosphate cements; Ceramics; Synthetic polymers;	Osteoconduction
Demineralised bone matrix;	Osteoinduction

- **Autografs, allografts and xenografts**

The traditional biological method of bone-defect management is grafting, which is a mechanism that takes a portion of tissue, removed from a donor site to the injured site, in order to reconstruct it. This method includes autografting, allografting and xenografting [23]. Autograft, the golden standard of bone grafting, provides optimal osteoconductive, osteoinductive and osteogenic properties, mechanical properties, and the lack of adverse immunological response. Nevertheless, it has several downsides, such as, the requirement of additional surgery for harvesting, the availability of grafts of sufficient size and shape and the risk of donor site morbidity, which may include fracture, long-lasting pain and infection [6,13,15]. Allografts refer to a tissue or organ graft between genetically different individuals of the same species. Allografts are the most frequently chosen bone substitute and they are availability in many forms, however, they carry the risk of transferring diseases or rejection. And xenograft bone refers to a tissue or organ graft that come from different species. It also has risks of disease transmission, infection and host rejection [24].

- **Bone substitute materials**

Many bone substitute materials intended to replace the need for autologous, allogeneic or xenogeneic bone have been evaluated over the last two decades (table 1.2).

Table 1.2 - General advantages and disadvantages of bone substitute materials [35].

Advantages	Disadvantages
○ Possibility of incorporating growth factors and stem cells that promote osteogenesis; ○ No local morbidity in the patient; ○ Can be molded to fill the defect; ○ Do not have problems in terms of quantity and availability;	○ Low neovascularization; ○ Unknown immune response;

Attending the synthetic materials, a wide range of bioceramic materials similar in composition to the mineral phase of bone are of clinical interest [37]. So, the inorganic materials, which have received most attention for bone repair applications are calcium phosphates, such as Hap and tricalcium phosphate (TCP)), bioactive glasses (BG), and their combinations [8,25].

Synthetic bone substitutes are commercially available in a wide variety of textures, sizes, shapes (blocks, granules, cements, gels and strips) and composition. On table 1.3 there are presented some examples [10,12,26].

Table 1.3 - Examples of bioactive ceramics on the market [6].

Bone Grafts Substitutes - Bioactive Ceramics		
Examples	**Type**	**Company**
Hydroxyapatite based		
Endobon	Sintered bovine Cancellous bone	Biomet /Merck/ Lorenz Surgical
Pro Osteon	Corraline Hap (granules or blocks)	InterPore, EUA
Osteograf	Crystalline hydroxyapatite	CeraMed, EUA
Bioactive glasses		
Bioglass®45S5	Bioactive glass: 90-95 % porosity Composition: 45 wt. (%) SiO_2, 24.5 wt. (%) Na_2O, 24.5 wt. (%) CaO and 6 wt. (%) P_2O_5	Novabone, EUA
Ceravital®	Group of glasses and glass-ceramics Composition: 40-50 wt. (%) SiO_2, 5-10 wt. (%) Na_2O, 30-35 wt. (%) CaO, 10-50 wt. (%) P_2O_5, 2.5-5 wt. (%) MgO and 0.5-3 wt. (%) K_2O	E. Pfeil & H. Bromer, Germany
Biogran®	Bioactive glass granules with the same composition as Bioglass®45S5	Orthovita, EUA

Endobon is a bovine-derived ceramic sintered and processed to eliminate all components, except the mineral ones [6]. It presents weak remodeling capacity and lack of immunogenicity. There has been great adhesion in European for orthopedic and craniofacial fractures and bone defects [6].

The most studied bioactive glass is the Bioglass®45S5. The abbreviation indicates that it contains 45 % in weight of SiO_2 (oxide creator) and the molar rate between $\frac{Ca}{P}$ is of 5:1. Since,

it has high value of this molar rate, it is responsible for its rapid connections to the tissues. The main disadvantages are low mechanical property and the little break resistance [27].

In general, the main disadvantages of the bone substitutes on the market are the lack of osteoinduction and osteogenesis properties.

1.3 - Drug Delivery System

Effectively, bone defects can occur in different locations of the bone, such as low load bearing bone (like maxillofacial bones) or load bearing bone (like bones of the extremities and of the spine) [28].

According to bone pathologies, there are three major repartitions, namely simple fractures, non-union defects and infections (that is one main cause for the development of non-union defects). So, drug delivery systems need to have different properties, considering the bone pathology, and need to be adapted to biomechanical demands. Furthermore, the type of defect also determinates the kind of drug delivery system that will be needed. This includes the size of the defect and the infection status of the defect. These factors are responsible for influencing the material used for the drug delivery system and the drug needed [28].

Surely, drug delivery systems for bone regeneration need to be osteoinductive and osteoconductive. Since the drug provides the osteoinductive action, the material that constitutes the drug delivery system, should account for the osteoconduction. This material may thereby act as a scaffold [28,29].

GENERAL REQUIREMENTS FOR DRUG DELIVERY SYSTEMS IN BONE REGENERATION ARE [28]:

1. Allow osseous integration;
2. Allow ingrowth of cells for bone regeneration and angiogenesis;
3. Allow cell attachment and proliferation;
4. Biocompatible;
5. Biodegradable (biomaterial should be metabolically degraded at a rate that corresponds to the rate of tissue restoration);
6. Controlled release of the drug;
7. Ease of handling;
8. Mechanical stability in case of load bearing (mechanical adaptation);
9. Non-toxic.

In general, a potential biomaterial to be used as drug carrier must have the ability to incorporate a drug, to retain it in a specific target site, and to deliver it progressively with time in the surrounding tissues [30].

Many drugs can be administered in two different ways, systemic or local administration. Systemic administration represents the route of administration of the substance into the circulatory system, affecting the entire body, while local administration refers the route, which affects locally. For instance, when the drugs are administered systemically, they could not

reach the bone tissue of interest. In this case, if it is to make effect, it is required a high dose of the drug in the body to ensure that eventually reaches the necessary location. Sometimes high doses of drugs associated with repeated administrations leads to toxic drug concentrations in the plasma [31,32].

Therefore, systemic administration could lead to the occurrence of unwanted effects, being the main route for administration of bone regeneration, inducing drugs locally. Then, localized release of drugs for bone repair is considered one of the impending areas for the clinical use [29]. In terms of local release profiles, it should demonstrate a high initial release rate, in order to respond to the high risk of infection from bacteria. Then, it should be followed by a sustained release at an effective level for inhibiting the occurrence of latent infection. In the case of orthopedic devices, it is important to combat bacteria during implantation [30,33].

Drug delivery systems are essential components for the controlled and prolonged release of drugs. In order to overcome the difficulties associated with traditional delivery methods, these systems are intended to provide a therapeutic agent in the appropriate quantity, place and time. So, the efficacy of the delivery system relies on a desired drug concentration level for long periods of time without reaching a toxic level, which can provoke adverse effects or inhibit bone formation, or dropping below the minimum effective level (figure 1.1). Thus, these systems provide a therapeutic action over an extended period of time, in order to optimize their effectiveness and to minimize side effects [32-35]. However, these systems still have some problems, such as the desired effect is not immediately achieved, the doctor does not have the possibility to adjust the dosage regimens and drug delivery systems are idealized based on the biological half-life of a normal population. If there is any change in the distribution of the drug, the therapeutic effect may be altered [32-35].

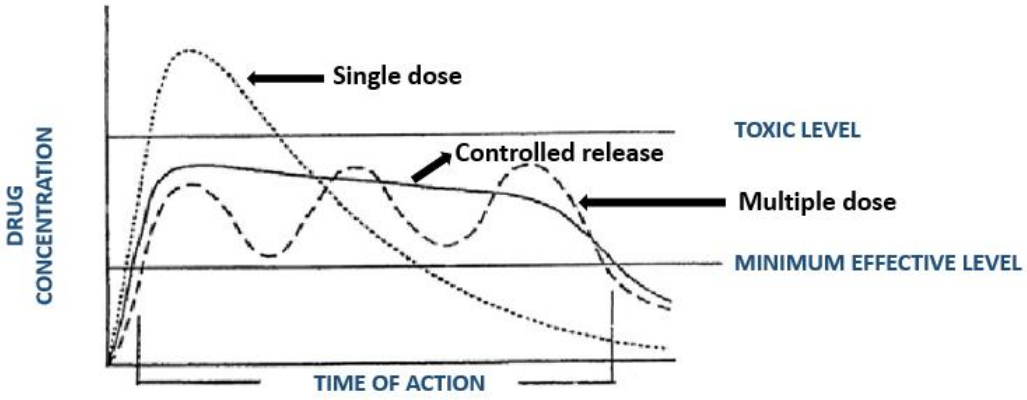

Figure 1.1 – Illustration of drug concentration following absorption of therapeutic agent as function of time [36].

1.3.1 - Hollow Microspheres as drug delivery system

Over the past few decades, there has been considerable interest in controlled drug delivery systems, which has led to a wide variety of materials and designs. The drug delivery systems in order to be administered on the human body, they should be the size of microparticles. So, microparticles have a size range between 1 to 1000 μm and they include pellets, microspheres and hydrogels [37]. According to microspheres, they could represent hollow devices, which can encapsulate material [32]. The material is surrounded by a membrane, which controls the release into the external environment.

Hollow microspheres are spherical microparticles and they have attracted considerable attention in recent years, on biomedical field, because of their interesting characteristics, such as large specific surface area, good flow ability and a wide range of potential applications in controlled release of drugs. Microspheres can be manufactured to have a uniform shape and size that helps to improve the delivery of the spheres to the specific target site and allows them to stay longer at the local of injured bone tissue and allows to reach the fracture local of reduced size. Microspheres can provide high bioavailability and they have a large specific surface area, which favors absorption. Besides these, hollow microspheres permit the encapsulation of a wide variety of drugs (including antibiotics, proteins and nucleic acids) [38-41]. The encapsulation of drugs can provide protection of them, especially sensitive agents as proteins, since they are rapidly destroyed by the body.

Microspheres have several applications, such as catalysts, bone fillers, controlled release of vaccines, stabilization of the encapsulation of therapeutic proteins and encapsulation of DNA for genetic problems [40,41].

In conclusion, using hollow microspheres can be advantageous because they allow an implantation via a minimally invasive route, since microspheres administration is simple (through the needle of a syringe); they can provide mechanical support to the target site of application; and they are more suitable for bone defect filling applications, due to their packing and predictable flow characteristics during injection, compared to irregular shaped microparticles.

Despite the advantages, hollow spherical structures have limitations that are associated with the techniques of their production. In terms of large-scale production there are still difficulties, such as long and/or complicated manufacturing processes, and high cost associated [16,40,41].

1.3.1.1 - Methods of producing hollow spheres

Hollow microspheres have shown a several growth in biomedical applications, due to some unique properties, having shown potential applications as fillers and controlled release capsules for drugs. For this purpose, it is necessary to know the different methods of producing hollow spheres, in order to be able to identify, which is the most appropriate for the purpose of this thematic of the dissertation [44-53].

- Templating method

The templating process is seen as a successful technique and is frequently used to produce hollow spheres of diverse materials. This method can be resumed in a couple of general steps (figure 1.2). First, a precursor material (compound that participates in a chemical reaction and produces another compound) is infiltrated into the void spaces of the material template, converting, the precursor, into the desired solid product. This means that the sphere forms a solid wall on the interface between the template and the surrounding continuous phase, once the precursor reacts with the surface of the dispersed templates. Finally, to create the porosity within the sample, the spheres are removed during or after precursor conversion. Afterwards, the hollow structure can be obtained by removing the template materials [42,43]. Template removal is the last step in the synthesis of these materials, and it has to be an appropriate method of removal, in a way that the physical and chemical properties of the product should not be affected. This fabricating process is versatile and is very simple, being able to control easily the fabrication, which it's used with many types of precursor materials. This method controls the structure, morphology, and particle size of nanomaterials through the template material (template). Besides its advantages, it is also limited because of the relatively high price in that the excess templates are utilized during the fabricating process [44-46].

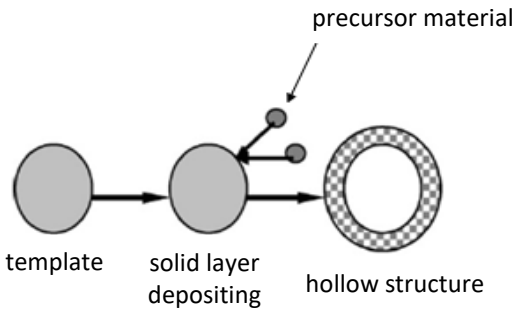

Figure 1.2- Schematic representation of process of hollow microspherical formation [47].

- Layer-by-layer self –assembly technique

Layer-by-layer (LbL) technique has been shown to be particularly interesting, in terms of the ability to readily adapt its properties, like size, composition, porosity, stability and surface functionality. This is a deposition fabrication technique. The mechanism of formation of LbL is similar to template method. The solid shell formation is made by gradual and alternative (of positively and negatively charged materials) deposition layers of the solute, which allows the introduction of multiple functionalities. There are a wide variety of materials that can be deposited by LbL including polymers, ceramics and biological molecules [48]. Then, it is important to remove the core materials to make it possible the formation of hollow structure. Though, it is a simple method, highly versatile approach and it presents a great control on the thickness of the microspherical shell, since it limits the time of deposition of solutes. Thus, this method can prepare hollow microspheres that have a uniform shell thickness and good spherical morphology. However the operation of this method can be quite complex and time-consuming [48-51].

- Emulsion processing

Emulsion technique consists on dispersion processing of a liquid in another immiscible liquid. This means that one liquid (the dispersed phase) is dispersed in the other (the continuous phase). The drops are used as “templates” and the size and shape of hollow structure are determined by those “templates”. The formation of hollow structures depends essentially on the initial conditions, otherwise some irregular morphologies may appear. This technique can also manufacture a good size of the final products because the whole process happens in the emulsion. So this method can develop a well structure in terms of uniform size distribution [41,47,52].

- Spray drying technology

The spray-drying process consists of spraying the slurry into the drying chamber by subjecting it to a controlled stream of hot air, generating the evaporation of the solvents, usually water (figure 1.3). This results in an ultra-rapid separation of the solids and soluble solutions contained with the minimum degradation of the product in drying, finishing the process with the recovery of the product already transformed into powder [47,53]. The spray-drying method makes possible to obtain materials with greater ease and flexibility. The structure and morphology of the products prepared by this method are influenced by the spray drying parameters (inlet air temperature, atomized pressure, feed rate) and also by slurry characteristics (slurry concentration, size of the primary powders) [47]. Therefore, it is necessary to introduce several factors responsible for influencing the intended morphology of the products. As disadvantages have high costs [40,47,53].

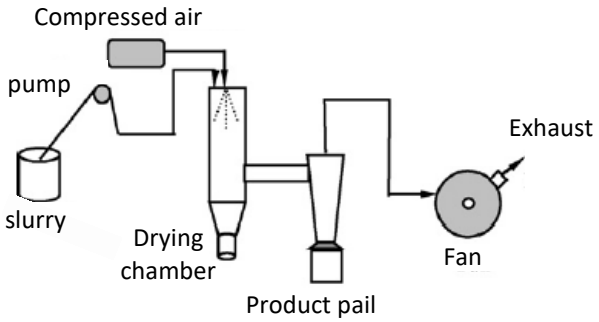

Figure 1.3- Flow chart of the prepared process by spray drying method [47].

1.3.1.2 – Porosity

Since bone is a porous tissue, there is a viable option for using porous hollow spheres to repair damaged bone [54]. In this way, porosity is defined as the percentage of void space (pores) in a solid, representing a morphological property independent of the material. Pores are important for bone tissue formation because they allow migration and proliferation of osteoblasts and mesenchymal cells, as well as vascularization. With the increased porosity, it is expected that the mechanical resistance of the material by itself decreases. But the existence of a porous surface with adequate pore dimensions on the body, actually, it can favor the growth of neoformed tissue, which will improve mechanical interlocking between the implant biomaterial and the surrounding bone tissue. This will provide a greater mechanical stability at the interface *in vivo* [54,55].

There are several aspects that should be considered to design porous materials for bone grafting. Hence, it is important: (1) the size of pores at the surface; (2) the size of porous network that connects the pores of the surface to allow areas of bone ingrowth to be found within the porous graft; (3) the porosity extension (percentage), the morphology and the capacity to form blood vessels and canaliculi within the porosities [50]. Pores should be open and interconnected, and for pore sizes that exceed 100 μm (minimum size for cell migration) bone will grow within the interconnecting pore channels near the surface and maintain its vascularity and long term viability, which is an essential requirement for osteoconduction [3,55]. Hence, microporosity results in larger surface area that increases cell adhesion and macroporosity allows bone growth, which allows vascularization within the bone substitute [54,56]. Both micro and macroporosity are decisive factors in increasing the rate of formation of new bone at the site of implantation. Besides these two factors (micro and macroporosity), the degree of interconnectivity of pores directly affect the diffusion of gases and nutrients present in physiological fluids, as well as the removal of residues [42,57]. In this manner, the implant aids as a structural bridge or scaffold for bone formation, which much allows communication between bone cells.

Thus, porous materials, like microspheres with porosity, exhibit superior cell attachment, cell proliferation, drug absorption and drug release kinetics compared to bulk microspheres [16].

Relatively to the creation of porosity, there are different approaches that include adding soluble porogenic agents, gas foaming, salt leaching, surfactant, resorbable polymers, even using three-dimensional printing, solid free fabrication process, phase separation and freeze-drying [30,56,57].

1.3.1.3 – Injectability

Bone substitutes are a good example of repairing bone tissue. Lately, in order to improve the clinical potential of those biomaterial bone substitutes, there has been an increasing interest on minimally invasive techniques, which one of the major techniques in orthopedics is the development of injectable systems. This type of technique avoid the increase in bone loss, trauma, and surgical time [58].

Therefore, injectable systems are advantageous because they can be easily molded into any shape of bone cavity, which it is beneficial in clinical situations that are difficult to access to the bone defect; they can shorten the surgical operation time; reduce postoperative pain and scar size; allowing patients to achieve rapid recovery; and reduce cost [58,59]. Thereby, the major advantage of injectable systems is injectability, since it refers to an essential requirement for a biomaterial, in a minimally invasive manner. These systems can be injected into the defect area using a syringe needle, which helps to reduce patient discomfort and shorten the healing time [60]. In this way, injectable systems are a good option for the delivery of the hollow microspheres if an adequate vehicle is chosen.

In terms of injectable systems, there are a variety of them and the systems that consist on the combination of a ceramic and a polymer have been developed for use in multiple orthopaedic applications. The polymers that act as vehicles must be easy to manipulate and must have a proper viscosity in order to allow the transportation of the particles. Different examples of ceramics have been used, such as Hap and TCP, as well as several polymers, including synthetic polymers (like as poly(ethylene glycol) (PEG)) or natural polymers, such as alginate, agar, collagen and chitosan, among others [59-63].

1.3.2 – Drugs in bone tissue engineering

Bone healing and remodeling are performed by a complex and coordinated effort of cells, bioactive factors and extracellular matrix [24]. Bone tissue has the capacity for auto-reconstruction, which combined with its binding proteins and the growth factors (acting as local regulators of cell function), enable the stimulation of proliferation, cell differentiation, migration and adhesion. However, in severe pathological conditions such as complicated fractures, trauma, treatment of bone tumors, joint replacement, congenital defects or spinal fusion, the damaged bone will not form or regenerate spontaneously [29]. Thus, an alternative to restore structural and functional bone integrity is the biochemical stimulation of local bone healing, being necessary to develop drug delivery systems for providing bioactive molecules and drugs to the injury site and, hence, extend their biological functionality [28].

Thus, the application of drugs in bone tissue engineering is very wide and a rapidly growing research in biomedical field.

1.3.2.1 – Antibiotics

Over the past few decades, it has been reported that many bacteria are the responsible for causing implant-related infections [64]. There are different bacteria responsible, such as *Staphylococcus aureus, Pseudomonas aeruginosa and Staphylococcus epidermidis*. However, the most relevant pathogens in orthopedic infections are coagulase-negative staphylococci, *Staphylococcus aureus* and *Staphylococcus epidermidis*. In the first three months after surgery, the infections are usually caused by virulent microorganisms such as *Staphylococcus aureus*, whereas delayed infections (3-24 months after surgery) are in most of the cases caused by low virulent microorganisms such as coagulase-negative staphylococci [65,66].

Thus, infections are a serious complication following surgeries, which are usually caused by the air of the operating room that may be contaminated; incomplete skin disinfection (or by the patient or by the operating staff) and contamination of the medical equipment; or even because there is already a bacteria present in the patient's body [67]. It is believed that approximately 60% of the prosthesis-related infections are caused by direct contamination during the operative procedures. It is, therefore, important to disinfect and take all the sterile performances properly. Beyond this, to minimize and combat the possibility of infections, it is important to administrate broad-spectrum antibiotics. These type of drugs should have a broad antibacterial spectrum, including Gram-positive and Gram-negative pathogens, sufficient bactericidal activity, low rate of primary resistant pathogens, minimal development of resistance during therapy, chemical and thermal stability [68].

Hence, attempts have been made to prevent and cure orthopedic infections by incorporating antibiotics in drug delivery systems, during surgical interventions. Relatively to drugs, it is important to know that if the drug is released too quickly, the entire drug could be released before the infection is stopped. On the other hand, if the release of the drug is delayed, the infection could increase. Therefore, this scenario will be difficult to control the

healing of the wound. In this way, there has been a major necessity to design drug release systems that are effective, in a way that they can easily release the drugs to the site of injury to prevent microbial infection and they should control the drug delivery over a long period of time for medical applications [69].

In terms of the most used antibiotic groups for the treatment of bone infections, stand out gentamicin, tobramycin, vancomycin, tetracyclines and cephalosporins.

Gentamicin is an aminoglycoside antibiotic and it is widely used in studies, being commonly employed in trauma, as treatment of osteomyelitis. It has a good efficacy in terms of controlled release of drug due to its broad-spectrum antimicrobial activity, high solubility and stability at high temperatures. These antibiotic has bacterial effects on aerobic Gram-negative and some *Staphylococcus* strains. Gentamicin works by interrupting protein synthesis. This drug has been loaded in several scaffolds in order to evaluate their ability as controlled-release carriers.

Tobramycin is an aminoglycoside antibiotic and comes from *Streptomyces tenebrarius*. It is used to treat various bacterial infections that are particularly caused by Gram-negative bacteria. *Pseudomonas* are especially sensitive to tobramycin. Tobramycin prevents the formation of a complex that will trigger a non-translation of mRNA into protein, which leads to a cell death. Tobramycin has a narrow spectrum of activity (refers to a specific group of bacteria). It is not active against Gram-positive bacteria, except for *Staphylococcus aureus* [70].

Vancomycin is a tricyclic glycopeptide produced by *Streptomyces orientalise* and is frequently used on the treatment of bone and joint infections. These antibiotic is usually used as a last resort medication for the treatment of serious and fatal infections by Gram-positive bacteria that do not respond to other antibiotics. Although, Gram-negative bacteria are insensitive to vancomycin. It is an antibiotic that works through inhibiting proper cell wall synthesis[70].

Tetracyclines have a broad-spectrum antibacterial activity. They exert their antibacterial activity by inhibiting the synthesis of microbial proteins. Most of medically relevant bacteria were proven to be sensitive to tetracyclines. However, many pathogenic organisms have been creating resistance due to the versatility and excessive use of these antibiotics. Tetracyclines represent a broad group, which include tetracycline, minocycline, and doxycycline. These antibiotics were widely used as topical therapy to treat periodontitis, but they were also used during bone grafting procedures because of their anti-collagenase, antibacterial and fibroblast-stimulatory properties [70].

Cephalosporins are a class of ß-lactam antibiotics and they represent a broad group, which can be divided into first, second, third and fourth generation agents, differing on the time of their discovery and their antimicrobial properties. These type of antibiotics have effect by acting on the cell wall synthesis [71].

1.3.2.2 – Anti-inflammatory agents

Besides antibiotics, it can be appropriate the incorporation of other drugs, such as anti-inflammatory agents, with potential application on orthopedic field. Implantation of engineered biomaterials cause local inflammation owing to the host immunoresponse, which therefore requires the use of anti-inflammatory agents, either steroids or non-steroids. Steroids agents are known as glucocorticoids, which are a type of corticosteroid hormones that have potent anti-inflammatory and immunosuppressive effects. Anti-inflammatory agents have been used because of their ability to suppress the immune response by inhibiting the formation and secretion of inflammatory mediators such as prostaglandins ((synthesis of a group of physiologically active lipid compounds that act only on the cells) and leukotrienes [72]. Non-steroidal anti-inflammatory drugs (NSAID) are widely used in treatment of pain, including bone fracture pain and orthopaedic post-operative pain. In this manner, it is described some examples of anti-inflammatory agents on table 1.4, including dexamethasone (DEX), acetylsalicylic acid (ASA) and ibuprofen (IBU) [30].

Table 1.4 – Different examples of anti-inflammatory agents.

ANTI-INFLAMMATORY AGENTS	CHARACTERISTICS
DEX	It is a corticosteroid that prevents the release of substances in the body that cause inflammation. DEX is used to treat many different conditions, including rheumatic problems, others arthritis, allergic disorders, skin conditions, lupus, psoriasis and breathing disorders. So, these corticosteroid helps to direct the differentiation of stem cells into the osteogenic lineage [30].
ASA	It is commonly known as aspirin and is a derivative of salicylic acid, a crystalline organic carboxylic acid. ASA has diverse therapeutic applications due to its analgesic, antipyretic, non-steroidal and anti-inflammatory properties. In terms of administration of ASA, it is mainly based on oral dosage form. It acts by inhibition of prostaglandin synthesis. This analgesic is widely used for the prevention and treatment of cardiovascular diseases, strokes and in various joint disorders, like as rheumatoid and other types of arthritis [73,74].
IBU	It is a non-steroidal anti-inflammatory drug class that is used for treating pain, fever and inflammation. IBU has been largely used orally, intravenously and even topically. These drug is also a potent inhibitor of prostaglandin synthesis [75]. This means that IBU is an inhibitor of the enzyme cyclooxygenase (COX), which plays an important role in the generation of inflammation.

References

[1] V. I. Sikavitsas, J. S. Temenoff, and A. G. Mikos, "Biomaterials and bone mechanotransduction," *Biomaterials*, vol. 22, no. 19, pp. 2581–2593, 2001.

[2] C. VanPutte, J. Regan, A. Russo, B. Manager, and A. Reed, *Seeley's Anatomy & Physiology 9th Lab Edition*. 2016.

[3] B. D. Ratner, A. S. Hoffman, F. J. Schoen, and J. E. Lemons, *Biomaterials science: an introduction to materials in medicine*. 2004.

[4] F. M. Chen and X. Liu, "Advancing biomaterials of human origin for tissue engineering," *Progress in Polymer Science*, vol. 53. pp. 86–168, 2016.

[5] L. B. Rodrigues, "Aplicações de biomateriais em ortopedia," *Estudos Tecnológicos em Engenharia*, vol. 9, no. 2, pp. 63–76, 2013.

[6] D. I. Ilan and A. L. Ladd, "Bone graft substitutes," *Operative Techniques Plastic and Reconstructive Surgery*, vol. 9, no. 4, pp. 151–160, 2002.

[7] T. Kokubo, "Bioactive glass ceramics: properties and applications," *Biomaterials*, vol. 12, no. 2, pp. 155–163, 1991.

[8] L. L. Hench, "Bioceramics: From Concept to Clinic," *Journal of the American Ceramic Society*, vol. 74, no. 7, pp. 1487–1510, 1991.

[9] J. Zhang *et al.*, "A simple and effective approach to prepare injectable macroporous calcium phosphate cement for bone repair: Syringe-foaming using a viscous hydrophilic polymeric solution," *Acta Biomaterialia*, vol. 31, pp. 326–338, 2016.

[10] M. Vallet-Regí, "Ceramics for medical applications," *Journal of the Chemical Society, Dalton Transactions*, no. 2, pp. 97–108, 2001.

[11] L. Wang *et al.*, "Injectable calcium phosphate with hydrogel fibers encapsulating induced pluripotent, dental pulp and bone marrow stem cells for bone repair," *Materials Science and Engineering C*, vol. 69, pp. 1125–1136, 2016.

[12] J. R. Jones, "Reprint of: Review of bioactive glass: From Hench to hybrids," *Acta Biomaterialia*, vol. 23, no. S. pp. S53–S82, 2015.

[13] B. H. Chen, K. I. Chen, M. L. Ho, H. N. Chen, W. C. Chen, and C. K. Wang, "Synthesis of calcium phosphates and porous hydroxyapatite beads prepared by emulsion method," *Materials Chemistry and Physics*, vol. 113, no. 1, pp. 365–371, 2009.

[14] W. Feng, L. Mu-Sen, L. Yu-Peng, and Q. Yong-Xin, "A simple sol-gel technique for preparing hydroxyapatite nanopowders," *Materials Letters*, vol. 59, no. 8–9, pp. 916–919, 2005.

[15] E. Charrière, J. Lemaitre, and P. Zysset, "Hydroxyapatite cement scaffolds with controlled macroporosity: Fabrication protocol and mechanical properties," *Biomaterials*, vol. 24, no. 5, pp. 809–817, 2003.

[16] K. M. Z. Hossain, U. Patel, and I. Ahmed, "Development of microspheres for biomedical applications: a review," *Progress in Biomaterials*, vol. 4, no. 1, pp. 1–19, 2015.

[17] S. Raynaud, E. Champion, D. Bernache-Assollant, and P. Thomas, "Calcium phosphate apatites with variable Ca/P atomic ratio I. Synthesis, characterisation and thermal stability of powders," *Biomaterials*, vol. 23, no. 4, pp. 1065–1072, 2002.

[18] P. V Giannoudis, H. Dinopoulos, and E. Tsiridis, "Bone substitutes: An update," *Injury*, vol. 36, no. 3, pp. S20–S27, 2005.

[19] W. R. Moore, S. E. Graves, and G. I. Bain, "Synthetic bone graft substitutes," *ANZ Journal of Surgery*, vol. 71, no. 6, pp. 354–361, 2001.

[20] L. Xiao, B. Wang, G. Yang, and M. Gauthier, "Poly(Lactic Acid)-Based Biomaterials : Synthesis , Modification and Applications," *Biomedical Science, Engineering and Technology*, pp. 247–282, 2006.

[21] B. D. Ulery, L. S. Nair, and C. T. Laurencin, "Biomedical applications of biodegradable polymers," *Journal of Polymer Science, Part B: Polymer Physics*, vol. 49, no. 12. pp. 832–864, 2011.

[22] M. M. Stevens, "Biomaterials for bone tissue engineering," *Materials Today*, vol. 11, no. 5. pp. 18–25, 2008.

[23] B. Stevens, Y. Z. Yang, A. MohandaS, B. Stucker, and K. T. Nguyen, "A review of materials, fabrication to enhance bone regeneration in methods, and strategies used engineered bone tissues," *Journal Biomedical Materials Research Part B*, vol. 85B, no. 2, pp. 573–582, 2008.

[24] T. N. Vo, F. K. Kasper, and A. G. Mikos, "Strategies for controlled delivery of growth factors and cells for bone regeneration," *Advanced Drug Delivery Reviews*, vol. 64, no.

12. pp. 1292–1309, 2012.
[25] M. N. Rahaman *et al.*, "Bioactive glass in tissue engineering," *Acta Biomaterialia*, vol. 7, no. 6. pp. 2355–2373, 2011.
[26] H. Yoshikawa and A. Myoui, "Bone tissue engineering with porous hydroxyapatite ceramics," *Journal of Artificial Organs*, vol. 8, no. 3. pp. 131–136, 2005.
[27] S. Sarin and A. Rekhi, "Bioactive glass: A potential next generation biomaterial," *SRM Journal of Research in Dental Sciences*, vol. 7, no. 1, p. 27, 2016.
[28] M. van Griensven, "Preclinical testing of drug delivery systems to bone," *Advanced Drug Delivery Reviews*, vol. 94. pp. 151–164, 2015.
[29] V. Luginbuehl, L. Meinel, H. P. Merkle, and B. Gander, "Localized delivery of growth factors for bone repair," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 58, no. 2, pp. 197–208, 2004.
[30] V. Mouriño and A. R. Boccaccini, "Bone tissue engineering therapeutics: controlled drug delivery in three-dimensional scaffolds," *Journal of the Royal Society Interface*, vol. 7, no. 43, pp. 209–27, 2010.
[31] U. Edlund and A. C. Albertsson, "Degradable polymer microspheres for controlled drug delivery," *Degradable Aliphatic Polyesters*, vol. 157, pp. 67–112, 2002.
[32] R. M. Mainardes and L. P. Silva, "Drug delivery systems: past, present, and future," *Current Drug Targets*, vol. 5, pp. 449–55, 2004.
[33] a Dubnika, D. Loca, and L. Berzina-Cimdina, "Functionalized hydroxyapatite scaffolds coated with sodium alginate and chitosan for controlled drug delivery," *Proceedings of the Estonian Academy of Sciences*, vol. 61, no. 3, p. 193, 2012.
[34] J. F. Coelho *et al.*, "Drug delivery systems: Advanced technologies potentially applicable in personalized treatments," *The EPMA Journal*, pp. 1–46, 2010.
[35] R. Langer and N. A. Peppas, "Advances in Biomaterials, Drug Delivery, and Bionanotechnology," *AIChE Journal*, 2003, vol. 49, no. 12, pp. 2990–3006.
[36] A. K. Bajpai, S. K. Shukla, S. Bhanu, and S. Kankane, "Responsive polymers in controlled drug delivery," *Progress in Polymer Science (Oxford)*, vol. 33, no. 11. pp. 1088–1118, 2008.
[37] H. Fu, M. N. Rahaman, D. E. Day, and R. F. Brown, "Hollow hydroxyapatite microspheres as a device for controlled delivery of proteins," *Journal of Materials Science: Materials in Medicine*, vol. 22, no. 3, pp. 579–591, 2011.
[38] K. K. Kim and D. W. Pack, "Microspheres for Drug Delivery," *BioMEMS and Biomedical Nanotechnology*, vol. I, pp. 19–50, 2006.
[39] Y. Bao, C. Shi, T. Wang, X. Li, and J. Ma, "Recent progress in hollow silica: Template synthesis, morphologies and applications," *Microporous and Mesoporous Materials*, vol. 227. pp. 121–136, 2016.
[40] Y. Jiao, Y. P. Lu, G. Y. Xiao, W. H. Xu, and R. F. Zhu, "Preparation and characterization of hollow hydroxyapatite microspheres by the centrifugal spray drying method," *Powder Technology*, vol. 217, pp. 581–584, 2012.
[41] J. H. Park, C. Oh, S. Il Shin, S. K. Moon, and S. G. Oh, "Preparation of hollow silica microspheres in W/O emulsions with polymers," *Journal of Colloid and Interface Science*, vol. 266, no. 1, pp. 107–114, 2003.
[42] Y. Xie, D. Kocaefe, C. Chen, and Y. Kocaefe, "Review of Research on Template Methods in Preparation of Nanomaterials," *Journal of Nanomaterials*, vol. 2016. 2016.
[43] Q. Zhang, W. Wang, J. Goebl, and Y. Yin, "Self-templated synthesis of hollow nanostructures," *Nano Today*, vol. 4, no. 6, pp. 494–507, 2009.
[44] Y. Gao, Q. Zhao, Q. Fang, and Z. Xu, "Facile fabrication and photoluminescence properties of rare-earth-doped Gd_2O_3 hollow spheres via a sacrificial template method," *Dalton Transactions*, vol. 42, no. Cvd, pp. 11082–91, 2013.
[45] J. B. Joo, P. Kim, W. Kim, J. Kim, N. D. Kim, and J. Yi, "Simple preparation of hollow carbon sphere via templating method," *Current Applied Physics*, vol. 8, no. 6, pp. 814–817, 2008.
[46] A. Stein, "Sphere templating methods for periodic porous solids," *Microporous Mesoporous Materials*, vol. 44–45, pp. 227–239, 2001.
[47] A. J. Wang, Y. P. Lu, and R. X. Sun, "Recent progress on the fabrication of hollow microspheres," *Materials Science and Engineering: A*, vol. 460–461, pp. 1–6, 2007.
[48] Z. Tang, Y. Wang, P. Podsiadlo, and N. A. Kotov, "Biomedical applications of layer-by-layer assembly: From biomimetics to tissue engineering," *Advanced Materials*, vol. 18, no. 24. pp. 3203–3224, 2006.

[49] G. Wu and X. Zhang, “Layer-by-Layer Assembly: From Conventional to Unconventional Methods,” *Multilayer Thin Films: Sequential Assembly of Nanocomposite Materials: Second Edition*, vol. 1, 2012, pp. 43–67.
[50] A. P. R. Johnston, C. Cortez, A. S. Angelatos, and F. Caruso, “Layer-by-layer engineered capsules and their applications,” *Current Opinion in Colloid and Interface Science*, vol. 11, no. 4. pp. 203–209, 2006.
[51] Y. Wang, A. S. Angelatos, and F. Caruso, “Template synthesis of nanostructured materials via layer-by-layer assembly,” *Chemistry of Materials*, vol. 20, no. 3, pp. 848–858, 2008.
[52] E. J. Windhab, M. Dressler, K. Feigl, P. Fischer, and D. Megias-Alguacil, “Emulsion processing - From single-drop deformation to design of complex processes and products,” *Chemical Engineering Science*, 2005, vol. 60, no. 8–9 SPEC. ISS., pp. 2101–2113.
[53] R. Sun, Y. Lu, and K. Chen, “Preparation and characterization of hollow hydroxyapatite microspheres by spray drying method,” *Materials Science and Engineering: C*, vol. 29, no. 4, pp. 1088–1092, 2009.
[54] G. Hannink and J. J. C. Arts, “Bioresorbability, porosity and mechanical strength of bone substitutes: What is optimal for bone regeneration?,” *Injury*, vol. 42, no. SUPPL. 2, 2011.
[55] A. Tampieri, G. Celotti, S. Sprio, A. Delcogliano, and S. Franzese, “Porosity-graded hydroxyapatite ceramics to replace natural bone,” *Biomaterials*, vol. 22, no. 11, pp. 1365–1370, 2001.
[56] V. Karageorgiou and D. Kaplan, “Porosity of 3D biomaterial scaffolds and osteogenesis,” *Biomaterials*, vol. 26, no. 27. pp. 5474–5491, 2005.
[57] H. H. Xu and C. G. Simon Jr., “Self-hardening calcium phosphate cement-mesh composite: reinforcement, macropores, and cell response,” *Journal of Biomedical Materials Research*, vol. 69, no. 2, pp. 267–278, 2004.
[58] L. Zhao, M. D. Weir, and H. H. K. Xu, “An injectable calcium phosphate-alginate hydrogel-umbilical cord mesenchymal stem cell paste for bone tissue engineering,” *Biomaterials*, vol. 31, no. 25, pp. 6502–6510, 2010.
[59] H. Liu, H. Li, W. Cheng, Y. Yang, M. Zhu, and C. Zhou, “Novel injectable calcium phosphate/chitosan composites for bone substitute materials,” *Acta Biomaterialia*, vol. 2, no. 5, pp. 557–565, 2006.
[60] G. H. Lee, P. Makkar, K. Paul, and B. T. Lee, “Incorporation of BMP-2 loaded collagen conjugated BCP granules in calcium phosphate cement based injectable bone substitutes for improved bone regeneration,” *Materials Science and Engineering: C*, vol. 77, pp. 713–724, 2017.
[61] J. L. Moreau and H. H. K. Xu, “Mesenchymal stem cell proliferation and differentiation on an injectable calcium phosphate - Chitosan composite scaffold,” *Biomaterials*, vol. 30, no. 14, pp. 2675–2682, 2009.
[62] K. L. Low, S. H. Tan, S. H. S. Zein, J. A. Roether, V. Mouriño, and A. R. Boccaccini, “Calcium phosphate-based composites as injectable bone substitute materials,” *Journal of Biomedical Materials Research*, vol. 94, no. 1. pp. 273–286, 2010.
[63] B. Chang, N. Ahuja, C. Ma, and X. Liu, “Injectable scaffolds: Preparation and application in dental and craniofacial regeneration,” *Materials Science and Engineering: R*, vol. 111. pp. 1–26, 2017.
[64] M. Zilberman and J. J. Elsner, “Antibiotic-eluting medical devices for various applications,” *Journal of Controlled Release*, vol. 130, no. 3. pp. 202–215, 2008.
[65] M. A. Rauschmann *et al.*, “Nanocrystalline hydroxyapatite and calcium sulphate as biodegradable composite carrier material for local delivery of antibiotics in bone infections,” *Biomaterials*, vol. 26, no. 15, pp. 2677–2684, 2005.
[66] M. Ribeiro, F. J. Monteiro, and M. P. Ferraz, “Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions,” Journal *Biomatter*, vol. 2, no. 4, pp. 176–194, 2012.
[67] D. Peres, I. Neves, F. Vieira, and I. Devesa, “Prosthesis infections after orthopedic joint replacement: the possible role of bacterial biofilms,” *Orthopedic Reviews (Pavia)*, vol. 27, no. 1, pp. 67–72, 2014.
[68] H. Van de Belt, D. Neut, W. Schenk, J. R. Van Horn, H. C. Van der Mei, and H. J. Busscher, “Infection of orthopedic implants and the use of antibiotic-loaded bone cements,” *Acta Orthopaedica Scandinavica*, vol. 72, no. 6. pp. 557–571, 2001.
[69] M. P. Ginebra, T. Traykova, and J. A. Planell, “Calcium phosphate cements as bone drug

delivery systems: a review," *Journal of Controlled Release*, vol. 113, no. 2, pp. 102–10, 2006.
[70] H. Lu, Y. Liu, J. Guo, H. Wu, J. Wang, and G. Wu, "Biomaterials with antibacterial and osteoinductive properties to repair infected bone defects," *International Journal of Molecular Sciences*, vol. 17, no. 3. 2016.
[71] S. R. El-Shaboury, G. A. Saleh, F. A. Mohamed, and A. H. Rageh, "Analysis of cephalosporin antibiotics," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 45, no. 1. pp. 1–19, 2007.
[72] J. M. Morais, F. Papadimitrakopoulos, and D. J. Burgess, "Biomaterials/tissue Interactions: Possible Solutions to Overcome Foreign Body Response," The *AAPS Journal*, vol. 12, no. 2, pp. 188–196, 2010.
[73] S. Das, R. Banerjee, and J. Bellare, "Aspirin loaded albumin nanoparticles by coacervation: Implications in drug delivery," *Trends in Biomaterials and Artificial Organs*, vol. 18, no. 2, pp. 203–212, 2005.
[74] Y. Tang and J. Singh, "Controlled delivery of aspirin: Effect of aspirin on polymer degradation and in vitro release from PLGA based phase sensitive systems," *International Journal of Pharmaceutics*, vol. 357, no. 1–2, pp. 119–125, 2008.
[75] K. D. Rainsford, *Ibuprofen: Pharmacology, therapeutics and side effects*, 2012.

Chapter 2

2 – Strategies to promote porosity on bioceramic spheres

Abstract

For a long time, bone disorders and injuries have been increasing, which affect millions of people worldwide. This impact on human health is mainly due to the increased average life expectancy. For this reason, it has been made efforts, in order to develop an ideal synthetic bone substitute that is capable of regenerate lesion bone.

The aim of this study was to produce porous bioceramic spheres, in order to fill injured locals and consequently, promoting bone tissue regeneration.

Porous bioceramics were fabricated by two different approaches. One consists on the addition of porogenic agents and the other consists on a syringe foaming method. Spheres produced by both approaches were examined by scanning electron microscopy, X-Ray diffraction and Fourier transform infrared spectroscopy. Relatively to pore forming agents approach, this is a simple technique, however, it did not evidence to be an effective process of producing open and interconnected porosity and it did not allow the creation of adequate pore size needed to osteoid growth. In this way, syringe foaming approach showed to be better than porogenic agents approach, since it produced open and interconnected porosity suitable for bone regeneration.

2.1 – Introduction

With the increase of aging population, bone defects caused by trauma or infections have been affecting the quality of life of millions of people. Bone defects can occur in locals that are not subjected to high mechanical loads (maxillofacial bones) or in locals that are subjected to load (bones of the extremities and bones of the spine) [1]. In the last years, efforts have been made, in order to solve this problematic, since autografts, allo or xenografts have presented disadvantages like, limited availability and risk of disease transmission [2,3]. Besides these alternatives, orthopaedic implants are not still presenting some characteristics, namely the ability to repair them self, the ability to maintain a blood supply and the capability of modifying their structure and properties in response to environmental factors, such as mechanical load [4].

Therefore, synthetic bone substitutes, including bioceramics, have been developed for bone regeneration, as bone fillers. Bioactive glasses (BG) is representative of bioceramics, being the material focus of this work. They represent an option for a bone graft, because they are biocompatible, bioactive and osteoconductive materials [4,5], allowing migration, bone cells attachment and proliferation. In this way, it will help to promote high levels of osteoconduction [6-8].

It is important to develop an ideal porous bone graft that has interconnected macro and microporous network, allowing cell penetration, tissue ingrowth and vascularization. Thus, pores between 50 and 150 μm determine osteoid growth [9,10].

Regarding bone grafts there is a significant interest in particles that are preferably spheres, because they have large specific surface area and can be introduced on irregular or reduced locals. Nowadays, there are several techniques able of producing porous bioceramics spheres [11]. In terms of enhancing macroporosity of bioceramics, there have been studied several strategies, including the addition of porogenic agents, the use of gas generating compounds, or even, the use of foaming agents [11,12].

Hence, in this study, it was explored two different approaches of creating porosity. A classical and simple method of introducing macroporosity is the use of organic agents (porogenic agents). The addition of these agents increases porosity, since they burn out at low heating rates, which helps to create macropores. There are several examples of porogenic agents, such as calcium carbonate ($CaCO_3$) [13,14], gelatine, sucrose, poly (vinyl alcohol) (PVA) [15,16], poly (ethylene glycol) (PEG) [17,18], fibers, among others [19]. Another strategy explored is a syringe foaming approach, which consists of a mixture between a paste of bioactive glass and a foam solution. This foam solution is constituted by a foaming agent that must be water soluble, nontoxic and should have good foaming ability and foam stability [20]. An example of foaming agents are the surfactants, because of their ability to stabilize the air-liquid interface in the foam solutions, which defines a porous bioceramic structure [21,22].

2.2 – Materials and Methods

2.2.1 - Raw materials

A silica bioactive glass with composition 64 % (w/w) SiO_2 – 26 % (w/w) CaO – 5 % (w/w) MgO – 5 % (w/w) P_2O_5 was prepared in the laboratory by the sol-gel method. For this synthesis, tetraethyl orthosilicate (TEOS, Sigma-Aldrich, Ref. 131903), ethanol (ETOH, Sigma-Aldrich, Ref. 16368), calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$, PanReac AppliChem, Ref. 141231.1211), nitric acid (HNO_3, Alfa Aesar, Ref. 44528), triethyl phosphate (TEP, Merck KGaA, Ref. 8.21141.1000) magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6H_2O$, Sigma-Aldrich, Ref. 63087) and ammonia solution (NH_3 ,Merck KGaA, Ref. 9617485) were used.

To perform syringe foaming approach, it was necessary Pluronic® F-127 powder (F-127, Sigma-Aldrich, Ref. P2443), sodium hypophosphite monohydrate powder (NaH_2PO_4, Sigma-Aldrich, Ref. S5012), sodium phosphate dibasic heptahydrate powder (Na_2HPO_4, Sigma-Aldrich, Ref. 431478), calcium chloride 2-hydrate ($CaCl_2.2H_2O$, PanReac AppliChem, Ref.131232.1211) and alginic acid sodium salt ($(C_6H_7O_6Na)_n$, PanReac AppliChem, Ref. A3249.0250)

2.2.2 – Bioactive glass production

For the present work, bioactive glass (BG) was produced by the sol-gel method, at room temperature. It was added 10 mL of ethanol (ETOH) in 12.5 mL of tetraethyl ortosilicate (TEOS), silica precursor. These starting reagents were mixed for 1 h. Then, it was prepared, in separately, 6 g of calcium nitrate in 20 mL of distilled water. When it was obtained a homogeneous solution, it was added to the previous formulation. This solution stay mixed for 1 h to initiate the hydrolysis of TEOS. After this time, it was added nitric acid (4 mL), which acts as a catalyst. After 1 h, it was added 1 mL of triethyl phosphate (TEP), phosphorus precursor. Once again, after 1 h past, it was added 1.25 g of magnesium nitrate (precursor of magnesium). This final solution was mixed for 1 h. Finally, it was added 4 mL of an ammonia solution, which was drop wise added, resulting in a white precipitate. After gel formation, gel is left at room temperature.

2.2.3 - Pore Forming agents approach

Relatively to the development of porosity, it was added pore forming agents, namely calcium carbonate ($CaCO_3$), two polymers (poly (vinyl alcohol) (PVA) and poly (ethylene glycol) (PEG)) and two type of fibers (Glucomannan and *Psyllium* fiber).

In order to analyze the effect of porogenic agents, different mixtures of these agents with gel obtained by sol gel method were prepared, under vigorous stirring (table 2.1). Control mixture was prepared without any porogenic addition.

Table 2.1 - Different quantities used for each one of porogenic agent in bioactive glass.

Porogenic agent	Weight Percentage (wt. (%))								
	0.5	0.75	1	1.5	2	2.5	5	7	10
$CaCO_3$	-	-	-	-	-	-	✓	✓	✓
PVA	-	-	-	-	-	-	✓	✓	✓
PEG	-	-	-	-	-	-	✓	✓	✓
***Psyllium* fiber**	✓	✓	✓	-	-	-	-	-	-
Glucomannan	✓	✓	✓	✓	✓	✓	-	-	-

2.2.4 – Syringe foaming approach

This approach consists in mixing the paste of BG with a foam, which was obtained by using a foaming agent. In this way, it is possible to obtain porous spheres of BG by forming an injectable formulation that seemed adequate for microfluidics. For syringe foaming approach, the gel obtained by sol gel method was firstly heated at 700 °C for 2 h (heating rate 0.5 °C/min.) and then, milled.

Firstly, it was necessary to create a liquid formulation containing 2.4 mL of Pluronic F-127 solution 10 % (w/v) (non-ionic surfactant used as foaming agent capable of producing a stable and injectable foam) and 0.2 mL of sodium phosphate monobasic solution 30 % (w/v) (NaH_2PO_4) (figure 2.1). The liquid solution was collected into a 10 mL syringe, which was attached to another syringe, by a connector, with 2.5 mL of air. The solution was manually injected from one syringe to the other, by pushing the plungers of the syringes, forming a foamed phase due to turbulence generated in the liquid, during 30 s. After obtaining a foam, the foam was kept into one of the syringes to subsequently added to a BG paste that is formed by 0.5 g of BG powder with 3 mL of alginate solution 2 % (w/v), optimized in order to occur crosslinking, and 1 mL of sodium phosphate dibasic solution 2.5 % (w/v) (Na_2HPO_4), used as a hard setting accelerator, to a third syringe. These both formulations were mixed during 30 s, in order to obtain BG formulation [7]. Control sample was prepared with BG powder and alginate solution 2 % (w/v).

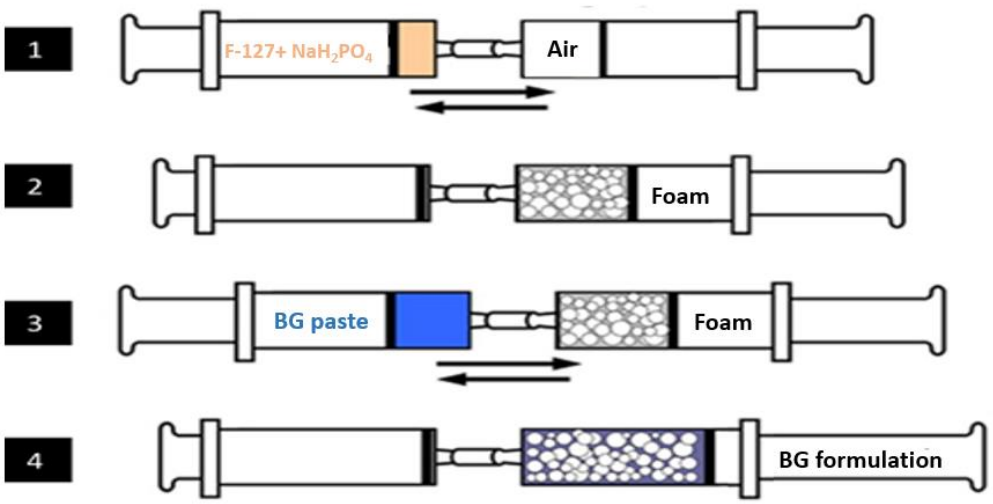

Figure 2.1 - Syringe foaming method, consisting of two syringes connected by a connector. (1) and (2) Foam of the liquid solution; (3) and (4) Obtaining bioactive glass formulation (adapted from [7]).

2.2.5 – Production of Microspheres

The method of producing microspheres was microfluidics. For this approach, it is necessary one syringe and one infusion pump. The infusion pump allows to control the flow rates and obtain reproducible spheres (spheres with the same size).

In the case of pore forming agents approach, after obtaining a homogeneous mixture, this mixture is incorporated into the syringe, which will form a drop and fall into the alginate solution 1 % (w/v) as receptor, producing microspheres. Afterwards, microspheres were immersed in calcium chloride 1 % (w/v), in order to reinforce the crosslinking, for 12 h, which was previously optimized. Then, they were washed with distilled water.

For syringe foaming approach, the formulation of BG is also incorporated into the syringe, falling into calcium chloride 5 % (w/v) receptor, forming spheres. After 12 h immersed in calcium chloride 5 % (w/v), the spheres were washed with ethanol, to reduce the electrostatic forces between the spheres.

Then, following the production of spheres, they are heated at 700 °C for 3 h (heating rate 0.5 ºC/min.) and after sintered at 1200 °C for 1 h (heating rate 4 ºC/min.).

2.3 - Physical-Chemical Characterization

Microspheres of BG were characterized by scanning electron microscopy (SEM), X-Ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR).

2.3.1 - Scanning electron microscopy (SEM)

The morphology of the samples was observed by a high resolution scanning electron microscope with X-Ray microanalysis, JEOL JSM 6301F/Oxford INCA Energy 350. The samples were mounted onto an aluminum stub using Araldite Glue (Devcon, Wellingborough, UK), in a specimen port and left to dry overnight. After drying, they were coated with a thin layer of gold/palladium (Au/Pd) by sputtering for 120 s and a current of 15 mA, using a SPI Module Sputter Coater.

2.3.2 - X-Ray diffraction (XRD)

To procedure on X-Ray diffraction, the samples were, initially, milled and dried at 50 °C. The samples were characterized by XRD, on MXP3V (Mac Science Ltd, Yokohama, Japan), using CuKα radiation at 40 kV and 30 mA, in order to identify the crystalline phases. XRD was collected within a 2θ range from 20 to 50° with a scanning speed of 0.02° and counting time of 4 s/step.

2.3.3 - Fourier transform infrared spectroscopy (FTIR)

For the identification of the functional groups present in the samples, a spectra was obtained using JADE 6.5. FTIR (FT/IR-6100 (Jasco Co., Tokyo, Japan). In order to do that, the samples were milled and dried at 50 °C. Then, they were mixed with potassium bromide (KBr) (ratio sample/KBr 1:200), being placed in a press, which was subjected to a unidirectional pressure for 2 minutes to form the pellet. Each pellet was placed on the equipment and the obtained spectra were done at a spectral resolution of 4 cm^{-1}, on the wavelength range of 400-4000 $cm^{-1}$and 200 scans were accumulated per sample.

2.4 – Results and Discussions

2.4.1 – Pore Forming approach

2.4.1.1 – Scanning electron microscopy (SEM)

As previously stated, it was produced porous microspheres by the addition of porogenic agents. For $CaCO_3$, PVA and PEG samples, the quantities used were bigger than for Glucomannan and *Psyllium* fiber samples. These different amounts of porogenic agents were added to ensure a good viscosity of the respective mixtures for the microfluidic method.

In order to visualize the microstructure of BG microspheres, they were characterized by SEM. The SEM figures indicated the presence of different morphologies, depending on the porogenic agent added. When the porogenic agents are heated at low heating rates, they burn out, leaving pores on the structure.

For PVA (figure 2.3) and PEG (figure 2.4) samples, the external surface showed to be more compact, whereas the external surface of $CaCO_3$ (figure 2.2) sample was more open and rougher with irregular macropores. It is possible to see that the heterogeneous surface of $CaCO_3$ spheres consists on small spherical pores.

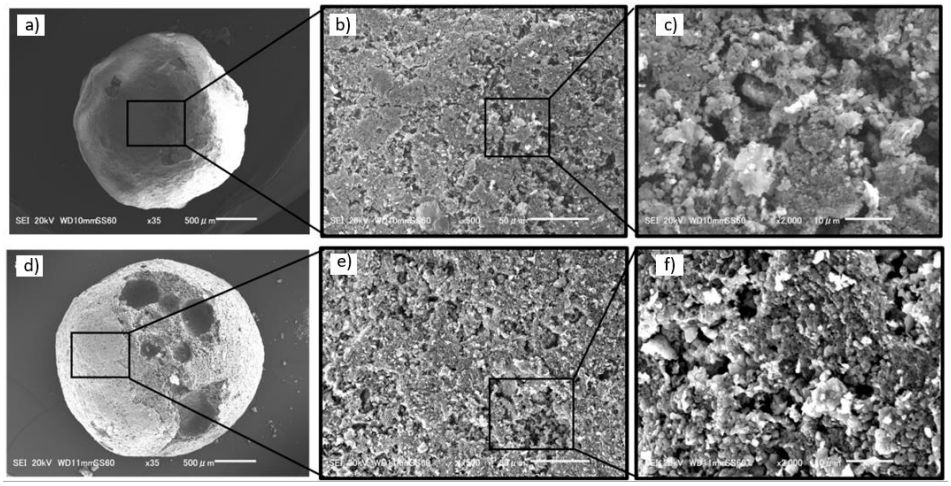

Figure 2.2 - SEM images of two samples: a),b),c) external surface of bioactive glass microsphere with 5 wt. (%) $CaCO_3$; d),e),f) external surface of bioactive glass microsphere with 10 wt. (%) $CaCO_3$.

BG microspheres with PVA, the mixture with the highest addition of the polymer, showed a more dense structure that can be explained with the development of a uniform polymer film, overlaying the silicates domain of the glass. This film resulted by the interaction between the hydrogen bonds and hydroxyl groups of PVA with remaining silanol present in the composition of glass surface. Thus, this could be the reason of the reduced occurrence of mesopores (figure 2.3f))[23].

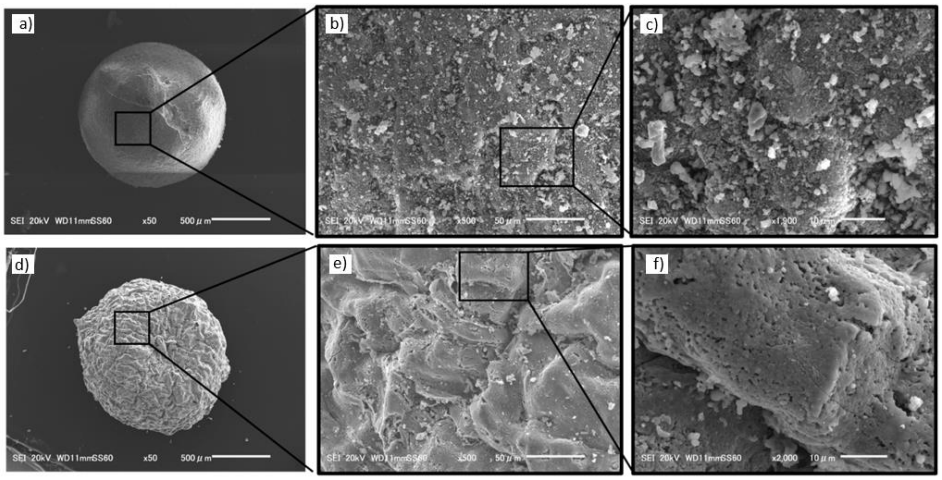

Figure 2.3 - SEM images of two samples: a),b),c) external surface of bioactive glass microsphere with 5 wt. (%) PVA; d),e),f) external surface of bioactive glass microsphere with 10 wt. (%) PVA.

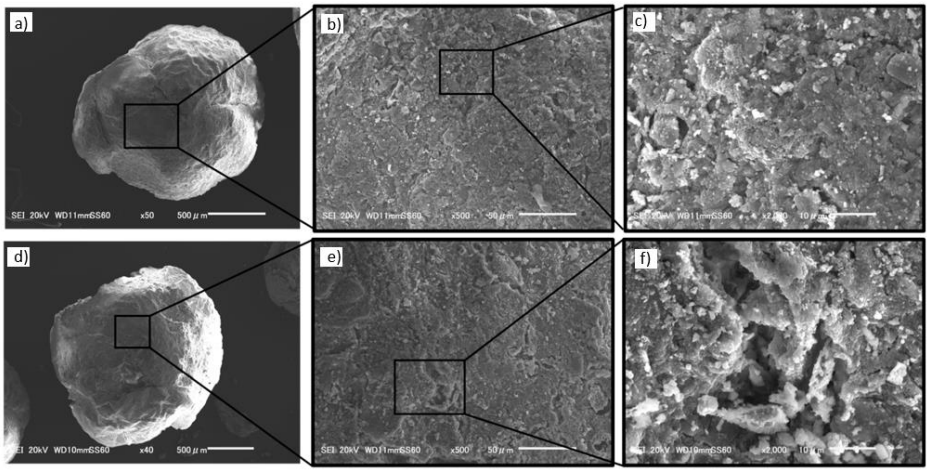

Figure 2.4 - SEM images of two samples: a),b),c) external surface of bioactive glass microsphere with 5 wt. (%) PEG; d),e),f) external surface of bioactive glass microsphere with 10 wt. (%) PEG.

For both Glucomannan and *Psyllium* fiber samples, they had a dense microstructure with low porosity (figures 2.5 - 2.7). However, for high resolutions, it was possible to detect the presence of micropores, which have a diameter approximately 4 μm (figure 2.5c)), on Glucomannan sample.

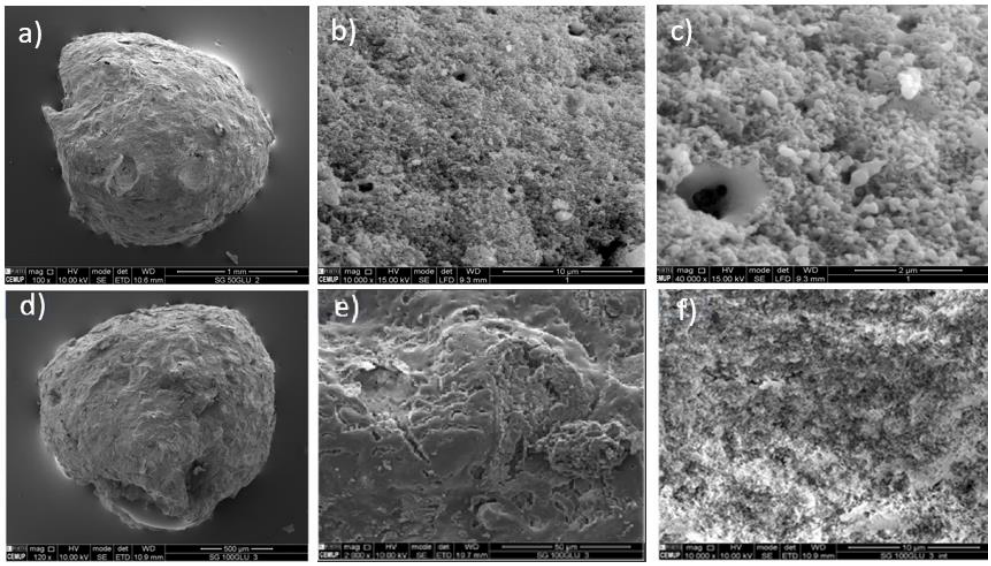

Figure 2.5 – SEM images of two samples: a),b),c) external surface of bioactive glass microsphere with 0.5 wt. (%) Glucomannan; d),e),f) external surface of bioactive glass microsphere with 1 wt. (%) Glucomannan.

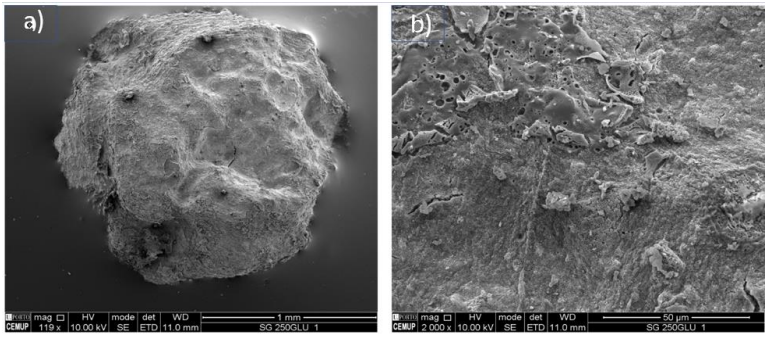

Figure 2.6 - a),b) external surface of bioactive glass microsphere with 2.5 wt. (%) Glucomannan.

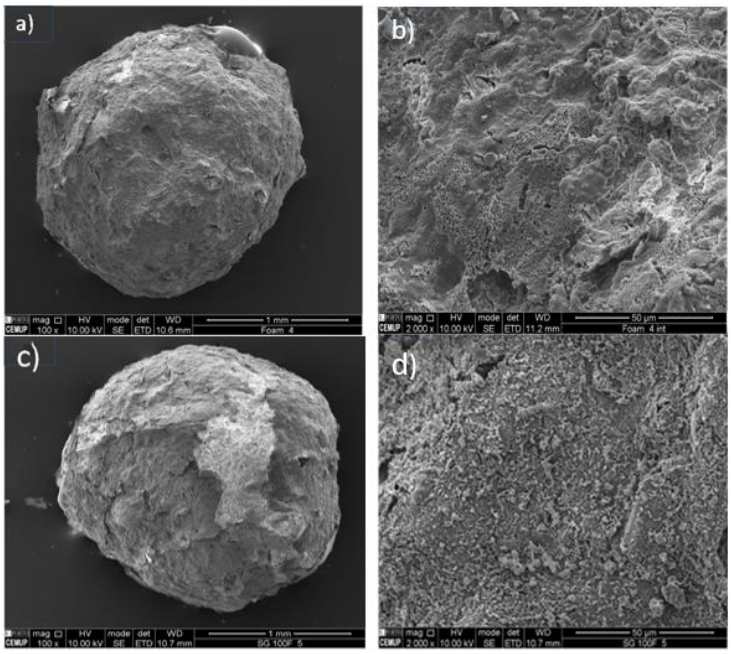

Figure 2.7 - SEM images of two samples: a),b) external surface of bioactive glass microsphere with 0.5 wt. (%) *Psyllium* fiber; c),d) external surface of bioactive glass microsphere with 1 wt. (%) *Psyllium* fiber.

Crosslinking is responsible for giving conformation and structure to the material, assuring in a spherical shape. This linking consists in the interaction between the ions of the mixtures, when they are dropped into the receptor.

For pore forming agents approach, it was performed a reverse crosslinking (figure 2.8), using the alginate as receptor. This crosslinking is weak and to enhance the crosslinking, it was necessary to immerse the spheres into calcium chloride solution.

As can be seen from SEM, the microspheres do not present a perfect spherical shape. This is due to the fact that the mixtures formed have big quantities of pore forming agents added. Although the viscosity of the mixtures were good to the microfluidics method, it seemed not to be adequate to assure the form spherical structures by the crosslinking stage.

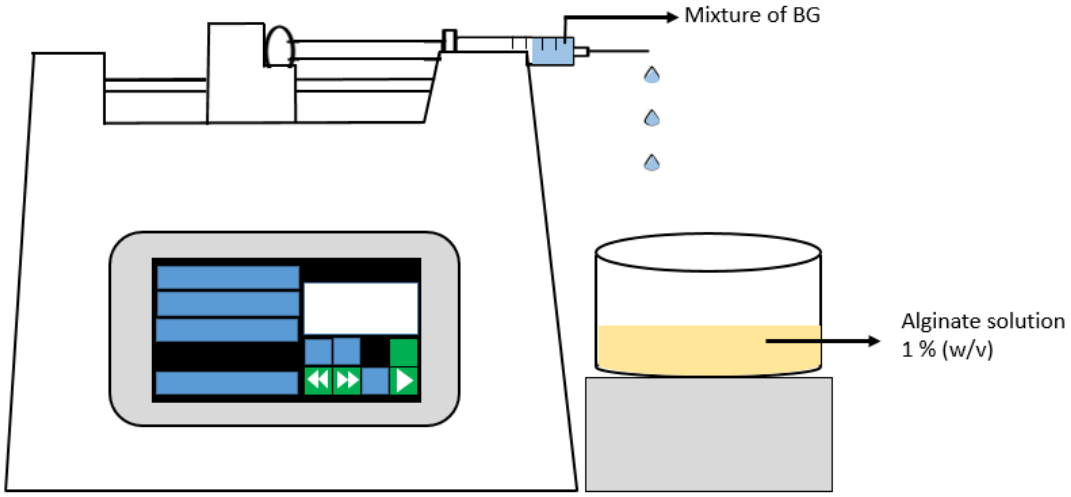

Figure 2.8 - Illustration of spheres production for pore forming agents approach.

2.4.1.2 – X-Ray diffraction (XRD)

BG samples were analyzed by XRD in order to identify the crystalline phases present in its structure. The positions of the peaks (according to 2θ) and their relative intensities allowed the identification of the present phases by comparison with datasheets. X-Ray diffraction patterns for the synthesized and thermally treated spheres in different conditions are presented below. This test was only performed on BG microspheres with 10 wt. (%) of $CaCO_3$, since they were the ones that evidenced better results, as confirmed by SEM images.

XRD of the figure 2.9, corresponding to control BG sample, shows the presence of Larnite ß-Ca_2SiO_4 (PDF- 33-0302).

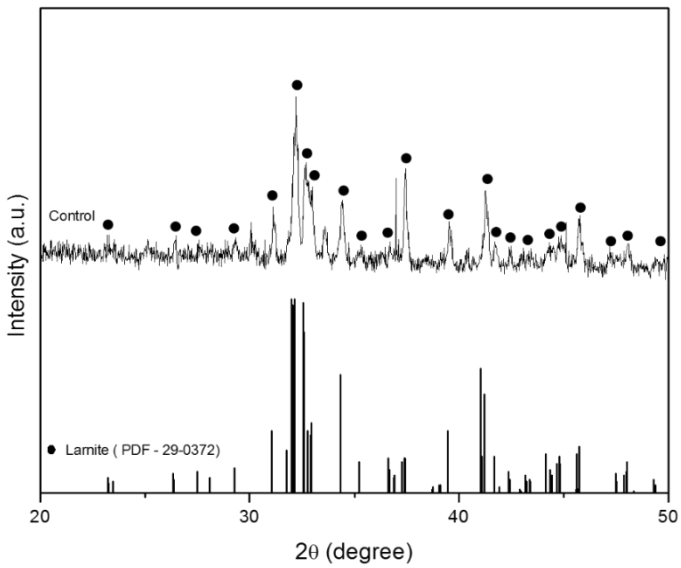

Figure 2.9 – X-Ray diffraction patterns of bioactive glass control sample obtained after thermal treatment.

From figure 2.10, corresponding to bioactive glass sample with 10 wt. (%) $CaCO_3$ sample, it is possible to see the presence of Wollastonite 1A,syn (Ca_2SiO_3), commonly known as Wollastonite (PDF – 29-0372), Calcium phosphate ($Ca_2P_2O_7$) (PDF-17-0499) and Magnesium phosphate ($Mg_3(PO_4)_2$)(PDF 48-1167).

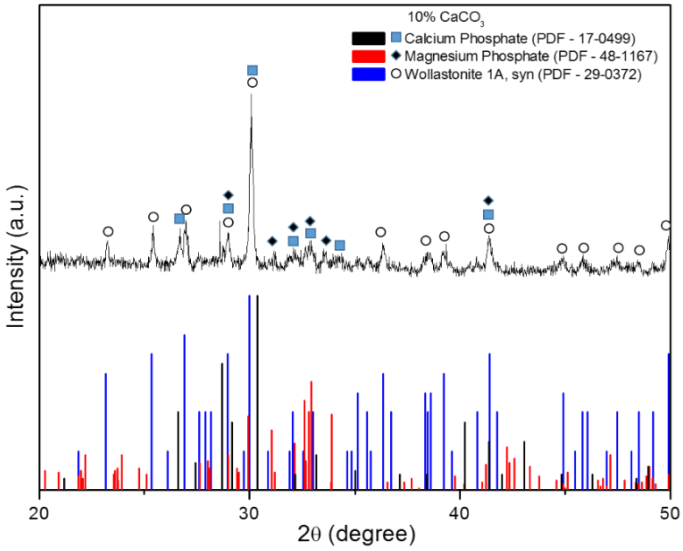

Figure 2.10 – X-Ray diffraction patterns of bioactive glass sample with 10 wt. (%) $CaCO_3$ obtained after thermal treatment.

2.4.2 - Syringe Foaming approach

2.4.2.1 – Scanning electron microscopy (SEM)

It was performed an analysis by SEM of the samples prepared by this approach (figure 2.11). It was possible to verify the presence of spherical macropores of various sizes randomly distributed. The macropores present a spherical geometry, which indicates that the air bubbles presented on the BG formulation, produced by this approach, reproduced the respective morphology. It was also possible to see a heterogeneous surface, with pores that seem to be interconnected.

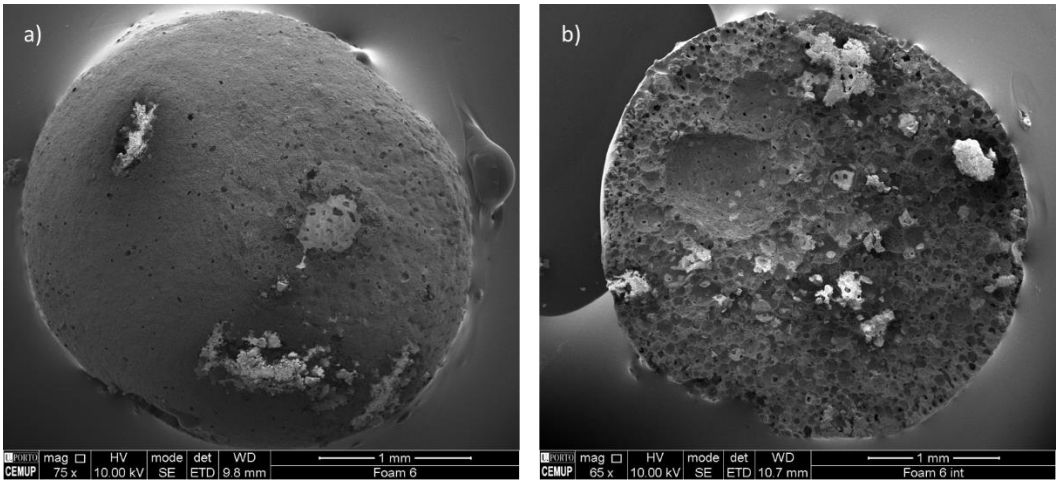

Figure 2.11 - a) external surface of bioactive glass sphere obtained by syringe foaming approach; b) sphere splitted.

The control samples were obtained from the mixture of BG powder with alginate solution 2 % (w/v) (figure 2.12). It was possible to create a homogeneous mixture by adding a solution of alginate to the milled sintered BG powder. Thus, the mixture between BG powder and alginate solution allowed to occur crosslinking, when it falls into the receptor, forming the microspheres with spherical shapes. Besides, the influence of the alginate's addition in BG samples was positive, since it helped to create interconnectivity of the pores. This happens because the alginate is a polymer that burns out at low heating rates.

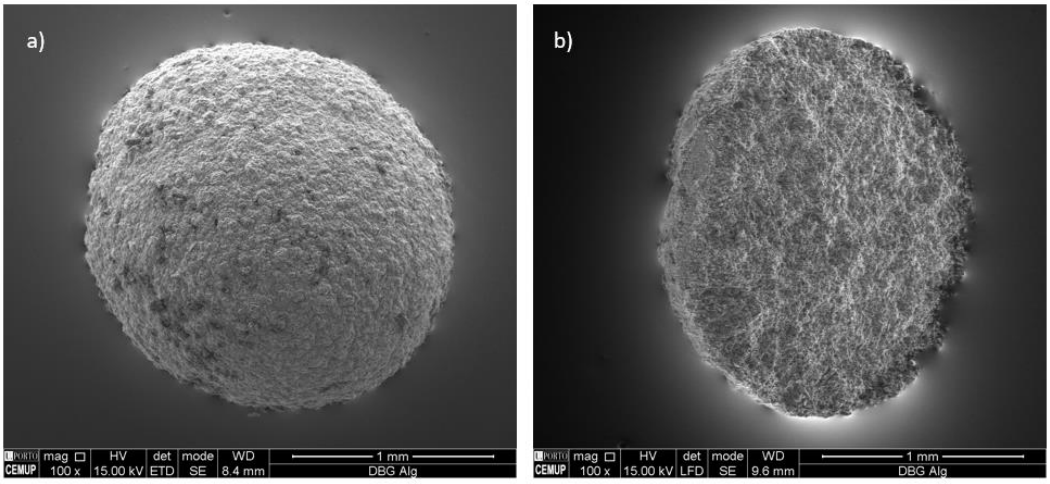

Figure 2.12 - a) external surface of bioactive glass control sample; b) sphere splitted.

2.4.2.2 – X-Ray diffraction (XRD)

BG microspheres samples were analyzed by XRD in order to identify the crystalline phases present in their structure. Figure 2.13 shows the XRD of the raw powder of bioactive glass, presenting Larnite (PDF-29-0372).

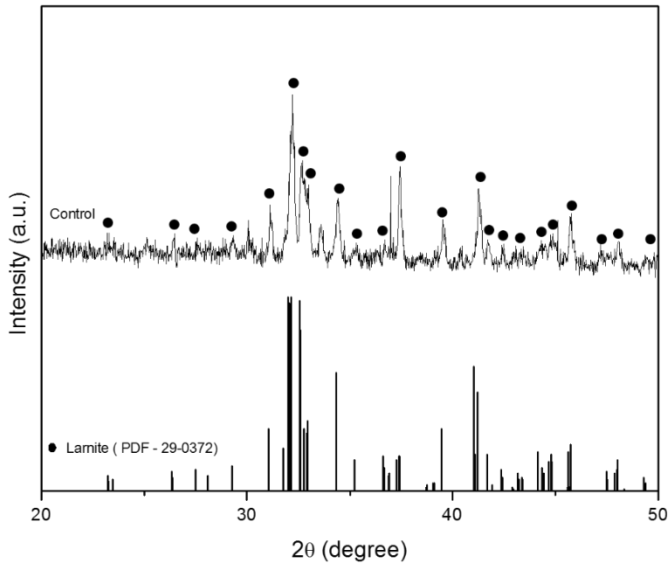

Figure 2.13 - X-Ray diffraction patterns of raw powder of bioactive glass after heat treated at 700 °C.

Figure 2.14 shows the XRD of the BG sample produced by syringe foaming method. This figure indicates the presence of Wollastonite-1A (PDF 00-029-0372), Larnite, syn (PDF 00-009-0351) and Diopside (PDF 00-011-0654). The raw powder of BG was obtained after a thermal treatment at 700 °C, being identified Larnite. With further increase of the temperature, it was possible to see the appearance of new phases. As the BG sample produced by syringe method was resulted of two thermal treatments (calcination at 700 °C and sintering at 1200 °C), it is possible to see a high crystallinity of the phases. In this way, it was possible to obtained Diopside and Wollastonite-1A, that is commonly known as Wollastonite [24].

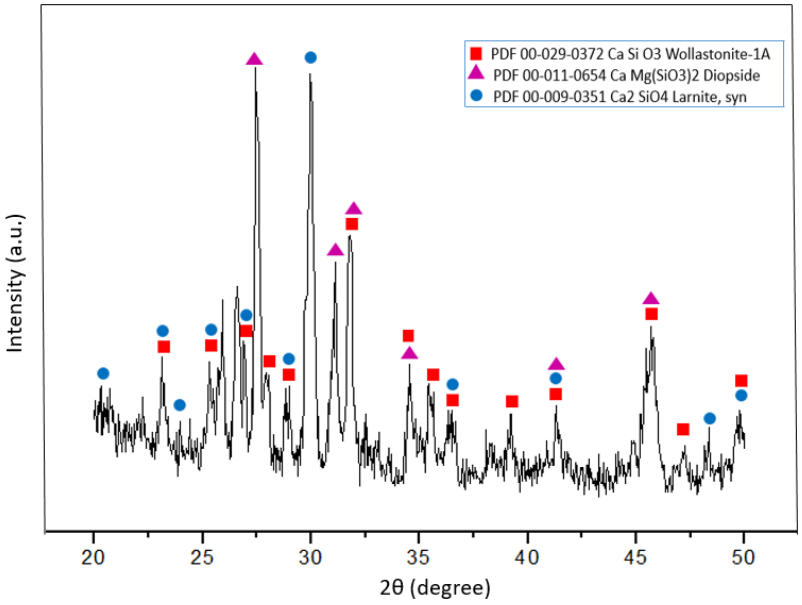

Figure 2.14 - X-Ray diffraction patterns of bioactive glass spheres prepared by syringe foaming method after thermal treatment.

2.4.2.3 – Fourier transform infrared spectroscopy (FTIR)

BG samples were analyzed by FTIR in order to identify functional groups. In this way, it is possible to verify if there are residues remaining after thermal treatment.

FTIR analysis of BG microspheres produced by syringe foaming method did not showed significant variations from control sample. On the figure 2.15, an intense band for 1084 cm^{-1} represents asymmetric Si-O-Si stretching in SiO_4. Peak at 971 cm^{-1} has been ascribed to the stretching vibration of silanol groups in pure silica (vibrational modes of Si–O-stretching) [23]. Additional peaks presented for 887 cm^{-1} was attributed to Si-O-NBO and for 789 cm^{-1} corresponds to symmetric Si-O-Si stretching vibration. Peak at 736 cm^{-1} represents the Si-O stretching. Finally, for peaks at 700 and 468 cm^{-1} were attributed to Si–O-Si stretching. These peaks are assigned to the presence of Larnite. The peaks at 971 cm^{-1} and 736 cm^{-1} could be associated to the presence of Wollastonite and 645 cm^{-1} could be associated to the presence of Diopside. These results are consistent with the results of the XRD, because the FTIR analysis could be ascribed to the presence of Larnite, Diopside and Wollastonite.

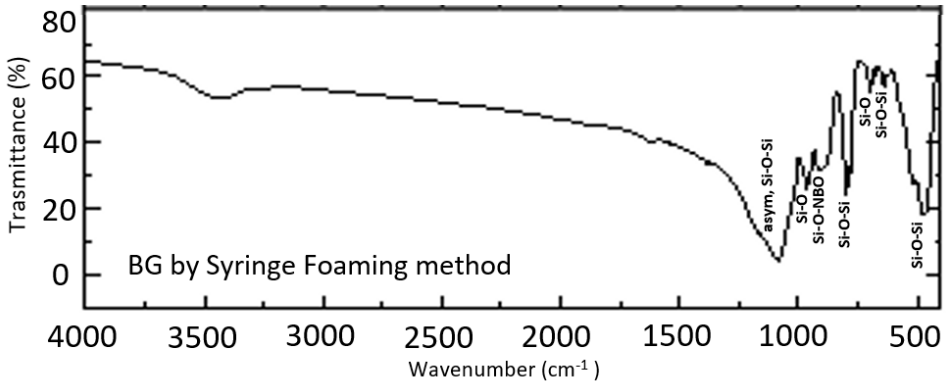

Figure 2.15 - FTIR of bioactive glass spheres produced by syringe foaming method.

2.5 – Conclusions

The main objective of this work was to produce porous ceramic microspheres, in order to fill bone defects. A silica bioactive glass was prepared in the laboratory by the sol-gel method, since this method produces glass with a high degree of purity. Besides this, it tends to produce glasses more bioactive that resorbs more quickly than melt derived glasses [4]. Among the several and different methods of producing microspheres, microfluidics was the chosen technique. Microfluidics manipulates and analyses fluids on microscale structures. This method allows the production of microspheres with a uniform size and it is advantageous, because it is simple to work and it is an effective strategy for a continuous and extensive production of microspheres.

To produce porous spheres, it was analyzed two approaches of creating porosity, namely the introduction of pore forming agents and syringe foaming method.

Overall, porogenic agent approach is an easy and simple process, since it consists in the homogenization of the mixture between the porogenic agents and the gel obtained by sol gel method. The addition of these agents helps to create pores, since they burn out. Besides this, the approach allows the preparation of mixtures that showed to be interesting for microfluidics. In terms of pore forming agents tested, calcium carbonate samples were the ones that exhibited a more spherical shape and presented a microstructure with more open and irregular macropores than PEG and PVA. Relatively to the fibers used, they did not show interesting results, because they evidenced a dense structure, with low macroporosity. Then, this approach did not produced spheres with an adequate spherical shape and an open and interconnected porosity, like it is desired.

Relatively to syringe foaming method, it showed to be an interesting technique of producing porous spheres for bone grafting, because it produces spheres with spherical shape with an open and interconnected structure pores, and pore sizes adequate for bone ingrowth.

In conclusion, syringe foaming method showed to be better than pore forming agents approach.

References

[1] M. van Griensven, “Preclinical testing of drug delivery systems to bone,” *Advanced Drug Delivery Reviews*, vol. 94. pp. 151–164, 2015.

[2] P. V Giannoudis, H. Dinopoulos, and E. Tsiridis, “Bone substitutes: An update,” *Injury*, vol. 36, no. 3, pp. S20–S27, 2005.

[3] R. A. Bhatt and T. D. Rozental, “Bone Graft Substitutes,” *Hand Clinics*, vol. 28, no. 4. pp. 457–468, 2012.

[4] M. M. Pereira, J. R. Jones, and L. L. Hench, “Bioactive glass and hybrid scaffolds prepared by sol–gel method for bone tissue engineering,” *Advances in Applied Ceramics*, vol. 104, no. 1, pp. 35–42, 2005.

[5] J. R. Jones, “Reprint of: Review of bioactive glass: From Hench to hybrids,” *Acta Biomaterialia*, vol. 23, no. S, pp. S53–S82, 2015.

[6] L. Wang *et al.*, “Injectable calcium phosphate with hydrogel fibers encapsulating induced pluripotent, dental pulp and bone marrow stem cells for bone repair,” *Materials Science and Engineering: C*, vol. 69, pp. 1125–1136, 2016.

[7] J. Zhang *et al.*, “A simple and effective approach to prepare injectable macroporous calcium phosphate cement for bone repair: Syringe-foaming using a viscous hydrophilic polymeric solution,” *Acta Biomaterialia*, vol. 31, pp. 326–338, 2016.

[8] M. Vallet-Regí, “Ceramics for medical applications,” *J. Chem. Soc. Dalt. Trans.*, no. 2, pp. 97–108, 2001.

[9] A. Tampieri, G. Celotti, S. Sprio, A. Delcogliano, and S. Franzese, “Porosity-graded hydroxyapatite ceramics to replace natural bone,” *Biomaterials*, vol. 22, no. 11, pp. 1365–1370, 2001.

[10] L. Cerroni, R. Filocamo, M. Fabbri, C. Piconi, S. Caropreso, and S. G. Condò, “Growth of osteoblast-like cells on porous hydroxyapatite ceramics: An in vitro study,” in *Biomolecular Engineering*, 2002, vol. 19, no. 2–6, pp. 119–124.

[11] M. P. Ginebra, M. Espanol, E. B. Montufar, R. A. Perez, and G. Mestres, “New processing approaches in calcium phosphate cements and their applications in regenerative medicine,” *Acta Biomaterialia*, vol. 6, no. 8. pp. 2863–2873, 2010.

[12] R. O’Neill *et al.*, “Critical review: Injectability of calcium phosphate pastes and cements,” *Acta Biomaterialia*, vol. 50. pp. 1–19, 2017.

[13] L. Wu, S. Bai, and Y. Sun, “Development of rigid bidisperse porous microspheres for high-speed protein chromatography,” *Biotechnology Progress*, vol. 19, no. 4, pp. 1300–1306, 2003.

[14] D. M. Wang, G. Hao, Q. H. Shi, and Y. Sun, “Fabrication and characterization of superporous cellulose bead for high-speed protein chromatography,” *Journal of Chromatography A*, vol. 1146, no. 1, pp. 32–40, 2007.

[15] T. Johnson, R. Bahrampourian, A. Patel, and K. Mequanint, “Fabrication of highly porous tissue-engineering scaffolds using selective spherical porogens,” *Biomedical Materials and Engineering*, vol. 20, no. 2, pp. 107–118, 2010.

[16] Y. Liu, W. Ren, L. Zhang, and X. Yao, “New method for making porous SiO2 thin films,” *Thin Solid Films*, vol. 353, no. 1, pp. 124–128, 1999.

[17] B. Patel, V. Gupta, and F. Ahsan, “PEG-PLGA based large porous particles for pulmonary delivery of a highly soluble drug, low molecular weight heparin,” *Journal of Controlled Release*, vol. 162, no. 2, pp. 310–320, 2012.

[18] D. Hutanu, “Recent Applications of Polyethylene Glycols (PEGs) and PEG Derivatives,” *Modern Chemistry and Applications*, vol. 2, no. 2, 2014.

[19] E. Chevalier, D. Chulia, C. Pouget, and M. Viana, “Fabrication of porous substrates: A review of processes using pore forming agents in the biomaterial field,” *Journal of Pharmaceutical Sciences*, vol. 97, no. 3. pp. 1135–1154, 2008.

[20] H. H. Xu and C. G. Simon Jr., “Self-hardening calcium phosphate cement-mesh composite: reinforcement, macropores, and cell response,” *Journal of Biomedical Materials Research Part A*, vol. 69, no. 2, pp. 267–278, 2004.

[21] C. C. Beozzo, M. A. Alves-Rosa, S. H. Pulcinelli, and C. V. Santilli, “Liquid foam templates associated with the sol-gel process for production of zirconia ceramic foams,” *Materials*, vol. 6, no. 5, pp. 1967–1979, 2013.

[22] E. B. Montufar *et al.*, “Foamed surfactant solution as a template for self-setting injectable hydroxyapatite scaffolds for bone regeneration,” *Acta Biomaterialia*, vol. 6, no. 3, pp. 876–885, 2010.

[23] H. S. Costa *et al.*, “Sol-gel derived composite from bioactive glass-polyvinyl alcohol,” *Journal of Materials Science*, 2008, vol. 43, no. 2, pp. 494–502.
[24] J. Ma, C. Z. Chen, D. G. Wang, X. G. Meng, and J. Z. Shi, “Influence of the sintering temperature on the structural feature and bioactivity of sol-gel derived SiO2-CaO-P2O5 bioglass,” *Ceramics International*, vol. 36, no. 6, pp. 1911–1916, 2010.

Chapter 3

3 – Production and characterization of porous hollow spheres for bone regeneration

Abstract

Porosity is an important factor when addressing bone grafts. These grafts should present micro and macroporosity that must be interconnected, since it will help bone ingrowth, when implanted *in vivo*. In this study it is proposed a strategy of creating interconnective pores in bioceramics microspheres (Bioactive glass – BG or Hydroxyapatite - Hap), using a syringe foaming method.

Bioceramics foamed formulations were characterized in rheological terms, demonstrating a good injectability for spheres' production. Finally, the spheres obtained were characterized using different techniques, such as scanning electron microscopy, X-Ray diffraction and Fourier transform infrared spectroscopy. From these analysis, it was possible to conclude that syringe foaming approach is an effective method of producing porosity that is open and interconnective and it was obtained adequate pore sizes for vascularization.

Therefore, a preliminary *in vitro* study was performed to evaluate drug load and release of an antibiotic, under different vacuum conditions. By the analysis of this study, it was possible to conclude that high vacuum is better than low vacuum condition. The obtained release of gentamicin sulfate displayed an interesting profile for combating bone infections.

3.1 – Introduction

Bone defects or injuries are remaining to be a big concern of limiting a significant percentage of the population. This occurrence is due to the fact that the average life expectancy is increasing as well as accidents and diseases.

In this context, there is a need to find alternatives to autografts and allografts. These grafts have been presented increasing limitations, such as reaction to foreign body, infections, risk of contracting diseases, among others [1]. In this way, the development of synthetic bone substitutes that requires minimally invasive surgical techniques, have been distinguished as a great advance on biomedical field. For instance, bioceramics, including bioactive glasses and calcium phosphates, have been the object of research for bone substitutes [2-4].

Bioceramics are widely used in biomedical applications because they have good biocompatibility, being the materials that best support bone integration (interaction between bone and implant)[5]. These properties allow migration, bone cells attachment and proliferation, promoting high levels of osseointegration and osteoconduction [4-7]. Therefore, bioactive glasses are biocompatible, bioactive, osteoconductive and even osteoinductive materials [8]. And hydroxyapatite is a commonly example of calcium phosphates, which has a chemical affinity with the mineral component of the bone [9].

The aim of this work is to develop and compare two different types of porous bioceramics (bioactive glass and hydroxyapatite) hollow spheres for bone defect filling that can also act as a drug delivery system of antibiotics, anti-inflammatory agents, for treatment of various infections, or even growth factors, to accelerate bone regeneration. The use of porous biomaterials has been an area of interest, because it is important to support bone cell and tissue growth [10]. Thus, it is necessary micropores (responsible for allowing nutrients and metabolic wastes to flow) as well as macropores, because they help migration and proliferation of bone cells as well as the angiogenesis. In terms of porosity size, pores between 50 and 150 μm determine osteoid growth and pores sizes that exceed 150 μm is essential for osteoconduction [11,12].

According to methods of enhancing macroporosity of bioceramics, there have been introduced several ones, including the addition of porogenic agents, the use of gas generating compounds, or even, the use of foaming agents [13,14].

In this study, it was performed a syringe-foaming approach, which consists in a mixture between the bioceramic paste (bioactive glass or hydroxyapatite) and a foam solution. For this foam solution is important to consider the selection of the foaming agent, which must be water soluble (to promote the formation of air bubbles), nontoxic and biocompatible, as well as associated with a good foaming ability and foam stability [15]. Surfactants are a commonly foaming agents, because of their ability to stabilize the air-liquid interface in the foam solutions, being the responsible of defining a porous bioceramic structure [16,17].

The purpose of the present work was to evaluate this syringe-foaming approach in terms of creating porosity in ceramic spheres and perform a preliminary release study of an antibiotic from the porous spheres. The need of associating an antibiotic to a bone graft is to avoid

postoperative infections. For the study, it was chosen gentamicin sulfate because of its wide spectrum.

3.2 – Materials and Methods

3.2.1 - Raw materials

The materials used in the present work to produce spheres were bioactive glass (BG) and hydroxyapatite (Hap). A silica bioactive glass with composition 64 % (w/w) SiO_2 – 26 % (w/w) CaO – 5 % (w/w) MgO – 5 % (w/w) P_2O_5 was prepared in the laboratory by the sol-gel method. For this synthesis, tetraethyl orthosilicate (TEOS, Sigma-Aldrich, Ref. 131903), ethanol (ETOH, Sigma-Aldrich, Ref. 16368), calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$, PanReac AppliChem, Ref. 141231.1211), nitric acid (HNO_3, Alfa Aesar, Ref. 44528), triethyl phosphate (TEP, Merck KGaA, Ref. 8.21141.1000) magnesium nitrate hexahydrate ($Mg(NO_3)_2{\cdot}6H_2O$, Sigma-Aldrich, Ref. 63087) and ammonia solution (NH_3 ,Merck KGaA, Ref. 9617485) were used. The hydroxyapatite powder was purchased ($Ca_5(OH)(PO_4)_3$, Sigma-Aldrich, Ref. 21223).

To perform syringe foaming approach, it was necessary Pluronic® F-127 powder (F-127, Sigma-Aldrich, Ref. P2443), sodium hypophosphite monohydrate powder (NaH_2PO_4, Sigma-Aldrich, Ref. S5012), sodium phosphate dibasic heptahydrate powder (Na_2HPO_4, Sigma-Aldrich, Ref. 431478), calcium chloride 2-hydrate ($CaCl_2.2H_2O$, PanReac AppliChem, Ref.131232.1211) and alginic acid sodium salt ($(C_6H_7O_6Na)_n$, PanReac AppliChem, Ref. A3249.0250)

3.2.2 – Bioactive glass production

For the present work, BG was produced by the sol-gel method, at room temperature. Figure 3.1 illustrates the steps of BG synthesis. Between each stage there was an interval of 1 h. TEOS, calcium nitrate, TEP and magnesium nitrate were used as precursors of the oxides wanted, while nitric acid was used as a catalyst. For the final stage, it was added an ammonia solution, which was drop wise added, resulting in a white precipitate. After gel formation, it was dried at room temperature, to remove the solvent from the gel structure. Afterwards, it was heat treated at 700 °C for 2 h (heating rate 0.5 °C/min.) and then milled.

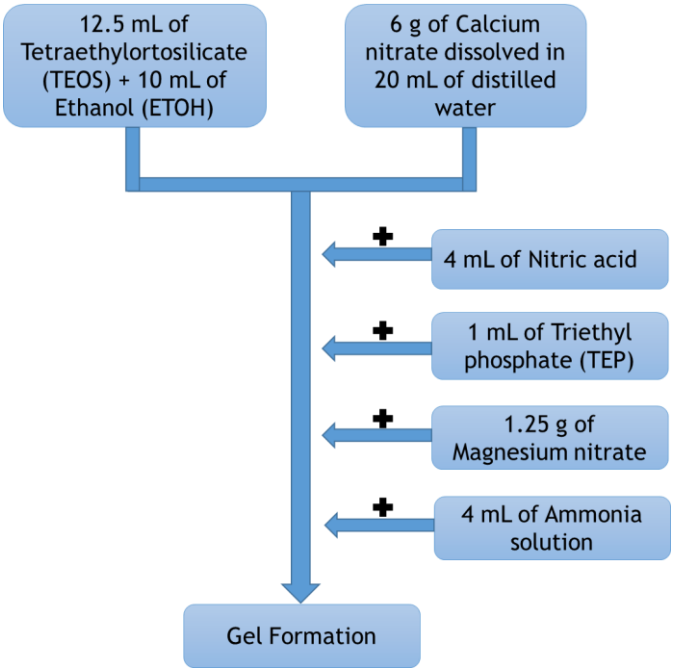

Figure 3.1 – Flow diagram with stages and reagents used on sol-gel procedure.

3.2.3 – Syringe foaming approach

This approach consists in mixing a paste (BG or Hap) with a foam, which is obtained by using a foaming agent. Then, it was produced porous microspheres of BG and Hap by forming an injectable foam using a syringe method.

Figure 3.2 demonstrates the four steps to produce the final formulation. The liquid formulation contains 2.4 mL of Pluronic F-127 solution 10 % (w/v) (non-ionic surfactant used as foaming agent capable of producing a stable and injectable foam) and 0.2 mL of sodium phosphate monobasic solution 30 % (w/v) (NaH_2PO_4). The liquid solution was collected into a 10 mL syringe, which was attached to another syringe by a connector with 2.5 mL of air. The solution was manually injected from one syringe to the other, by pushing the plungers of the syringes, forming a foam due to turbulence generated in the liquid, during 30 s. After obtaining foam, the foam was kept into one of the syringes. Subsequently, it was added to a third syringe a paste of BG or Hap, which is formed by a mixture of powder (0.5 g BG or 1.5 g Hap) with 3 mL of alginate solution 2 % (m/v), optimized in order to occur crosslinking, and 1 mL of sodium phosphate dibasic solution 2.5 % (w/v) (Na_2HPO_4), used as a hard setting accelerator. Finally, the paste of BG or Hap was mixed with the foam formulation during 30 s, in order to obtain a BG or Hap formulation [6]. Control samples were prepared with BG or Hap powder and alginate solution 2 % (w/v).

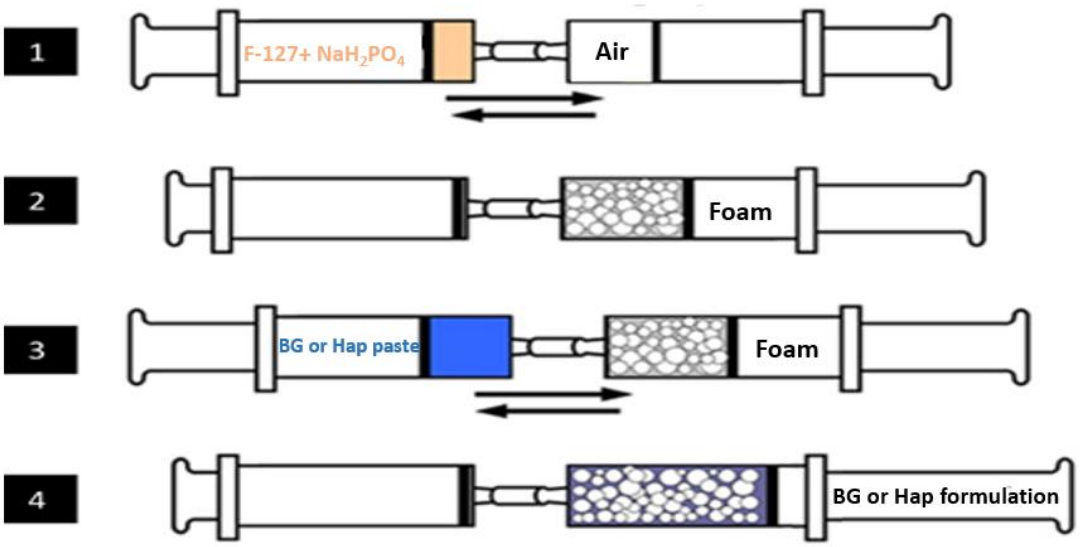

Figure 3.2 - Syringe foaming method, consisting of two syringes connected by a connector. (1) and (2) Foam of the liquid solution; (3) and (4) Obtaining bioactive glass or hydroxyapatite formulation (adapted from [6]).

3.2.4 - Production of Hollow Microspheres

In a previous work, authors have already shown the efficiency of using microfluidics to prepare microspheres. For hollow microspheres production, it is necessary two syringes and two infusion pumps with adjustable and independent flow rates. The infusion pump permits to control the flow rates, being able to produce reproducible spheres. One syringe is filled with the formulation formed (BG or Hap), as outer fluid, and the other with 5.7 g of polystyrene (PS) dissolved in 100 mL of dichloromethane (DCM - CH_2Cl_2), as inner fluid. These two syringes are connected by a coaxial device, where the two immiscible formulations are infused through both the syringes in a way that, when they get out, it forms a single emulsion drop. The formulation (BG or Hap) forms the shell of the sphere and the mixture between PS and DCM forms the core. Consequently, these drops will fall into a receptor (calcium chloride 5 % (w/v)). Crosslinking will be responsible for giving the sphere's conformation. After 12 h immersed in calcium chloride solution, the spheres were washed with ethanol, to reduce the electrostatic forces between the spheres. Then, following production of spheres, they were submitted to a heat treatment (700 °C for 3 h, using a heating rate of 0.5 °C/min. followed by 1200 °C for 1 h with a heating rate of 4 °C/min.).

3.3 - Physical-Chemical Characterization

3.3.1 - Rheological Characterization

In order to better understand which one of the formulations developed are the best to produce microspheres by microfluidic systems, they were characterized in terms of rheology.

Thus, dynamic viscoelastic spectra was measured by a rheometer (Malvern Instruments, Bohlin Germini HR^{nano} Rotational, Rotonetic^{TM} Drive, United Kingdom) with parallel plate's fixture. The diameter of the plates was 40 mm, using a Gap of 1000 µm. The appropriate values of the strain amplitude were determined in order to ensure that all measurements were performed within linear viscoelastic region (LVR), such that elastic modulus (G') and viscous modulus (G'') were independent of strain amplitude. Oscillatory and viscosity measurements were performed. For oscillatory measurements, the modulus G' and G'' of gel derived from BG and Hap were measured in function of angular frequency. For BG a range from 0.1 to 200 Hz was used and for Hap the range was from 0.1 to 100 Hz, at controlled constant temperature 25 °C. For viscosity measurements, the viscosity and shear stress, in function of shear rate, of both formulations (BG and Hap) and thixotropy were determined. Thixotropy was characterized by the recovery time of the viscosity after a steep decrease from a high to a low shear rate.

3.3.2 – Scanning electron microscope (SEM)

The morphology of the samples was observed by a high resolution scanning electron microscope with X-Ray microanalysis, JEOL JSM 6301F/Oxford INCA Energy 350. The samples were mounted onto an aluminum stub using Araldite Glue (Devcon, Wellingborough, UK), in a specimen port and left to dry overnight. After drying, they were coated with a thin layer of gold/palladium (Au/Pd) by sputtering for 120 s and a current of 15 mA, using a SPI Module Sputter Coater.

3.3.3 – Porosity analysis

The total porosity introduced in the porous microspheres samples ($Ø_{BG}$=1.85±0.04 mm and $Ø_{Hap}$=2.11±0.08 mm) was evaluated.

Archimedes' method is a simple and rapid method of getting information about the closed porosity of the tested samples. In order to evaluate the open and total porosity of the different samples, it was necessary to assess the following densities.

Archimedes' density formula:

$$\rho(\text{Archimedes}) = \frac{mass\ of\ sphere}{(mass\ of\ sphere - mass\ of\ sphere\ immersed) \times \rho(\text{water})} \qquad \text{(eq.1)}$$

Calculation of closed porosity:

$$P(\text{closed Porosity})\ (\%) = \left(\frac{1}{\rho\ \text{Archimedes}} - \frac{1}{\rho\ \text{material}}\right) \times 100 \qquad \text{(eq. 2)}$$

, where ρ material is the theoretical density of material used. In the case of BG, the density is 3 g/cm^3 and for Hap density was considered 3.1 g/cm^3.

It is important to quantify the open pores. For open porosity, it was necessary to do some calculations, being able to reach the following equation:

$$\mathrm{P(open\ Porosity)\ (\%)} = (1 - \frac{\rho\ \mathrm{geometric}}{\rho\ \mathrm{Archimedes}}) \times 100 \qquad \text{(eq. 3)}$$

, where ρ geometric is giving by:

$$\rho\ \mathrm{geometric} = \frac{mass\ of\ a\ sphere}{volume\ of\ a\ sphere} \qquad \text{(eq. 4)}$$

In this method, the total porosity of the samples are calculated according to:

$$P(\mathrm{Total\ Porosity})(\%) = \left(1 - \frac{\rho\ \mathrm{geometric}}{\rho\ \mathrm{material}}\right) \times 100 \qquad \text{(eq. 5)}$$

3.3.4 - X-Ray diffraction (XRD)

The samples were characterized by X-ray diffraction (XRD), on MXP3V (Mac Science Ltd, Yokohama, Japan), using CuKα radiation at 40 kV and 40 mA, in order to identify the crystalline phases. XRD was collected within a 2θ range from 20 to 50° with a scanning speed of 0.04° and counting time of 4 s/step. For this analysis the samples were milled and dried at 50 °C.

3.3.5 – Fourier transform spectroscopy (FTIR)

For the identification of the functional groups present in the samples, a spectra was obtained using Frontier spectrophotometer (Perkin Elmer). In order to do that, the samples were milled and dried at 50 °C. Then, they were mixed with potassium bromide (KBr) (ratio sample/KBr 1: 200), being placed in a press, which was subjected to a unidirectional pressure for 2 minutes to form the pellet. Each pellet was placed on the equipment and the obtained spectra were done at a spectral resolution of 4 cm^{-1}, on the wavelength range of 400-4000 $cm^{-1}$and 64 scans were accumulated per sample.

3.4 – Preliminary Drugs studies

3.4.1 - Drug Loading

In order to evaluate the incorporation capacity of drugs on hollow microspheres, it was performed encapsulation studies, only for BG sample, varying vacuum condition. It was chosen for a first drug study, an antibiotic. Hollow microspheres of BG were loaded with gentamicin sulfate solution by immersing ± 30 mg into the solution, under low conditions with 100 rpm and high vacuum condition, for 24 h. These vacuum conditions help to create a pressure difference, which forces the solution to flow. Then, the spheres were washed with distilled water and frozen and, posteriorly, lyophilized, to ensure the removal of water. After being lyophilized, the spheres were weighted, in order to measure the amount of loaded gentamicin.

$$DL\ (\%) = \frac{\text{Weight of the microspheres with gentamicin} - \text{Weight of the microspheres}}{\text{Weight of the microspheres}} \quad \text{(eq.6)}$$

3.4.2 - Drug Release

The release of gentamicin sulfate from BG microspheres was performed in phosphate buffer saline (PBS, pH=7.4) at 37 °C, simulating body temperature. The microspheres were completely immersed in 20 mL of PBS solution with orbital shaking at 100 rpm, 37 °C. The release medium was collected at predetermined time intervals and then replaced by a fresh PBS solution (2 mL) each time. The collected release medium was performed by the absorbance method using a UV-1800 spectrophotometer (Shimadzu, Japan) at the wavelength of 252 nm.

3.5 – Statistical analysis

The results of closed, open and total porosity and drug load were presented as mean ± standard deviation, for n = 6. Statistical analysis was performed using one-way ANOVA followed by Tukey multiple comparison test, using the software *GraphPad Prism 7*. Differences were considered significant at $p < 0.05$.

3.6 – Results and Discussions

3.6.1 – Rheological Characterization

Several formulations of BG and Hap were developed and tested. Attending to the levels of crosslinking and injectability behavior, it was studied in more detail the ones presented in the tables 3.1 and 3.2.

Table 3.1 – Formulations tested of bioactive glass samples.

Formulations	Powder of BG (g)	Solution of Na_2HPO_4 2.5 % (w/v) (mL)	Solution of Alginate 2 % (w/v) (mL)	Solution of F-127 10 % (w/v) (mL)	Solution of NaH_2PO_4 30 % (w/v) (mL)
1	1.5	1	3	-	-
2	1.0	1	3	-	-
3	0.5	1	3	-	-
4	0.5	1	3	2.4	0.2

Table 3.2 – Formulations tested of hydroxyapatite samples.

Formulations	Powder of Hap (g)	Solution of Na_2HPO_4 2.5 % (w/v) (mL)	Solution of Alginate 2 % (w/v) (mL)	Solution of F-127 10 % (w/v) (mL)	Solution of NaH_2PO_4 30 % (w/v) (mL)
1	1.5	1	2	-	-
2	1.5	1	3	-	-
3	1.5	1	3	2.4	0.2

Figure 3.3 a) presents the oscillatory measurements of BG formulations and it shows a significant difference of the elastic (G') and viscous (G'') modulus presented for different addition of powder.

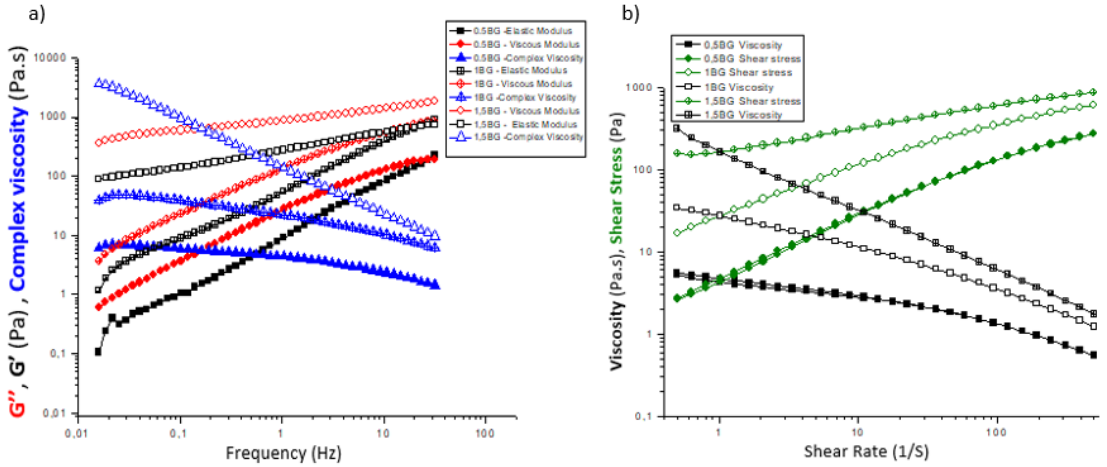

Figure 3.3 - a) Angular frequency dependence of elastic (G') and viscous (G'') modulus and complex viscosity for different quantities of bioactive glass powder added; b)Shear rate dependence of viscosity and shear stress of the respective formulations.

Thus, for formulations with big addition of powder, it is expected an increase of both modulus (G' and G''). For low values of angular frequency, there is a predominance of the viscous character (G'' > G'), regardless the amount of powder added. This allows to assume that the formulation exhibits an initial liquid-viscous behavior. However, by increasing the angular frequency, G' grows faster than G'', crossing at a cross-over frequency (ωc), reaching the point of gelation. Thus, for higher frequencies than ωc, G' prevails at G'', whereby the system exhibits a predominantly elastic character. Relatively to viscosity values, this is concordant with the quantity of added powder. So, viscosity values are superior for 1.5 g of BG powder added (formulation 1). The addition of powder increases the viscosity of BG formulation, as it shows in figure 3.3b). So, formulation 3, with 0.5 g of BG powder, is ideal for a microfluidic system. Besides this, the ratio between this quantity of powder and the quantity of alginate (3 mL) was found to be good to form spheres.

Thixotropy is a time-dependent property that indicates the capacity of the paste to recovery its structure, when shear rate decreases. Thixotropy results (table 3.3) shows that

high quantities of powder represent high thixotropy values, which means that for these thixotropy values, the structure recovery takes more time. Thus, the ideal quantity of powder for syringe foaming method is the one that has the lowest amount (0.5 g of BG), since it presents the lowest viscosity. For low values of viscosity, it is easier to deform, when a shear rate is applied.

In order to prove the surfactant's effect, it is possible to verify, analyzing figure 3.4a), that the addition of surfactant will decrease the formulation's complex viscosity, when it is added to 0.5 g of BG powder formulation. These results are supported with the representation of both modulus and with shear stress and viscosity behaviors. Since BG formulation with surfactant (formulation 4) is more fluid, the recovery structure is easier and faster than for formulation 3, being this last one more thixotropic (table 3.3).

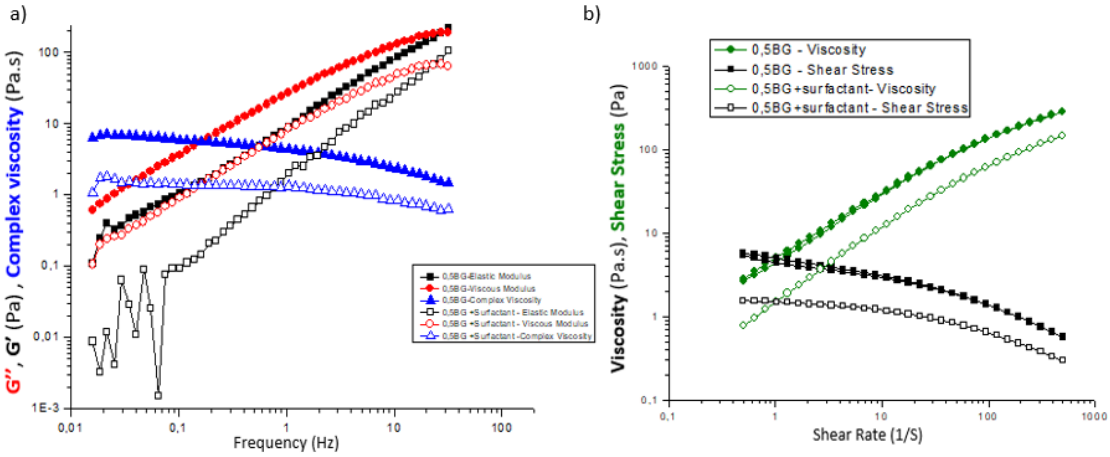

Figure 3.4 - a) Angular frequency dependence of elastic (G') and viscous (G'') modulus and complex viscosity for formulation 3 and formulation 4 of bioactive glass; b)Shear rate dependence of viscosity and shear stress of the respective formulations.

Table 3.3 – Thixotropic values of the different bioactive glass formulations analyzed.

Formulations of BG	Thixotropic values
1	300.8
2	39.7
3	6.87
4	1.99

Hap formulations were analyzed, in terms of alginate and surfactant's effect on the microfluidic system used to produce spheres (table 3.2). For Hap formulations, two quantities added (2 and 3 mL) of alginate solution 2 % (w/v) were studied. The results were the expected (figure 3.5) as the high addition of alginate turned the formulation more fluid, which represented a fast recovery-time of the structure. So, the addition of 3 mL of alginate solution 2 % (w/v) is better for microfluidic system. Using the formulation with 3 mL of alginate solution 2 % (w/v) and adding surfactant, the formulations will be more fluid, which is confirmed in figure 3.6 and by thixotropic values (table 3.4).

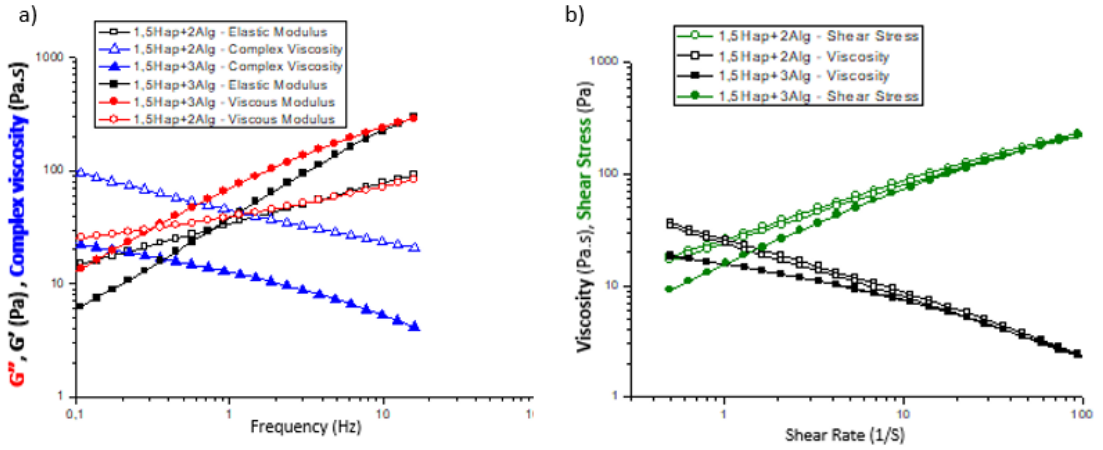

Figure 3.5 - Angular frequency dependence of elastic (G') and viscous (G'') modulus and complex viscosity for formulation 1 and formulation 2 of hydroxyapatite; b)Shear rate dependence of viscosity and shear stress of the respective formulations.

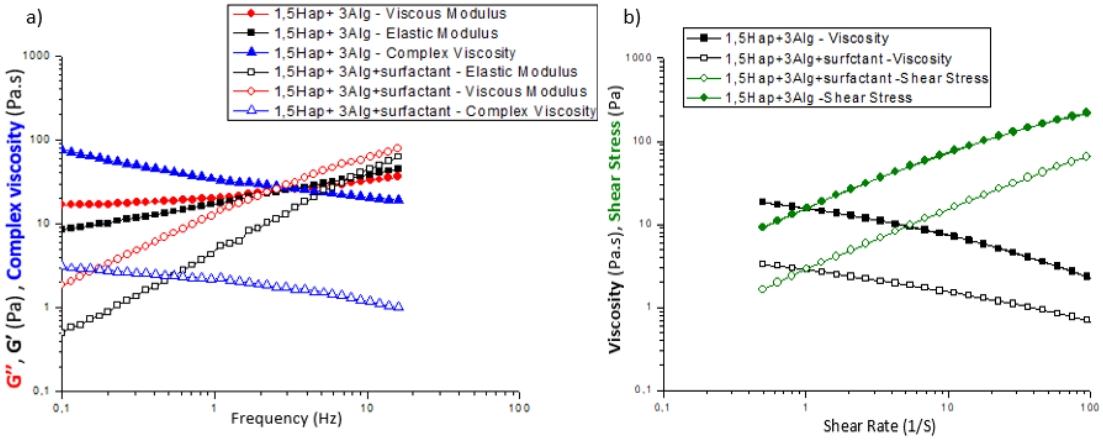

Figure 3.6 - Angular frequency dependence of elastic (G') and viscous (G'') modulus and complex viscosity for formulation 2 and formulation 3 of hydroxyapatite; b) Shear rate dependence of viscosity and shear stress of the respective formulations.

Table 3.4 - Thixotropic values of the different hydroxyapatite formulations analyzed.

Formulations of Hap	Thixotropic values
1	247.3
2	169.5
3	7.12

Since this work uses an injectable approach of producing spheres, it was possible to conclude the best quantities of powder and alginate for the two different formulations (BG and Hap), by rheological analysis (table 3.5). In this way, the formulations vary on the quantity of powder added of the material in question. To prepare the formulation of Hap, it was used 1.5 g of Hap powder, because it was the quantity necessary to occur crosslinking. In the case of BG, it was used 0.5 g of powder, because is the most ideal for a microfluidic system. The solution of alginate 2 % (w/v) helps to create interconnectivity of the pores.

Table 3.5 - Final quantities for bioactive glass and hydroxyapatite formulations.

Powder		Solution of Na_2HPO_4 2.5 % (w/v) (mL)	Solution of Alginate 2 % (w/v) (mL)	Solution of F-127 10 % (w/v) (mL)	Solution of NaH_2PO_4 30 % (w/v) (mL)	Air (mL)
BG (g)	Hap (g)					
0.5	1.5	1	3	2.4	0.2	2.5

The air is an important element since it helps to create formulations with much more bubbles than the formulations with only surfactant.

3.6.2 – Scanning electron microscope (SEM)

The microstructure of BG and Hap hollow microspheres produced by syringe method with the optimized formulation were observed by SEM. For the hollow spheres of the two biomaterials (figures 3.7 and 3.8), it was verified the presence of spherical macropores of various sizes randomly distributed. Both materials exhibit a highly porosity, with pores that seem to be interconnected. Works have been demonstrated that pore interconnectivity seems to play the most important role in bone grafts, when compared with total porosity [20].

The control samples consisted in a mixture of powder (BG or Hap) with alginate solution 2 % (w/v). It was possible to create a homogeneous and viscous mixture by adding a solution of alginate to the BG or Hap powder that allowed to occur crosslinking, when the mixture falls into the receptor. In this way, it was possible to form microspheres with perfect spherical shapes, since crosslinking is responsible for giving conformation and structure to the material. Besides this, the influence of the alginate's addition in BG or Hap samples was positive, since it helped to create interconnectivity of the pores. This happens because the alginate is a polymer that burns out at low heating rates.

For BG and Hap spheres structure, it was identified, by Image J software, pores with sizes between 30 μm and 90 μm. Therefore, both materials are in accordance with the appropriated pore size requirements for osteoid growth [11,12,21].

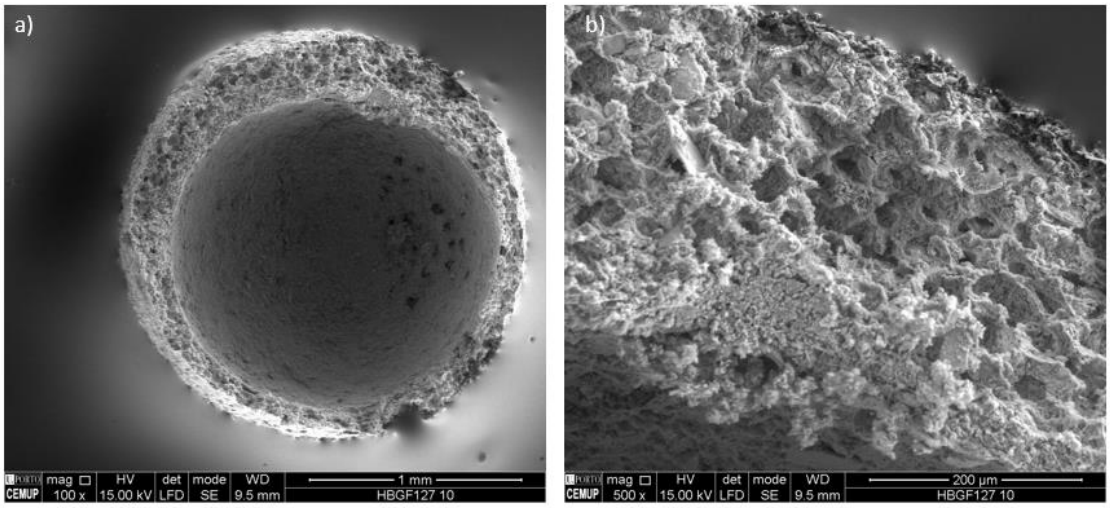

Figure 3.7 - Cut of a bioactive glass hollow spheres obtained by syringe foaming approach.

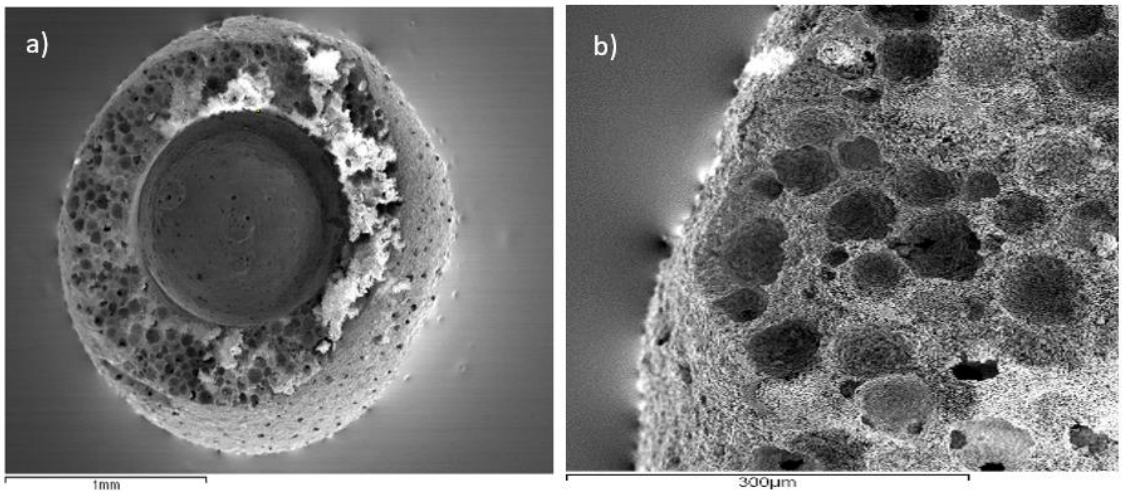

Figure 3.8 - Cut of a hydroxyapatite hollow spheres obtained by syringe foaming approach.

In order to evaluate more accurately the pore size and size distribution, quantitative analysis is being carried out by mercury intrusion porosimetry (MIP).

3.6.3 – Porosity analysis

The porosity percentage of microspheres for both materials was evaluated. For this, it was made some calculations, where the theoretical density (ρ) of the Hap used was considered as 3.1 g/cm^3. Although there has not been done a quantitative phase analysis, since $\rho_{Hap} = 3.16$ g/cm^3 and $\rho_{\beta-\mathrm{TCP}} = 3.07$ g/cm^3, an estimated value 3.1 g/cm^3 was considered.

Figure 3.7 shows the results about closed, open and total porosity. Analyzing the total porosity, both materials, BG and Hap porous microspheres, evidenced significantly differences when compared to the respective control spheres, which is constituted only by the respective powder and alginate solution. Therefore, porosity of BG spheres produced by syringe method

(83.7±1.1) is significantly higher than the control sample (65.8±1.2). And the porosity of Hap samples produced by syringe foaming approach (76.1±1.6) is significantly higher than the control sample (52.9±1.3).

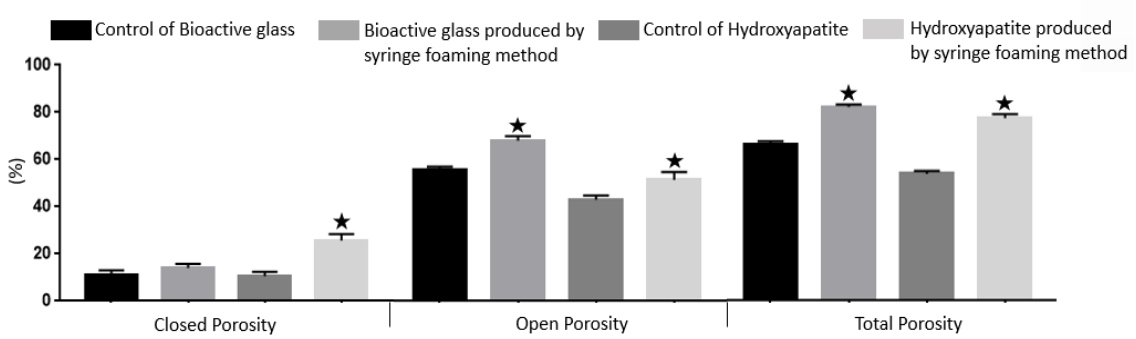

Figure 3.9 - Percentage of closed, open and total porosity of the bioactive glass and hydroxyapatite spheres produced by syringe foaming method.

Figure 3.7 demonstrates also the results relatively to the open porosity. Relatively to BG samples prepared by syringe method (69.6±3.2) a significantly higher porosity than the control samples (54.8±1.5) was obtained. The Hap spheres obtained by syringe approach show significantly higher porosity (49.5±3.3) than the control sample (42.2±1.6).

Finally, according to closed porosity results of BG porous spheres produced by syringe foaming method (14.1±1.9), they are not significantly different from the control sample (11.0±2.1). Relatively to closed porosity of Hap microspheres produced by syringe foaming method (26.6±2.8), they are significantly different from the control sample (constituted only by Hap powder and alginate) (10.7±1.9). Hap porous spheres produced by syringe foaming approach are statistically higher than the control sample.

Generally, for porous grafts with open porosity of >50% is considered to be the minimum requirement to permit bone ingrowth [22].

Thus, both materials prepared by the syringe method showed high values of total porosity, being the most of them open. These results confirm that, independently of the material used, the addition of surfactant and air caused a statistically increase on total porosity values, when compared to the control. These results support the SEM analysis, since it showed the production of the desired porosity.

3.6.4 – X-Ray diffraction (XRD)

BG and Hap spheres were analyzed by XRD in order to identify the crystalline phases present in their structure. The positions of the peaks (according to 2θ) and their relative intensities allowed the identification of the present phases by comparison with datasheets. X-Ray diffraction patterns for the synthesized and thermally treated spheres are presented below. Figure 3.10 shows the XRD of the raw powder of bioactive glass, presenting Larnite (PDF-29-0372).

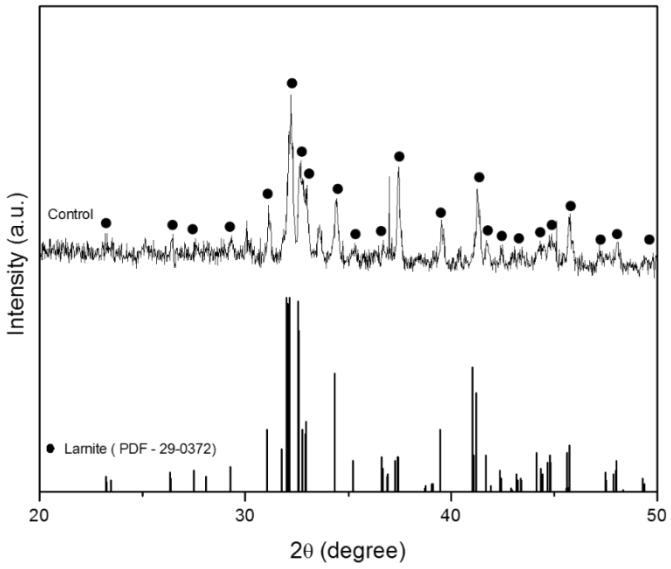

Figure 3.10 - X-Ray diffraction patterns of raw powder of bioactive glass after thermal treatment.

Figure 3.11 demonstrates XRD of BG sample produced by syringe foaming method with the optimized formulation. This figure indicates the presence of Larnite, syn (PDF 00-009-0351), Wollastonite-1A (PDF 00-029-0372) and Diopside (PDF 00-011-0654). The raw powder of BG was obtained after a thermal treatment at 700 °C, identifying Larnite. With further increase of the temperature, it was possible to see the appearance of new phases. As the BG sample produced by syringe method was resulted of two thermal treatments (calcination at 700 °C and sintering at 1200 °C), it is possible to see a high crystallinity of the phases. In this way, it was possible to obtained Diopside and Wollastonite-1A that is commonly known as Wollastonite [23].

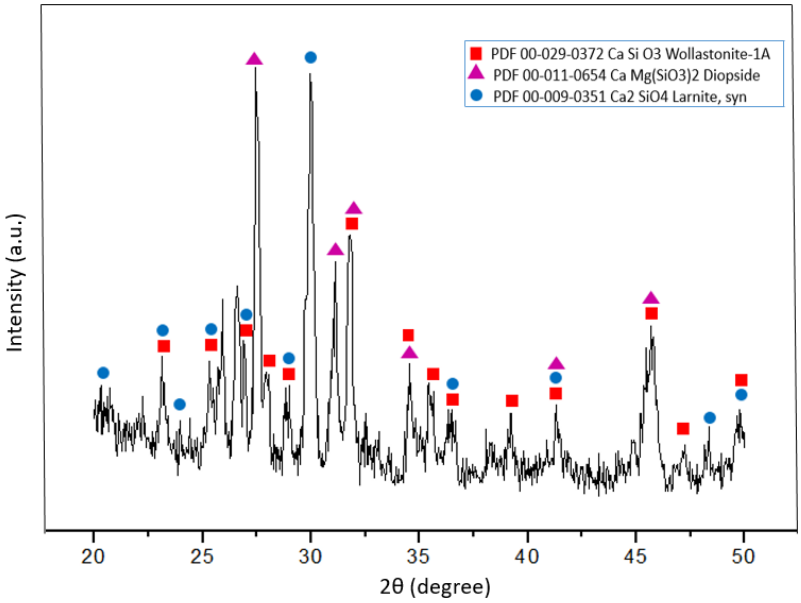

Figure 3.11 - X-Ray diffraction patterns of bioactive glass spheres by syringe foaming method after thermal treatment.

Figure 3.12 represents XRD of Hap raw powder used and figure 3.13 demonstrates the XRD of Hap spheres produced by syringe foaming method, performed with the optimized formulation. It is possible to see on figure 3.12 the presence of Whitlockite, syn (PDF 00-009-0169) and Carbonated Hydroxyapatite (PDF 01-072-7532).

Analyzing the different phases presented, it is possible to conclude that the Hap spheres produced by syringe foaming approach present differences from the phases of the respective raw material. Relatively to Hap sample produced by syringe method (figure 3.13), it was possible to see carbonate hydroxyapatite, which is an hydroxyapatite with CO_3^{2-} groups that could substitute the OH^- groups or the PO_4^{3-} groups and depending on the substitution they could be classified as apatite A or B, respectively. In this work, the thermal treatment up to 1200 °C led to the appearance of a small amount of free calcium oxide, whose content grows as the temperature increases. Carbonate hydroxyapatite can improve the bioactivity of the bone substitutes, since the carbonates are constituents of bone structure [24,25].

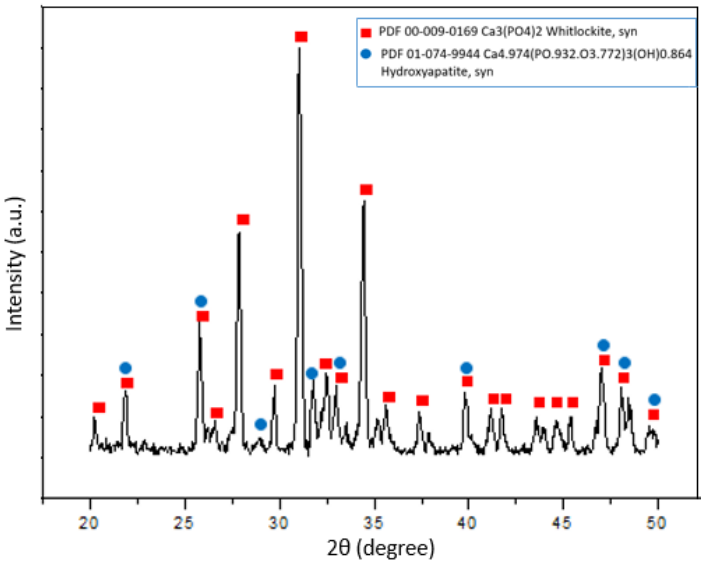

Figure 3.12 - X-Ray diffraction patterns of raw powder of hydroxyapatite.

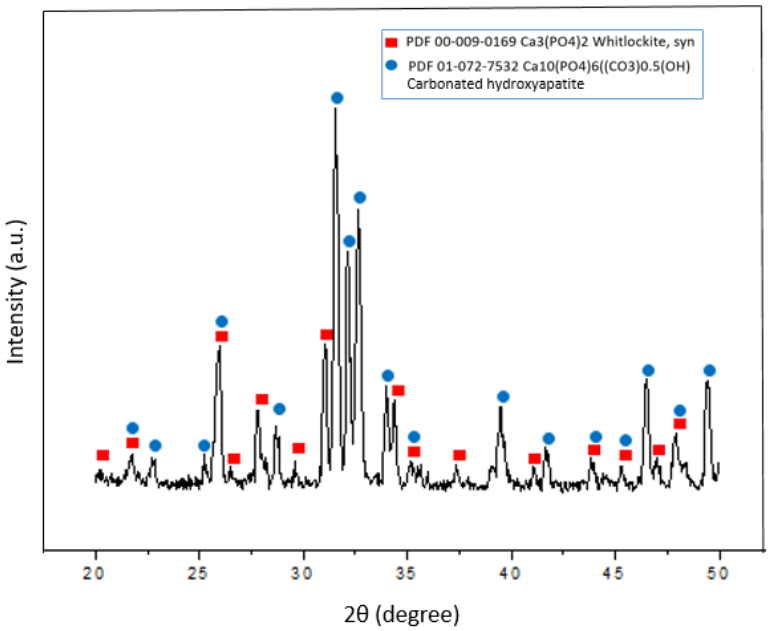

Figure 3.13 - X-Ray diffraction patterns of hydroxyapatite spheres by syringe foaming method after thermal treatment.

3.6.5 – Fourier transform infrared spectroscopy (FTIR)

BG and Hap microspheres samples were analyzed by FTIR in order to identify functional groups, enabling to verify if there are residues after thermal treatment.

FTIR spectra of BG spheres produced by syringe foaming method (figure 3.14 a)) presents an intense band for 1084 cm^{-1}, which represents asymmetric Si-O-Si stretching in SiO_4. Peak at 971 cm^{-1} has been ascribed to the stretching vibration of silanol groups in pure silica (vibrational modes of Si-O-stretching) [26]. Additional peaks presented 887 cm^{-1} was attributed

to Si-O-NBO and 789 cm^{-1} corresponds to symmetric Si–O–Si stretching vibration. Peak at 736 cm^{-1} represents the Si-O stretching. Finally, peaks at 700 and 468 cm^{-1} were attributed to Si–O–Si stretching. These peaks are assigned to the presence of Larnite. The peaks at 971 cm^{-1} and 736 cm^{-1} could be associated to the presence of Wollastonite and 645 cm^{-1} could be associated to the presence of Diopside. These results are consistent with the results of the XRD analysis, since bands identified by FTIR analysis could be ascribed to the presence of Larnite, Diopside and Wollastonite.

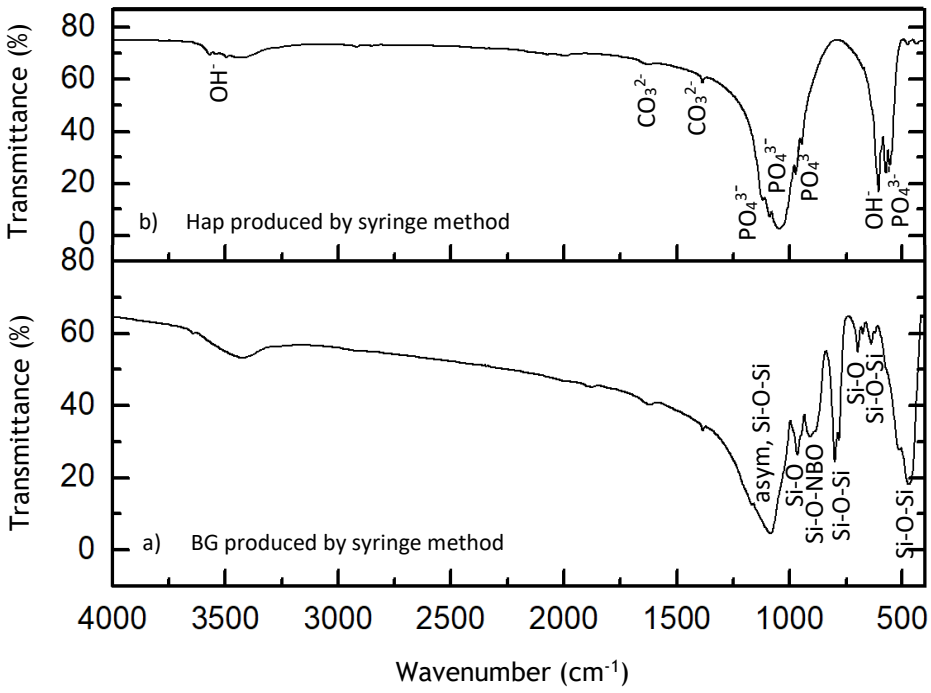

Figure 3.14 - FTIR spectra of a) bioactive glass spheres produced by syringe foaming method, b) hydroxyapatite spheres produced by syringe foaming method.

The FTIR spectrum on figure 3.14 b) shows absorption peaks characteristic of Hap. For Hap sample produced by syringe foaming method, the peaks at 3573 cm^{-1} and 641 cm^{-1} can be assigned to stretching vibration of O-H bonds (hydroxyl stretch) [23]. The CO_3^{2-} bands that are assigned on the rage of 1650 to 1300 cm^{-1}, they refer to surface carbonate ions, suggesting that the Hap sample is carbonated. So, at 1631 cm^{-1} and 1387 cm^{-1} are assigned to carbonate CO stretching vibrations, referring to the distribution of the CO_3^{2-} [27].

There are an intense PO_4^{3-} peak at 1048 cm^{-1}. For PO_4^{3-}, they are also presented at 1086 cm^{-1}, 959 cm^{-1}, 600 cm^{-1} and 570 cm^{-1} [27]. A tenuous peak identified at 479 cm^{-1} could be attributed to Ca-O binding.

Therefore, visualizing FTIR spectra of Hap microspheres produced by syringe foaming method, it is possible to conclude that the obtained results are in agreement with XRD analysis. The bands identified by FTIR results could be ascribed to the presence of Whitlockite and carbonated Hydroxyapatite.

3.7 – Preliminary Drug Studies

Nowadays, it has been reported that many bacteria are the responsible for causing postoperative bone infections. There are different bacteria responsible, however the most relevant pathogens in orthopedic infections are coagulase-negative staphylococci, *Staphylococcus aureus* and *Staphylococcus epidermidis* [28,29]. So, the success of surgical interventions on the implantation of a bone substitute is the prevention from bacterial infections. For this reason, antibiotics are often provided.

In this way, it is important the use of a drug delivery system that are able to prevent bacterial infection on the site of injury [30].

For preliminary drug studies, it was used gentamicin sulfate, as an antibiotic for the treatment and prevention of bone infections. This antibiotic is a broad-spectrum antimicrobial, having a good efficacy in terms of controlled release of drug [31]. According to literature, it was chosen the BG hollow microspheres for these studies, because they encapsulate more than Hap hollow microspheres. BG spheres present silanol (Si–OH) groups [32], which can improve the interaction with drugs, achieving a high control on drug loading and release processes [33,34].

3.7.1 – Drug Loading

In this way, it was analysed the best vacuum condition to impregnate the hollow BG spheres. For the drug loading study, the spheres were weighed before and after they were immersion into a gentamicin solution, under low conditions with 100 rpm and high vacuum conditions. Figure 3.15 shows the gentamicin sulfate loading achieved, which indicates that for high vacuum condition the percent loading is higher than for low vacuum condition, as expected. This happens due to high pressure difference created, allowing the drug to enter the spheres. For high vacuum, it was loaded 28 mg of gentamicin and for low vacuum was 12 mg.

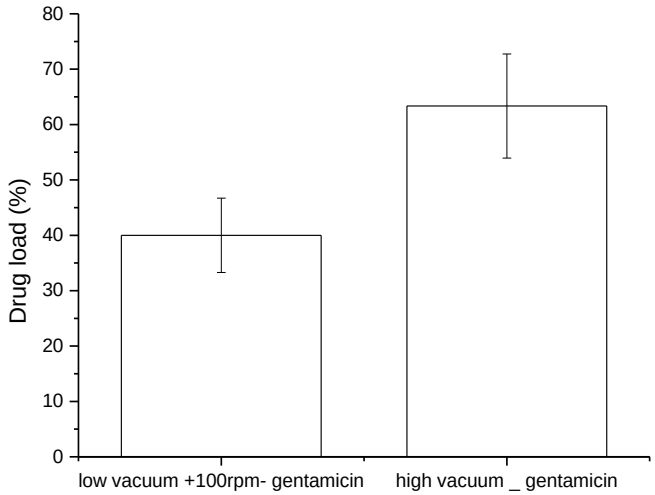

Figure 3.15 – Drug loading results of gentamicin sulfate in bioactive glass microspheres.

3.7.2 – Drug Release

The concentrations of gentamicin sulfate released from hollow microspheres were analyzed using spectrophotometry. Figure 3.16 shows the *in vitro* release of gentamicin from BG hollow microspheres carried out at 37 °C in PBS pH=7.4.

Both vacuum conditions exhibited a similar release behavior, having a high release rate in the first 24 h, followed by a slower release. For high vacuum, the loaded drug was released in 170 h (7 days) from hollow microspheres and for low vacuum, the drug release lasted for approximately 75 h (3 days).

For high vacuum condition, the microspheres presented a rapid release of encapsulated gentamicin in the initial 24 h. This initial behavior is due to the drug that is adsorbed on the pores of the sphere's surface. After the 24 h passed, the release of gentamicin presented an attenuated release, because it was located in the core, which turns more difficult to be release due to the shell.

It is possible to see on figure 3.16 that the drug release of low vacuum was faster than for high vacuum condition.

Then, it is possible to verify that high vacuum conditions prolong the effective drug release for more 4 days than for low vacuum, representing the desired profile in order to combat infections [35].

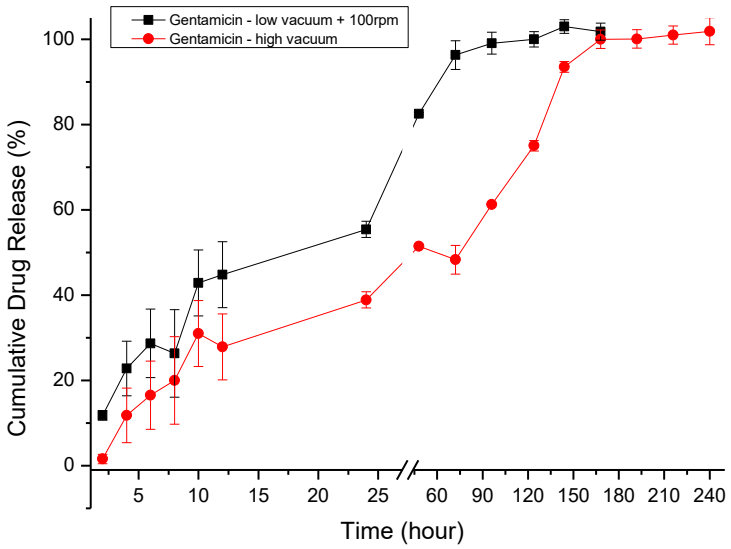

Figure 3.16 – Cumulative Drug Release (%) profile of gentamicin sulfate in bioactive glass hollow microspheres.

For a local drug delivery system having the potential of being effective on the elimination of infections, it should be provided a sustained antibiotic concentration above the minimum inhibitory concentration (MIC) [36]. Figure 3.17 shows the concentration of gentamicin sulfate that was released from the BG hollow spheres. In this way, the final concentration in the release for high vacuum was 900 mg/L. This value remained above the MIC of gentamicin sulfate, since the therapeutic concentration is 8 mg/L for more resistant

microorganisms [37]. On the first 3 h of action, the concentration release of gentamicin was sufficient to kill resistant bacteria (figure 3.17).

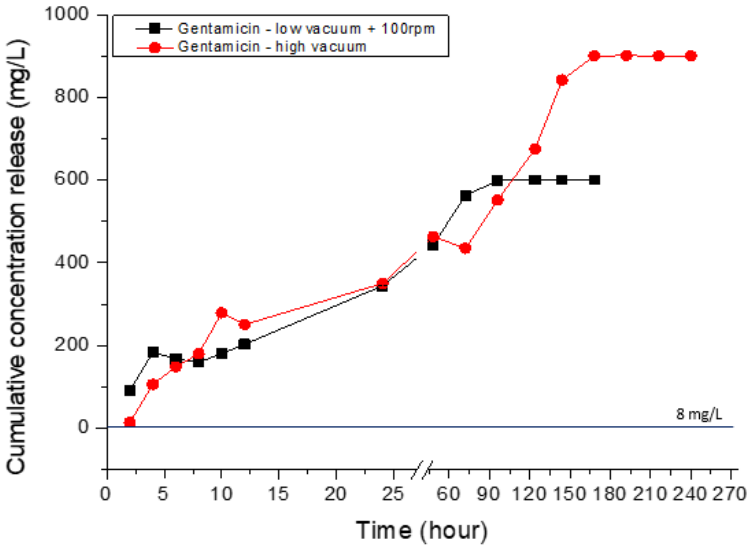

Figure 3.17 - Cumulative Drug Release (mg/L) profile of gentamicin sulfate in bioactive glass hollow microspheres (8 mg/L: therapeutic concentration for more resistant microorganisms).

3.8 – Conclusions

Effectively, syringe foaming method showed to be an interesting and successful technique of producing porous spheres as bone graft, because it produces open and interconnected pores with the desired pore sizes. This method demonstrated to be an easy and versatile technique, in order to be reproduced for clinical applications. The use of Pluronic-F127 as a foaming agent was positive, because it works as a surfactant, forming air bubbles, and allows to inject easily for the production of spheres. The addition of surfactant turns both formulations more fluid, which was proved by rheological tests, allowing the pastes of bioactive glass (BG) and hydroxyapatite (Hap) to be extruded completely from the syringe. Thus, both formulations indicated a good injectability, which is beneficial for production of the spheres with spherical shape.

The results obtained by SEM showed the presence of spherical pores of various sizes randomly distributed on BG and Hap spheres. In this way, spheres of BG and Hap were obtained with a high interconnected porosity. For both biomaterials, pore diameters ranging from 30 to 90 µm, which are good for fulfilling the requirements for bone ingrowth.

Finally, the calculation of closed, open and total porosities confirms that, independently of the material used, the addition of surfactant and air are advantageous, creating spheres with the desired quantity of pores, when compared to the control, supporting SEM results. Both materials showed high values of total porosity, presenting superior values for open porosity, when compared to respective controls.

It was assessed the capacity of gentamicin sulfate loading and released profile of BG hollow microspheres, varying the vacuum conditions. In this way, for high vacuum condition the percent loading was higher than for low vacuum condition. Relatively to the releasing behavior of the drug, it was verified that for high vacuum condition exhibited a slow and sustainable release during approximately 7 days, after an initial rapid release. It was achieved a concentration of 900 mg/L of the gentamicin sulfate, assuring a good concentration that is above MIC. This profile is useful to be applied for infection situations, since it is required to administrate an immediate high dose of an antibiotic and then, a prolonged and controlled dosage. Thus, an interesting profile was obtained without having to do surface functionalization.

References

[1] K. J. Burg, S. Porter, and J. F. Kellam, "Biomaterial developments for bone tissue engineering," *Biomaterials*, vol. 21, no. 23, pp. 2347–2359, 2000.

[2] D. I. Ilan and A. L. Ladd, "Bone graft substitutes," *Operative Techniques in Plastic and Reconstructive Surgery*, vol. 9, no. 4, pp. 151–160, 2002.

[3] T. Kokubo, "Bioactive glass ceramics: properties and applications," *Biomaterials*, vol. 12, no. 2, pp. 155–163, 1991.

[4] L. Wang *et al.*, "Injectable calcium phosphate with hydrogel fibers encapsulating induced pluripotent, dental pulp and bone marrow stem cells for bone repair," *Materials Science and Engineering: C*, vol. 69, pp. 1125–1136, 2016.

[5] L. B. Rodrigues, "Aplicações de biomateriais em ortopedia," *Estudos Tecnológicos em Engenharia*, vol. 9, no. 2, pp. 63–76, 2013.

[6] J. Zhang *et al.*, "A simple and effective approach to prepare injectable macroporous calcium phosphate cement for bone repair: Syringe-foaming using a viscous hydrophilic polymeric solution," *Acta Biomaterialia*, vol. 31, pp. 326–338, 2016.

[7] M. Vallet-Regí, "Ceramics for medical applications," *Journal of the Chemical Society Dalton Transactions*, no. 2, pp. 97–108, 2001.

[8] M. M. Pereira, J. R. Jones, and L. L. Hench, "Bioactive glass and hybrid scaffolds prepared by sol–gel method for bone tissue engineering," *Advances in Applied Ceramics*, vol. 104, no. 1, pp. 35–42, 2005.

[9] J. R. Jones, "Reprint of: Review of bioactive glass: From Hench to hybrids," *Acta Biomaterialia*, vol. 23, no. S. pp. S53–S82, 2015.

[10] M. M. Stevens, "Biomaterials for bone tissue engineering," *Materials Today*, vol. 11, no. 5. pp. 18–25, 2008.

[11] L. Cerroni, R. Filocamo, M. Fabbri, C. Piconi, S. Caropreso, and S. G. Condò, "Growth of osteoblast-like cells on porous hydroxyapatite ceramics: An in vitro study," *Biomolecular Engineering*, vol. 19, no. 2–6, pp. 119–124, 2002.

[12] A. Tampieri, G. Celotti, S. Sprio, A. Delcogliano, and S. Franzese, "Porosity-graded hydroxyapatite ceramics to replace natural bone," *Biomaterials*, vol. 22, no. 11, pp. 1365–1370, 2001.

[13] R. O'Neill *et al.*, "Critical review: Injectability of calcium phosphate pastes and cements," *Acta Biomaterialia*, vol. 50. pp. 1–19, 2017.

[14] M. P. Ginebra, M. Espanol, E. B. Montufar, R. A. Perez, and G. Mestres, "New processing approaches in calcium phosphate cements and their applications in regenerative medicine," *Acta Biomaterialia*, vol. 6, no. 8. pp. 2863–2873, 2010.

[15] H. H. Xu and C. G. Simon Jr., "Self-hardening calcium phosphate cement-mesh composite: reinforcement, macropores, and cell response," *Journal of Biomedical Materials Research Part A*, vol. 69, no. 2, pp. 267–278, 2004.

[16] C. C. Beozzo, M. A. Alves-Rosa, S. H. Pulcinelli, and C. V. Santilli, "Liquid foam templates associated with the sol-gel process for production of zirconia ceramic foams," *Materials*, vol. 6, no. 5, pp. 1967–1979, 2013.

[17] E. B. Montufar *et al.*, "Foamed surfactant solution as a template for self-setting injectable hydroxyapatite scaffolds for bone regeneration," *Acta Biomaterialia*, vol. 6,

no. 3, pp. 876–885, 2010.
[18] D. Campoccia, L. Montanaro, and C. R. Arciola, “The significance of infection related to orthopedic devices and issues of antibiotic resistance,” *Biomaterials*, vol. 27, no. 11. pp. 2331–2339, 2006.
[19] Y. Li *et al.*, “Mesoporous bioactive glass as a drug delivery system: Fabrication, bactericidal properties and biocompatibility,” *Journal of Materials Science: Materials in Medicine*, vol. 24, no. 8, pp. 1951–1961, 2013.
[20] J. X. Lu *et al.*, “Role of interconnections in porous bioceramics on bone recolonization in vitro and in vivo,” *Journal of Materials Science: Materials in Medicine*, vol. 10, no. 2, pp. 111–120, 1999.
[21] M. Gutierres *et al.*, “Bone ingrowth in macroporous Bonelike® for orthopaedic applications,” *Acta Biomaterialia*, vol. 4, no. 2, pp. 370–377, 2008.
[22] Q. Fu, E. Saiz, M. N. Rahaman, and A. P. Tomsia, “Bioactive glass scaffolds for bone tissue engineering: state of the art and future perspectives,” *Materials Science and Engineering: C*, vol. 31, no. 7, pp. 1245–1256, 2012.
[23] J. Ma, C. Z. Chen, D. G. Wang, X. G. Meng, and J. Z. Shi, “Influence of the sintering temperature on the structural feature and bioactivity of sol-gel derived SiO2-CaO-P2O5 bioglass,” *Ceramics International*, vol. 36, no. 6, pp. 1911–1916, 2010.
[24] W. Feng, L. Mu-Sen, L. Yu-Peng, and Q. Yong-Xin, “A simple sol-gel technique for preparing hydroxyapatite nanopowders,” *Materials Letters*, vol. 59, no. 8–9, pp. 916–919, 2005.
[25] A. Ślósarczyk, Z. Paszkiewicz, and C. Paluszkiewicz, “FTIR and XRD evaluation of carbonated hydroxyapatite powders synthesized by wet methods,” *Journal of Molecular Structure*, vol. 744–747, no. SPEC. ISS., pp. 657–661, 2005.
[26] H. S. Costa *et al.*, “Sol-gel derived composite from bioactive glass-polyvinyl alcohol,” *Journal of Materials Science*, vol. 43, no. 2, pp. 494–502, 2008.
[27] I. Rehman and W. Bonfield, “Characterization of hydroxyapatite and carbonated apatite by photo acoustic FTIR spectroscopy,” *Journal of Materials Science: Materials in Medicine*, vol. 8, no. 1, pp. 1–4, 1997.
[28] M. A. Rauschmann *et al.*, “Nanocrystalline hydroxyapatite and calcium sulphate as biodegradable composite carrier material for local delivery of antibiotics in bone infections,” *Biomaterials*, vol. 26, no. 15, pp. 2677–2684, 2005.
[29] M. Ribeiro, F. J. Monteiro, and M. P. Ferraz, “Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions,” *Biomatter*, vol. 2, no. 4, pp. 176–194, 2012.
[30] M. P. Ginebra, T. Traykova, and J. A. Planell, “Calcium phosphate cements as bone drug delivery systems: a review.,” *Journal of Controlled Release*, vol. 113, no. 2, pp. 102–10, 2006.
[31] B. Microspheres, “Loading of Gentamicin Sulfate into Poly (Lactic-Co-Glycolic Acid) Biodegradable Microspheres,” *Journal of Dental School*, vol. 33, no. 2, pp. 145–151, 2015.
[32] W. Xia and J. Chang, “Well-ordered mesoporous bioactive glasses (MBG): A promising bioactive drug delivery system,” *Journal of Controlled Release*, vol. 110, no. 3, pp. 522–530, 2006.
[33] M. Vallet-Regi, I. Izquierdo-Barba, and M. Colilla, “Structure and functionalization of mesoporous bioceramics for bone tissue regeneration and local drug delivery,” *Philosophical Transactions of the Royal Society A*, vol. 370, no. 1963, pp. 1400–1421, 2012.
[34] J. R. Jones *et al.*, “Bioactive glass scaffolds for bone regeneration and their hierarchical characterisation,” *Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine*, vol. 224, no. 12, pp. 1373–1387, 2010.
[35] M. Vallet-Regí, F. Balas, and D. Arcos, “Mesoporous materials for drug delivery,” Angewandte *Chemie - International Edition*, vol. 46, no. 40. pp. 7548–7558, 2007.
[36] V. L. Cook, A. L. Bertone, J. J. Kowalski, S. P. Schwendeman, A. J. Ruggles, and S. E. Weisbrode, “Biodegradable Drug Delivery Systems for gentamicin release and treatment of synovial membrane infection,” *Veterinary Surgery*, vol. 28, no. 4, pp. 233–241, 1999.
[37] M. Bohner, J. Lemaître, P. Van Landuyt, P. Y. Zambelli, H. P. Merkle, and B. Gander, “Gentamicin-loaded hydraulic calcium phosphate bone cement as antibiotic delivery system,” *Journal of Pharmaceutical Sciences*, vol. 86, no. 5, pp. 565–572, 1997.

Chapter 4

4 - General Conclusions and Future Perspectives

4.1 – Conclusions

The increase of bone defects has led to a greater demand for alternatives of bone promotion, trying to avoid progressively the technique of grafting, better known and commonly used.

Hence, as it is the purpose of this dissertation, bioceramics based on calcium and phosphorus were considered to be the most suitable material due to its similarity with the constitution of the inorganic matrix of the bone. Regarding the porosity optimization of the microspheres, it was studied two approaches of creating open and interconnected porosity with the pore sizes needed to bone ingrowth. The porogenic agent approach is an easy technique, however it demonstrated to be a poorly effective process, in terms of producing open and interconnective pores. Contrary to this approach, it was developed the syringe foaming method as other approach to create porous microspheres. This method has proved to be an efficient method, because it reproduced microspheres with macroporosity that are interconnective.

Finally, to ensure a better bone regeneration, an alternative is the association of drugs. For that, it was performed a preliminary study with an antibiotic, in order to combat postoperative infections, caused by bacteria. In this way, it was possible to verify that high vacuum condition permits an initial fast release followed by an attenuated and controllable release, during for 7 days. Hence, it was obtained an interesting release profile with a final concentration of 900 mg/L and a duration that is similar to the required time for the use of antibiotics in the combat of infections that commonly appear in the postoperative period.

4.2 - Future Perspectives

Considering the increase need for controlled drug delivery systems and the potential of hollow bioceramic spheres in this area, it is important to continue with this scope of work. As future prospects, some other studies should be done:

- Perform antibacterial evaluations, in order to understand if the quantity of the antibiotic chosen is properly for combating the bacteria in question.

- Test others drugs, like anti-inflammatory agents, as they are widely used on the treatment of pain, including bone fracture pain and orthopaedic post-operative pain. Anti-inflammatory agents represent an interesting drug group, since they contribute to the healing process of bone fracture.

- Perform injectability studies of the microspheres, since it is intended to be administered in a minimally invasive way. For this, it must be chosen a suitable vehicle of transportation for injectable microspheres. Options of these type of vehicles that have been tested are polymers, such as chitosan, sodium alginate, hyaluronic acid, among others. These studies allow to analyze the necessary extrusion force to inject the material.

- Evaluate biological behavior *in vitro*. These studies assess cell metabolic activity, when they are in contact with the biomaterial.