UNIVERSITAT DE BARCELONA

Final Degree Project

Biomedical Engineering Degree

"Synthesis of Sr-Mg-Zn co-substituted hydroxyapatite"

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ABSTRACT

Nowadays, many developed nations have aging population which has led to an up growth of demand of artificial joints prostheses. With the increasing needs of bone implants, the academic community is focusing on improving the mechanical and biological properties of implants.

Bone is mainly composed by hydroxyapatite, and that is the reason why hydroxyapatite is known as a stable material for coating orthopaedic implants surface. Hydroxyapatite provides an excellent biocompatibility to the implant and enhances osseointegration. However, once the use of Hap has extended, some limitations and complications have arisen such as low biological interaction. Modifying hydroxyapatite by ionic substitution, in order to resemble compositions of hydroxyapatite and bone, has emerged as a solution.

The ionic substitution influences all the properties of synthetic hydroxyapatites. In particular, in this project, the ionic substitution of hydroxyapatite has been achieved by two strategies. **Sol-gel methodology**, which the substitution is achieved by addition of elements during the synthesis of hydroxyapatite. And, **ball-milling**, where the substitution is done after the synthesis using mechanical milling to induce ionic substitution.

In this bachelor thesis, the study has been focused on synthetizing strontium, magnesium and zinc tri-substituted hydroxyapatite with both methods. Moreover, a proper characterization of samples has been conducted in order to study the effect of ion substitution on its structure. Furthermore, cytotoxicity assays of samples obtained against cells have been performed with the aim of studying whether they were toxic.

On the other hand, this project gives an insight into several fields that surround the proposed solution, such as the background and state of art of the hydroxyapatite synthetize and coating methods, as well as the current market situation and regulations of this material. Moreover, the economic feasibility of the project it has also been studied.

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1. INTRODUCTION

Nowadays, many developed nations have aging population as a result of life expectancy has grown over last decades. This huge amount of elderly people has led to an up growth of the demand of artificial joints prostheses and dental implants.

An implant is a medical device manufactured to replace a missing biological structure and to support or enhance a damaged organ or tissue. Implants are made of biomaterials, which can be polymeric, ceramic or metallic such as titanium. The academic community is now focusing on improving the mechanical and functional properties of these biomaterials [1].

Metallic implants are commonly used in orthopaedic and dental industry. Titanium alloys are widely used on this field due to its interesting properties such as their excellent mechanical strength, biocompatibility (usually defined as the ability of a material to perform with an appropriate host response depending on its application) and anticorrosion property [2].

Moreover, titanium alloys spontaneously form a coherent and protective oxide layer (TiO_2) of few nanometers thick on its surface providing an excellent corrosion resistance and improving its bioactivity [3].

Although titanium alloys have great properties, sometimes the oxide layer formed has not the appropriate thickness or roughness, resulting in a lack of bioactivity of the implant and a lower interaction between the prosthesis and the bone, which may induce several complications, such as fibrous encapsulation and finally with the implant rejection.

Fibrous encapsulation is the formation of scar tissue on the surface of the implant produced by fibrocytes [4]. The formation of this tissue prevents the direct contact between the implant and the bone, and this can cause a poor osseointegration.

Osseointegration is the direct structural and functional connection between living bone and the surface of the implant [5]. The implant must have the ability to bond with the surrounding tissue. An insufficient osseointegration can result in implant failure [6].

Researchers have proved that the mechanical properties of the implant and its biological interaction between the metal surface and human body are two factors which affect the implant's osseointegration and biocompatibility [7].

The surface of an implant plays a critical role in osseointegration, as it is the part which is in constant contact with bone and body. Properties of the implant's surface, such as roughness or chemical composition, will determine the quantity and quality of bone formation around the implant [2]. Therefore, the improvement and modification of Ti-based surface has become an interesting solution by which the osseointegration of the implant can be enhanced and the failure implant rates can be reduced.

There are different methods that by modifying the surface of the implant, an improvement in osseointegration, and consequently, a better viability of the implant can be achieved.

One possible strategy to enhance osseointegration is increasing surface roughness, which has been evidenced an improvement in cell adhesion and proliferation [7]. Another method, such as anodization emerged as a possible technique in order to modify implants surfaces. In this process, oxide films are deposited on the surface of the implant, achieving a uniform bioactive ceramic layer which improves the adhesion of cells [8].

However, the surface modification of titanium alloys with Hydroxyapatite (Hap) coatings is the most used solution in order to enhance osseointegration. Hap is a bio-ceramic material with a chemical composition $Ca_{10}(PO_4)_6(OH)_2$.The use of hydroxyapatite has widely extended due to its chemical similarity content with bones.

Bones are hard tissues which plays a very important role in mobility and stability. Those tissues are made of apatite, which is the name of a group of calcium phosphate materials. Hydroxyapatite, which is an apatite type, is the major component of bone and tooth enamel.

Beyond the several applications that Hap can have, the main use of Hap is its use for coating the surface implant with the aim of stimulate bone healing and improving osseointegration [7].

There are several reasons for coating implants with hydroxyapatite. Firstly, the use of HA-coated implants has been reported to stimulate bone healing, resulting in improvement of rate initial implant integration. Moreover, the layer of HA on the top of the implant enhance stability. The use of HA-coated implants reduce recovery time and speed rehabilitation [9].

Once the use of Hap coatings on implants has been extended, some limitations and complications have arisen such as slow rate of biological interaction, due to the high stability of hydroxyapatite structure. Scientists demonstrated that such complication may be associated with the composition differences between synthetized

and natural apatite's. Biological apatite's have minor elements in its structure, such as magnesium or sodium, which play an important role in its biological properties.

Therefore, scientists have focused on modifying the structure and composition of hydroxyapatite in order to achieve a greater similarity to the bone's composition. These modifications were performed by ionic substitutions of calcium ions different by cations such as Strontium, Magnesium and Zinc to improve the crystallographic, chemical, biological, morphological, thermal, mechanical, and physical properties of synthetic Hap.

2. OBJECTIVES

The main purpose of this project is to synthetize and characterize multi-substituted hydroxyapatite powders. In this study, the hydroxyapatite will be synthetized and substituted with two methods: ball milling and sol-gel process. The hydroxyapatite will be substituted with magnesium and strontium to improve osteogenesis of bone tissues and zinc to add antibacterial properties. The synergy of the ion substitutions obtained will be studied by the bioactivity against Primary Human Osteoblasts.

To sum up, the main objectives of this project will be displayed below:

- Synthetize hydroxyapatite with Magnesium, Strontium and Zinc substitution by two methods: sol-gel and ball milling.
- Compare Sol-gel and Ball-Milling as methods to obtain substituted hydroxyapatite by powder characterization:
    - Study powder morphology and composition homogeneity
    - Study crystal structure and particle size
- Determine the cytotoxicity of the powders against Primary Human Osteoblasts following ISO 10993.
- Study feasibility of the obtained powders to be used in plasma spray processes.
- Give an overview of the economic viability of the project
- Identify and analyse the main limitations as well as expose some possible improvements.

Moreover, apart from check which methods enhances the most osseointegration, this project it has also been useful to be able to experience the economic feasibility of each method, as well as determine the time needed to synthetize hydroxyapatite in each process and also check its difficulty degree.

In addition, it has also been a purpose to help and try to provide knowledge to the study group, as well as expand my knowledge of material engineering by working in a laboratory and using specialized equipment intended for characterization such as electron microscopes.

3. SCOPE AND SPAN

The scope of this project has been limited by time. The project includes the study and synthesis of hydroxyapatite by two different methods: sol gel and ball-milling. The structure of the obtained powders has been characterized. Moreover, cytotoxicity of powders against primary human osteoblast cells has been tested

It has been assessed the modification of the structure of hydroxyapatite experiments by incorporating these ions by studying the crystallographic phase composition. The last part of this project is the attempt to coat Ti-6Al-7Nb substrates by plasma spraying using the powders produced.

The main limitations of this project are the high number of samples to be synthesized and the time spent on it. Approximately 2 days are needed per each sample. Time was a limiting factor, because was not possible to achieve the enough powder fluidity to coat the titanium samples. An optimization of the agglomeration/particle size reduction is needed for both samples.

3.1. FORMATION OF PLACE PROJECT

The study has taken place at the Materials Science and Physical Chemistry Department at Physics and Chemistry Faculty of the University of Barcelona, specifically at the CPT, that stands for Thermal Projection Centre.

In order to study the cytotoxicity powders against cells it has been needed the collaboration of the IMIM that stands for Hospital Del Mar d'Investigacions Mèdiques.

4. BACKGROUND

4.1. State of the situation

Titanium and derived alloys have been intensively used as implant materials due to their very good mechanical and corrosion resistance and biocompatibility.

Titanium alloys are the most commercialized to fabricate orthopaedic implants. Ti-6Al-4V alloy was the first alloy registered as metallic implant due to its fatigue strength and biocompatibility [10]. However, Challa et al.[3] in 2013 have demonstrated that the presence of vanadium in the alloy cause toxicity due to the amount of ion release. Therefore, free-vanadium alloys such as Ti-6Al-7Nb gained attention [11]. However, other materials such hydroxyapatite started to be used in order to enhance osseointegration and improving the biocompatibility of the metallic implants.

4.1.1. Structure and properties of hydroxyapatite

Bone is a specialized mineralized connective tissue which its 33% of weight corresponds to the organic matrix. The organic matrix is mainly composed by hydroxyapatite, which constitutes the 67% of bone. For this reason, Hap has been and is being investigated as a possible material for coatings and composites.

Calcium phosphates are a family of ceramic materials containing calcium ions (Ca^{2+}) and orthophosphates (PO_4^{3-}). However, among these ceramic materials, synthetic hydroxyapatite is the material which its use has extended due to is the major component of bones [12].

Synthetic Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is a bioceramic formed by calcium, phosphorus and hydrogen atoms. Hap is a type of apatite, which are a group of phosphate minerals. The structure of Hap is hexagonal unit cell, with a lattice parameters of *a* =b= 9.432 Å and *c* = 6.881 Å [13]. Its formed mainly by calcium and phosphate ions and has a stoichiometric Ca/P ratio of 1.67. In *Figure* 1, a scheme of the structure of Hap is displayed:

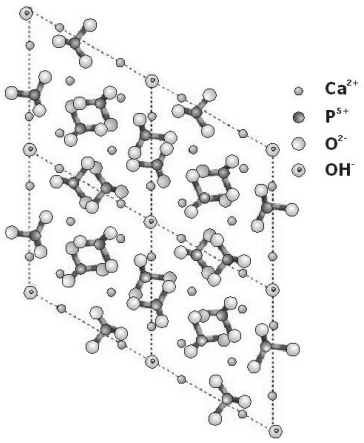

Figure 1: Crystallographic structure of hydroxyapatite[14].

Hydroxyapatite is the most stable calcium phosphate under physiological conditions, such as temperature or composition of body fluids. Moreover, it is a biocompatible ceramic material, therefore, it does not produce toxic and immunological response when is exposed to body fluids and interacts with the surrounding medium. Lastly, as hydroxyapatite has a great similarity the mineral phase of the bone, it can enhance the adhesion and proliferation of surrounding cells.

In *Table 1*, the main properties of hydroxyapatite are displayed:

Properties	Value
Density(g/cm^{-3})	3.15
Young module (GPa)	90
Hardness (Mohs)	5
Fusion temperature (ºC)	1660
Thermal Expansion coefficient (K^{-1})	$11 \cdot 10^{4}$

Table 1: Main properties of hydroxyapatite.

Hap it is a fragile and a brittle material. However, its properties are highly-dependent on its grain size, density, which depend on in which conditions the hydroxyapatite has been synthetized.

Even though hydroxyapatite has greater similarities with bone composition, biological apatites have a different composition, crystal size and morphology from synthetic hydroxyapatite [15]. In *Table 2*, the differences and similarities between hydroxyapatite and human tooth are exposed:

Composition of hydroxyapatite and human tooth emanel		
Constituent	**Human tooth enamel(wt%)**	**Hydroxyapatite(wt%)**
Ca	36.4	39.9
P	17.8	18.5
Ca/P	1.58	1.67

Table 2: composition of human tooth enamel and hydroxyapatite.

4.1.2. Synthesis methods

The hydroxyapatite has been widely studied since 1926. Since then, a huge amount of applications has emerged. Moreover, several methods for the synthesis of hydroxyapatite has been discovered. They can be classified in to three main groups, as it is displayed in *Figure 2*:

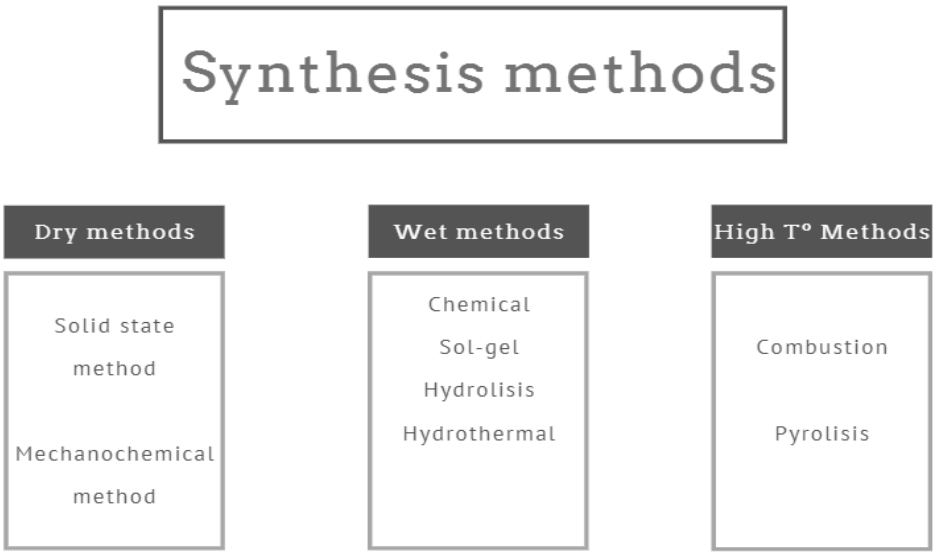

Figure 2: Scheme of the main synthesis methods of hydroxyapatite.

- **Dry methods**: there are two different methods, which are solid-state and mechanochemical methods. In the dry method, the precursor chemicals, which are calcium and phosphate, are in a dry form [16]. Both precursors are mixed to synthetize the Hap.
    - Solid state method: consist on the decomposition reaction of mixed solid reactants, in this case, calcium and phosphate precursors, by heating with the aim of producing new solids and gases [17]. The mechanism of this method is by solid diffusion.
    - **Mechanochemical method**: technique which employs compression, shear, or friction via grinding and milling to induce a chemical transformation [18]. The most popular technique is ball milling, in which the solids can be grinded at different frequencies and at different times [19].

- **Wet methods:** these methods consists on the use of aqueous solutions during the synthesis of Hap [16].
    - Chemical precipitation: undergoes different processes. Calcium and phosphates are mixed, according to a certain molar ratio [20]. During this mixture, pH and temperature are adjusted [21]. A heat treatment is needed to stabilize the hydroxyapatite crystalline structure.
    - Hydrothermal: methods which implies the reaction in aqueous media of calcium and phosphate precursors under high temperature and pressure conditions [22].
    - Hydrolysis: the least used method for synthetizing hydroxyapatite. It consists on a water ionization process which induces the diffusion of hydroxide ions. The hydrolysis of calcium phosphates may induce the formation of Hap (non-stoichiometric).

- **Sol-gel method:** this method involves the formation of a colloidal sol, which consist on a suspension of colloidal particles in a liquid, which will subsequently turn into a gel [23]. Sol gel is the chemical process that consist on the transition from a liquid to a solid. It requires a drying process and a thermal treatment in order to produce hydroxyapatite.

- **High-temperature methods:** those methods consist on producing Hap using high-temperatures in order to decompose the precursor's materials [16]. Combustion and pyrolysis are examples of high temperature methods. Those techniques are rarely used.

In this project were selected ball milling and sol-gel methods to produce the substituted hydroxyapatite powders.

4.1.3. Main applications of hydroxyapatite

Hydroxyapatite has several applications on the biomedical field:

- Bone filling
- Coating implant surface
- Drug-delivery system

Firstly, treatment of large bone defects caused by tumours, traumas or infections normally requires bone grafting surgery [24]. Inorganic composite material can mimic real bones; therefore, Hap can have a potential use on **bone filling,** due to it provides scaffold for bone in growth.

Moreover, another recent application is using the hydroxyapatite nanoparticles as a drug-delivery system [25]. The porous and nanocrystalline Hap has a great interest as a drug delivery system due to its nontoxicity and biocompatibility. It's mainly used as skeleton drug delivery system in bone disorders, such as osteoporosis.

Finally, **coating with hydroxyapatite** the implant surfaced has been founded as a potential and interesting application of the hydroxyapatite, due to it will enhance osseointegration, chemical stability and biocompatibility to the orthopaedic implant. Hap coatings can be achieved through different techniques [26].

4.1.4. Substituted hydroxyapatite

In orthopaedics and dentistry, the use of calcium-phosphate based bioceramics, such Hap for coating implants, has shown potential application due to is high biocompatibility and its similar composition to natural bone.

Biological apatites are calcium-deficient and less stoichiometric, with a Ca/P ratio of 1.5. Biological apatites belong to a family of minerals called apatites, which respond to the composition of $Ca10\text{-}x(HPO_4)_x(PO_4)_{6\text{-}x}(OH)_{2\text{-}x}$. Its chemical structure facilitates the exchange and substitution of other elements. Biological apatites have have minor elements, such as carbonate, magnesium, sodium

which play an important role in the biological properties of the apatite and modify its properties, such as increasing its solubility.

The bioactive behaviour of stoichiometric Hap can be improved by ion substitution. This substitution can be achieved either by cations (Sr^{2+}, Pb^{2+}, Mg^{2}), which will occupy the position of Ca^{2+}, and by anions, such as CO_3^{2-} [27].

Depending on the ionic substitutions, different properties will be enhanced. In this section some examples of ionic substitution will be explained.

- In the case of **Silicon substitution**, it has been demonstrated that the bone and cartilage growth may be increased due to the presence of Si will lead to an increase in bone mineral density [28].
- Secondly, **Silver substituted hydroxyapatite** has great benefits. The incorporation of silver ions in hydroxyapatite has an effective antimicrobial agent. Consequently, it may prevent orthopaedic and dental implant infections [29].
- Moreover, using **magnesium** might enhance bioactivity and biocompatibility of the Hap due to this element is present on teeth and bones. A suitable presence of Mg might lead to an increase of bone mass and will enhance the proliferation of osteoblasts [30].
- **Strontium substitution** have similar effects than magnesium substitution, due to the presence of Sr induce osteoclast apoptosis, and therefore, maintain bone formation and osteoblast proliferation. Zhu et al., 2018 proved that Sr substituted Hap show enhanced biocompatibility and bioactivity [30].
- Furthermore, **zinc** is an abundant element on bone tissues. The presence of zinc might promote bone formation, cell differentiation and proliferation of osteoblast. It might also reduce the inflammatory response. In addition, HA-Zn coating showed moderate antibacterial properties against Gram- and Gram+ bacteria [31], [32].

In the following *Table*, the main properties provided by each ion substitution are displayed:

	Function and properties of each substitution				
	Silicon	Silver	Magnesium	Strontium	Zinc
Antibacterial properties					
Osteoblast proliferation					

Table 3: Main properties given by each ion.

To conclude, it has been demonstrated that multi-substituted Hap show similarity with the inorganic phase of bones and it may induce a synergic effect might have advantageous effects on bone regeneration [33].

4.1.5. Coating methods

Since the early 1980s, the hydroxyapatite has been often used as a coating material of the metal (Ti) substrate due to it provides bioactivity and osseointegration to the implant [34]. Different coating methods have been emerged.

The production methods of Hap coatings can be divided in two groups [34]: wet chemical deposition techniques and physical deposition techniques. Each coating method will reproduce different layer characteristics.

Physical deposition techniques

- Thermal Spraying techniques: the coating material is heated and sprayed on the substrate. Includes high temperature and velocity. Depending on the heat method, there are different thermal spray techniques, for example plasma spraying [35].

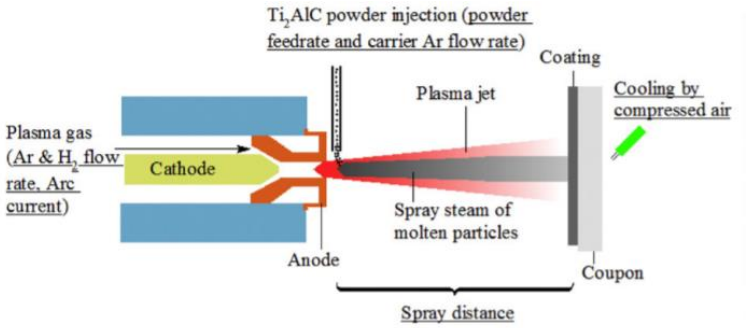

Figure 3: Schematic view of a plasma spraying gun and the powder deposition method [36].

- Electrophoretic deposition: consist on a coating method that consists in depositing charged colloidal Hap particles onto a substrate with an opposite charge. Particles are moved and driven by an electric field [37].

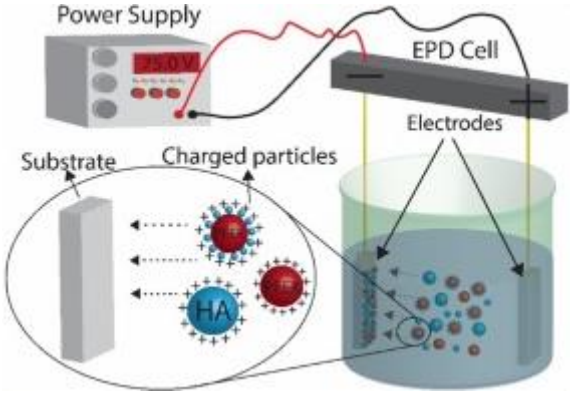

Figure 4:Scheme of the electrophoretic deposition process [38].

- Physical vapour deposition: they are also called sputtering techniques. Those methods use a high energetic beam of ions or electrons projected onto a calcium phosphate target. The calcium and phosphate ions will be deposited onto the substrate surface.

Wet chemical deposition techniques: Chemical deposition techniques coat the substrate with solutions or suspensions. Some techniques are explained below:

- Chemical vapour deposition technique: consists in the exposure of the substrate to volatile precursors which will react or decompose on the substrate surface [39].
- Biomimetic deposition techniques: this method consist in mimicking natural manufacturing methods to generate biological apatites.
- Sol-gel method: there are two different deposition techniques.
    - *Spin coating*, which the material (sol) is dissolved and dispersed into a solvent, and then the sol in dropped into the substrate surface. A spinning is performed in order to produce the layer. The step processes can be seen in *Figure 5:*

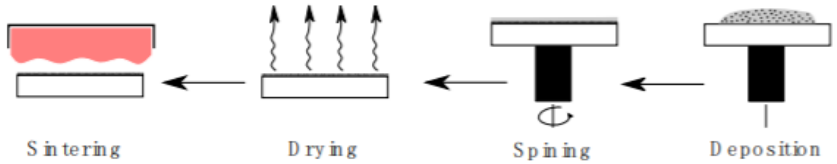

Figure 5: Spin coating methodology process [40].

    - *Dip coating* consists in the immersion of the substrate into a solution containing the coating precursors (the hydroxyapatite sol-gel). The substrate is soaked at a constant speed and the coating is deposited during the substrate withdrawal. The different steps during the coating can be observed in *Figure 6:*

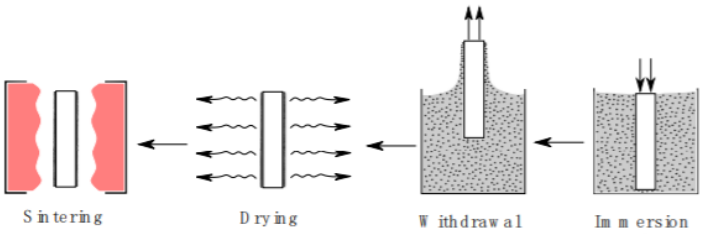

Figure 6: Dip coating methodology process [40].

In this project has been chosen plasma spraying as a deposition method because is the most commonly used commercial method to produce hydroxyapatite coatings on orthopaedical metallic implants.

4.2. State of art

As the number of surgeries as joint replacements has increased substantially, researchers have been focusing into enhancing implant osseointegration and decrease the recovery time to improve the quality of life of patients.

In order to increase biocompatibility it is necessary to study the synergy between synthesis methods and coating procedures to improve not only the mechanical properties, but also the reactivity of the bioactive coatings to increase cell-implant interaction that results into a better osseointegration [41].

5. MARKET ANALYSIS

Behind every successful product or project, a market analysis is essential in order to analyse the current situation and its future perspectives. The economy, the going market trends and the traits of costumer's expenditure will be analysed by searching and studying the incidence and frequency of use of hydroxyapatite-coated implants.

It is a fact that the number of articles, patents and designs discussing about the use of calcium-based compounds is increasing due to the high interest of those materials. This rising interest from scientist in these materials, such as hydroxyapatite, has led to an increase in interest of powerful companies.

Because of the market analysis, it will be possible to evaluate the economic situation and identify the principal companies which are investigating on the modification of surface implants with hydroxyapatite.

5.1. Current situation

Hydroxyapatite has several applications in the biomedical field. In the beginning, Hap was used for bone grafting. As years passed, hydroxyapatite started to be used as a scaffold in order to enhance osseointegration.

The hydroxyapatite market can be segmented depending on its application. Hap can be used in orthopaedic field, in dental care and in plastic surgery. Hap is used as a material for coating implants, for bone filling and for bone grafts.

In 2019 the hydroxyapatite market was led by the orthopaedic group. The global population is aging rapidly due to the increasing life expectancy and the reduced birth rate on countries. Such increase in the number of senior people has led to a rise of orthopaedic surgeries.

Normally, hydroxyapatite is used as a material to coat implant surfaces needed in orthopaedic surgeries. This market segment is expected to grow. The most common orthopaedic surgeries are knee and hip replacements. The number of hip replacements performed in the United States have increased substantially. Over more than ten years of study, the number of surgeries doubled.

The principal companies who are working on implants and researching on the benefits of use of hydroxyapatite are FLUIDINOVA (Portugal), SofSera Corporation (Japan), Berkeley Advanced Biomaterials (US), Taihei Chemical Industrial Co. Ltd. (Japan), SigmaGraft (US), CAM Bioceramics (Netherlands) among others.

5.2. Evolution and future perspectives

As there is an increase in elderly people and in prevalence of chronic diseases, the implant global market as well as the hydroxyapatite market is projected to grow in the next years.

During last years, the demand of implants, as well as the use of bioactive ceramics, such as hydroxyapatite in implant surgeries has increased rapidly.

The medical implants market was estimated at USD 89,230 million in 2020, and expected to increase with a CAGR of 6.5 during the next 6 years[42].

The use of Hap coatings on orthopaedic and dental implants has been known as early as the 1980s. However, recently, nano-structured hydroxyapatite is gaining attention due to is expected to have better bioactivity. Studies have suggested that thinner Hap layers, in the nanometer range, reported increased cellular response than thicker Hap layers [43]. Moreover, it's been studying important application of nano-structured Hap, such as drug delivery. Using Hap as therapeutic drug/antigens/protein carrier provides slow rate of release and protection from degradation [44]. Furthermore, there are more trends of use of hydroxyapatite which include applications such as its use for purification of antibodies on an industrial scale, as an artificial blood vessel or trachea, as well as a catheter made of a Hap composite.

6. CONCEPTION ENGINEERING

6.1. Proposed solution

The aim of this study is to compare two different methods to achieve the substitution of hydroxyapatite powders: **ball milling method** and **sol-gel method**. The substitution will be done using magnesium, strontium and zinc as target cations for the substitution.

Finally, the powders obtained will be used to coat Ti-6Al-7Nb samples using **plasma spray** as a coating technique.

6.2. Selection of substitution methods.

There a wide variety of methods of hydroxyapatite synthesis, as well as there are several coating methods. In this section, it will be argued the reasons why the sol-gel and ball-milling methods have been chosen to synthetize the substituted hydroxyapatite powders.

- The sol-gel method has been because it is a process in which hydroxyapatite can be synthetized from a cheap calcium and phosphate sources and it does not require expensive equipment or advanced technology. Also, the substitution can be achieved before the hydroxyapatite synthesis by the incorporation of the ions into the sol solution using non-expensive salts as precursors of the ions to substitute. In addition, with this method, uniform and crystalline hydroxyapatite can be synthesized.
- Ball milling has been chosen as a technique of substitution of hydroxyapatite because it is method that can easily implemented at an industrial level. The ionic substitution can be achieved by using commercial Hap powders to be mixed with the oxide of the atoms selected for the substitution.

6.2.1. Alternative synthetic and substitution methods.

There are other techniques, for example, chemical precipitation method, which have great advantages such as is a low cost and easy technique, however, the hydroxyapatite obtained normally has a poor uniformity and particles may be agglomerated.

Moreover, high temperature synthetizing techniques has been rejected due to they provide a poor control over the processing parameters[16]. Therefore, crucial parameters such as crystallinity and homogeneity of Hap cannot be controlled.

6.3. Selection of coating methods

The second objective of this Bachelor thesis was to coat the surface of Titanium samples with the Hap previously synthetized.

- **Plasma spray coating method has been selected** because plasma spray is the most used coating technique to produce commercial orthopedical implants as knee or hip implants. This method has great advantages such as high deposition rate, high adhesion strength and it is possible to form a layer of larger thickness than other techniques.

Other coating techniques that could be used are:

- Sol gel-deposition method that can be achieved by two strategies, dip and spin coating. Both are simple and cost effective. A great advantage with respect to plasma spray is that those methods do not use high temperatures, and therefore, no amorphous coating will appear. The biggest disadvantage is the low adhesion strength of the coating obtained that is not suitable for application where frictional forces are applied to the substrate.
- Electrophoretic deposition: This technique showed great benefits such as the layer thickness can be adjusted through the applied voltage. In addition, it allows to form homogenous and uniform layers. However, the main drawback is the shrinkage and cracking that often appear during the coating process.

To sum up, plasma spray has used for coating titanium substrates due to its apparently high efficacy and deposition rate, as well as because the equipment was available in the laboratory.

7. DETAILED ENGINEERING

7.1. Sample code

The code used for each sample follows a pattern:

HA_METHODOLOGY_ELEMENT_NUMBER_CONCENTRATION%_Tº_HOURS

- **HA** corresponds to hydroxyapatite code.
- **Methodology** can be either sol gel which will be denoted as “SG” or ball milling, which will be written as “BM”.
- **Element** that will be used in order to substitute the hydroxyapatite.
- **Number** will correspond to the number of specific samples performed to differentiate the sample replicates.
- **Concentration** will correspond on the molar percentage of the ion substitution.
- **Temperature and hours** will determine which thermal treatment has been done.

An example of sample code will be the following:

“HA_SG_Mg_PA001_2,5%_600ºC_1h”

The sample was obtained by sol-gel methodology. The hydroxyapatite is substituted with Magnesium element with a molar concentration of 2.5%, 600ºC and 1 hours is the temperature and time of the thermal treatment that the sample has been exposed to.

7.2. Experimental procedure

In this section the synthesis process of the Hydroxyapatite powders will be explained. In this Bachelor thesis, two different synthesis methods of powders have been considered: Sol-gel method and Ball-milling process

7.2.1. Sol-gel synthesis of hydroxyapatite

The methodology of this procedure was adapted from [45]. The calcium precursor was calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$, Sigma-Aldrich, CAS : 13477-34-4) and the phosphorus precursor was triethyl phosphite ($(C_2H_5O)_3P$, Sigma-Aldrich, CAS: 122-52-1).
Briefly, 21.92g (0.0928 mol) of calcium nitrate tetrahydrate was dissolved in 50mL of absolute ethanol (CH_3CH_2OH, Sigma-Aldrich, CAS: 64-17-5) and added dropwise to 10mL of triethyl phosphite (0.058 mol) at a dropping rate of 1mL/min. The reaction was carried out maintaining a molar ratio (Ca/P)=1.6. The prepared solution was then placed in a water bath set at 70±10ºC and aging overnight closely capped. The gelation was done drying the solvent in the same water bath until a viscous precursor was obtained. The precursor was then dried in an oven at 90ºC overnight a powder sample was obtained. Finally, the powder was thermally treated at 600ºC for 1h in a furnace with oxidant atmosphere.

For the substituted-Hap preparation the methodology explained previously was adapted to maintain the molar ratio (Ca+M/P)=1.6, where M is referring to metallic ions (Zn^{2+}, Mg^{2+} and Sr^{2+}) to substitute calcium atoms on the hydroxyapatite structure.
The metallic salts used are Zinc Chloride ($ZnCl_2$, Sigma-Aldrich, CAS: 7646-85-7), Strontium Chloride ($SrCl_2$ anhydrous ≥99.9%, CAS: 10476-85-4**)** and Magnesium Chloride ($MgCl_2$ anhydrous ≥98%, Sigma-Aldrich, CAS: 7791-18-6). The salts were dissolved in absolute ethanol and added dropwise simultaneously than the calcium nitrate solution to the triethyl phosphite. The prepared solution as placed in a water bath set at 70±10ºC and aging overnight closely capped. The gelation was done drying the solvent in the same water bath until a viscous precursor was obtained.
All the samples were dried in an oven at 90ºC overnight followed by a thermal treatment at 600ºC for 1 hour in a furnace with oxidant atmosphere in order to stabilize the crystal structure.
Once the powders were obtained, they were washed with Mili-Q water and a vacuum pump in order to eliminate ions that could interfere with a correct characterization of the samples. Finally, samples were dried in an oven at 90ºC for 2 hours.

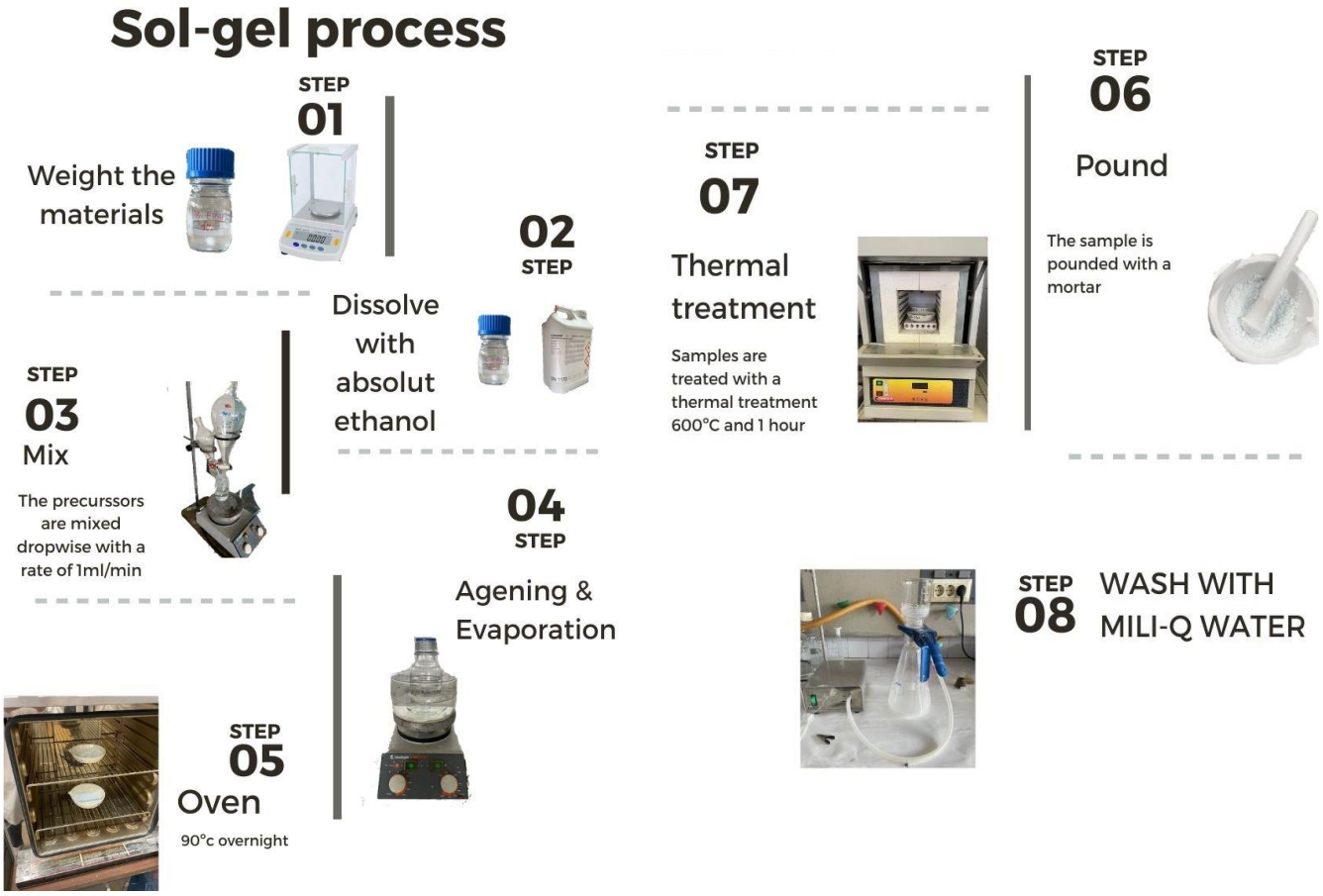

Figure 7: Sol-gel methodology scheme.

In the *Table 4*, all the different synthetized samples are displayed, indicating the quantity in grams of each component.

Composition of sol-gel samples					
Sample code	Nitrate calcium (g)	Tryethyl phosphite(ml)	$MgCl_2$ (g)	$SrCl_2$ (g)	$ZnCl_2$ (g)
HA_SG_PA007	**21.92**	**10**			
HA_SG_Mg_PA002_2,5%	**21.36**	**10**	**0.356**		
HA_SG_Sr_PA002_2,5%	**21.36**	**10**		**0.533**	
HA_SG_Zn_PA001_2,5%	**21.36**	**10**			**0.48**
HA_SG_Mg_Zn_PA001_2,5%	**21.36**	**10**	**0.149**		**0.244**
HA_SG_Sr_Zn_PA001_2,5%	**21.36**	**10**		**0.266**	**0.244**
HA_SG_Sr_Mg_Zn_PA001_2,5%	**21.36**	**10**	**0.0995**	**0.177**	**0.162**

Table 4: Composition of each component in samples obtained by sol-gel.

7.2.2. Ball Milling synthesis of hydroxyapatite

The methodology of this procedure was adapted from the protocol of Dra. Bose [12]. It has been chosen this methodology due to the availability of the equipment.

The powders were prepared by mixing commercial Hap ($Ca_{10}(PO_4)_6(OH)_2$, Sigma-Aldrich, CAS: 1306-06-5) with the oxide elements desired for the substitutions, Strontium Oxide (SrO, Sigma-Aldrich , CAS: 1314-11-0), Zinc Oxide (ZnO, Sigma-Aldrich , CAS: 1314-13-2) and Magnesium oxide (MgO, Sigma-Aldrich , CAS: 1309-48-4). The metallic oxides were mixed with commercial Hap in different concentrations: 1%, 2.5% and 5% molar, using a 1:1 milling media to powder ratio at 240 rpm during 6h. The mixture was performed in a PM 400 MA-type ball-milling machine.

In the following table all the different obtained samples are displayed, indicating the quantity of each component:

Composition of ball-milling made samples					
Sample code	Zirconia balls (g)	Commercial Hap	MgO_2 (g)	SrO_2 (g)	ZnO_2 (g)
HA_BM_MgO_2,5%	**25.05**	**25**	**0.05**		
HA_BM_SrO_2,5%	**25.128**	**25**		**0.128**	
HA_BM_ZnO_2,5%	**25.1**	**25**			**0.1012**
HA_SG_Mg_Zn_2,5%	**25.076**	**25**	**0.025**		**0.051**
HA_SG_Sr_Zn_2,5%	**25.115**	**25**		**0.065**	**0.051**

Table 5: Quantity of component in each sample produced by ball-milling.

HA_SG_Sr_Mg_Zn_2,5%	**25.187**	**25**	**0.033**	**0.087**	**0.067**

7.3. Characterization methodology

The samples obtained by sol-gel and ball-milling methodology have been characterized its chemical composition with IR, the homogeneity of the elemental composition with SEM, its crystalline structure with DRX and its mean particle size with laser scattering. Moreover, cytotoxicity assays have been performed.

7.3.1. X-Ray Diffraction

The measured samples were powders, which have been placed in cylindrical standard sample holders of 16 millimetres of diameter and 2.5 millimetres of height. An X-Ray Diffraction test with PANanalytical X'Pert PRO MPD alpha1 powder diffractometer in Bragg-Brentano θ/2θ geometry of 240 mm of radius and Cu $K\alpha_1$ radiation (λ = 1.5406 Å) was performed. The θ/2θ scan was from 4 to 120º 2θ with step size of 0.026º and the measuring time was 150 seconds. In each measurement, three repeated consecutive scans were performed.

To quantify the amount of beta tetra calcium phosphate founded in each sample the methodology described by Landi, et al.[46] was used. The Relative Intensity Ratio (RIR) can be estimated using the intensity peaks of (2 1 1) and (0 2 10) of HAP and B-TCP, respectively. In this project the formula used is *Equation 1*.

Equation 1. RIR

$$RIR = \frac{I_{\mathrm{B-TCP}}}{I_{\mathrm{HAP}} + I_{\mathrm{B-TCP}}} * 100$$

Where I_{B-TCP} corresponds to the intensity peak, measured in number of counts, of the peak situated in the plane (0 2 10) of the beta-tricalcium phosphate. The I_{HAP} corresponds to the hydroxyapatite intensity peak situated in the plane (2 1 1).

Furthermore, Landi describe a method to characterize the degree of crystallinity of the samples using the *Equation 2*:

Equation 2. Degree of crystallinity

$$\mathrm{Xc} \approx 1 - \frac{\mathrm{V}_{112/300}}{I_{3\,0\,0}}$$

Where $V_{112/300}$ corresponds to the intensity hollow between (1 1 2) and (3 0 0) peaks. And $I_{3\,0\,0}$ is the number of counts of the peak (3 0 0) intensity plane. This method will be used to verify that if by adding ions, the crystallinity of the samples is reduced.

In order to measure both parameters, a baseline and a smoothing of the spectrum was done.

7.3.2. Infrared spectroscopy (IR)

A FTIR spectrometer (Perkin Elmer- Spectrum two) has been used in order to chemically characterize the samples.

7.3.3. SEM characterization

A secondary electron microscope has been used in order to measure the homogeneity and morphology of the composition. The SEM used

An energy dispersive X-ray spectroscopy has been used in order to detect the chemical composition of the analysed sample, using an elemental mapping of the samples to verify the homogeneity of the sample.

It has been worded with a voltage of 20000KV and with a working distance of 20mm.

7.3.4. Laser scattering characterization

A laser scattering characterization has been performed in order to measure the particle size distribution of the sample. The particle-size distribution of the HA-powders were studied using laser diffraction LS 13 320 XR (Beckman Coulter®) in a dry powder system with a Fraunhofer as an optical model.

7.3.5. Cytotoxicity protocol

Primary Human Osteoblasts (HOB) isolated from femoral trabecular bone following the protocol described by Nacher[10] from a knee joint after an arthroplasty were used as a cell line. The Parc de Salut Mar Ethics Committee approved the study and the use of primary osteoblastic cells. An initial cell density of $1x10^4$ cell/well were incubated during 24h at 37ºC in 5% CO_2 humidified chamber in 96-well culture plates.

The cell viability with the powder was done according ISO 10993-5:2009 performing the test with a confluent cell monolayer. The extraction vehicle selected was a culture medium with serum (DMEM, Dulbecco) as its supports cellular growth and can extract both polar and non-polar substances.

Briefly, 0.1 g of Hap-powder was mixed with 1mL DMEM and incubated for 48±2 h at 37±1ºC in a 5% CO_2 humidified chamber. At the end of the incubation period, the Hap-powder was removed by centrifugation and the extracts were used at various concentrations (100%, 50% and 10%).

A cytotoxic effect was considered positive if the reduction of cell viability was **higher than 30%** and cell proliferation was quantified by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega). After the control of systematic cell seeding and growth errors the culture medium was removed and discarded; it is mandatory to eliminate all the culture medium since can contain reductive chemicals that can react and reduce the MTT (3-(4,5-dimethylthiazol-2-yl).2,5-diphenyltetrazoliumbromid) reagent.

The proliferation was quantified by the reduction of MTT that can be determined by spectroscopic measurements at 570 nm wavelength. After the elimination of the culture medium, 50 µL of MTT solution (1mg/mL of MTT in DMEM) was added to each well and the solution was allowed to incubate during 2h before remove it. Lastly, a 100 µL of isopropanol was added to each well and the absorbance of the solution was measured in a microplate reader equipped with a 570 nm filter.

One positive control and one negative control were introduced in the assay. The positive control corresponds to commercial hydroxyapatite to be able to compare with ball milling results and hydroxyapatite produced by sol-gel to compare with the different samples made by this method. For each type of sample, the assay was repeated three times, therefore, for each sample three different absorptions were obtained. An average of these three was made and the standard deviation of each of them was calculated. The cell viability of each sample was obtained using the following equation:

Equation 3. Calculation of cell viability by ISO 10993-5.

$$\%cell\ viability = \frac{As}{Ac} \times 100$$

Where “As” is the absorbance obtained of the measured sample and “Ac” corresponds to the absorbance of the control.

7.3.6. Plasma-spray coating

Powders obtained by ball milling and sol-gel methodology were used. The Hap coatings were sprayed onto a Ti-6Al-7Nb surface with an *A3000 F4 Plasma Technics* spray. In *Figure 8*, it can be seen the plasma spray machine used.

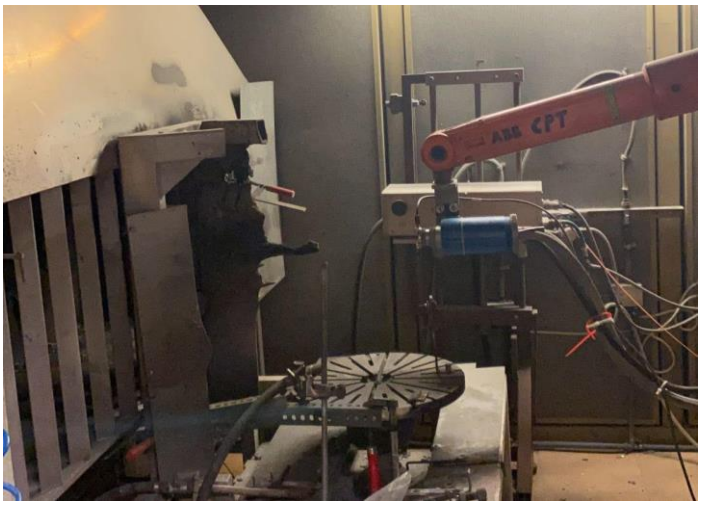

Figure 8: Plasma Spray machine

The Hap coating conditions are described in *Table 6*:

General conditions for deposition of Hap coatings with plasma spray	
Power (KW)	68(40-60)
Argon flow (L/min)	50
Hydrogen flow (L/min)	1
Current (A)	500
Voltage(V)	51.5
Projection distance(mm)	80
Number of steps	5

Table 6: General conditions for deposition of Hap coatings with plasma spray.

Before spraying the hydroxyapatite towards the surface, roughness of sample's surface must be increased in order to enhance the adhesion of coating. A shoot peening of titanium alloy surfaces with alumina particles has been conducted.

7.4. Results

Although during the work in the laboratory more samples have been synthesized, with different concentrations and different ions, only the tri-substituted hydroxyapatite samples at a concentration of 2,5% molar will be described in this work.

7.4.1. Selection of thermal treatment conditions for sol-gel samples.

Before starting the characterization and synthesis of the substituted hydroxyapatite, it is essential to choose the optimal heat treatment that the powders will receive in order to avoid the destabilization of Hap crystalline structure.

Sol-gel sample were subjected to different heat treatments, at 600ºC, 700ºC, 800ºC, 900ºC and 1000ºC. In the *Figure 9*, the DRX spectrum of each sample are displayed. The peaks corresponding to each phase has also been specified. The peaks appearing in blue colour corresponds to Hap and the peaks in red are the specific of beta tetracalcium phosphate (TCP). Two diffraction papers have been used in order to detect each phase. For **B-TCP**, the reference code of the diffraction paper is **00-009-0169**. (Peaks related to this phase will be highlighted in **red**). For **Hap**, the reference code of the diffraction paper corresponds to **01-084-1998**, which will be highlighted in **blue**.
As it can be observed, the intensity of the peak corresponding to Hap, which corresponds to the plane (1 2 1), in the red square, decreases as the temperature increases.

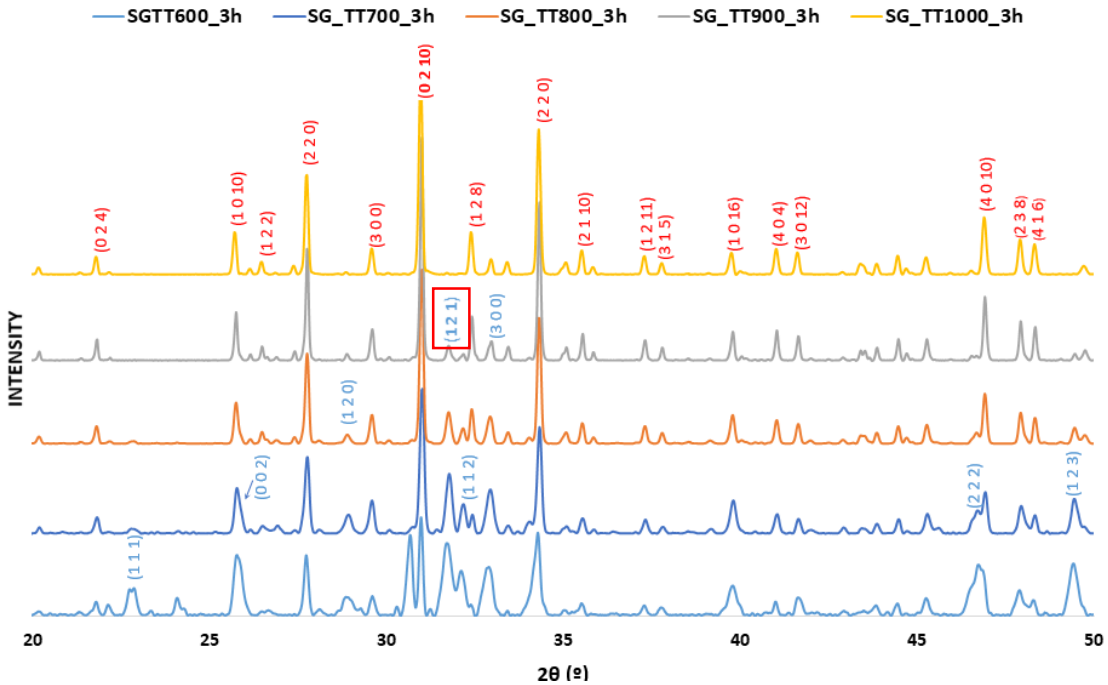

Figure 9: DRX spectrum of samples treated at different temperatures.

In the following table, the RIR of B-TCP has been measured for each temperature. As it can be observed, the B-TCP RIR ratio increases with temperature, therefore, **as temperature increase, less hydroxyapatite phase and more B-TCP is obtained.**

RIR of samples depending on thermal treatment conditions		
Sample code	Temperature (Cº)	RIR (%)
HA_SG_PA007_600_3h	600	58.6
HA_SG_PA007_700_3h	700	72.2
HA_SG_PA007_800_3h	800	86.3
HA_SG_PA007_900_3h	900	91.3
HA_SG_PA007_1000_3h	1000	91.4

Table 7: RIR parameter is measured for samples with different thermal conditions.

Furthermore, once the 600ºC temperature was selected as the optimal temperature for thermal treatment, the time needed was also studied. A DRX was conducted for two samples treated at 600ºC but at different time durations (1 hour and 3 hours).

In Figure 10, DRX spectrums of hydroxyapatite obtained by sol-gel methodology thermally treated at 600ºC and 1 and 3 hours are displayed. The peak which corresponds to Hap phase (1 2 1), which is pointed in *Figure 10*, increased its intensity when duration of the heat treatment is reduced.

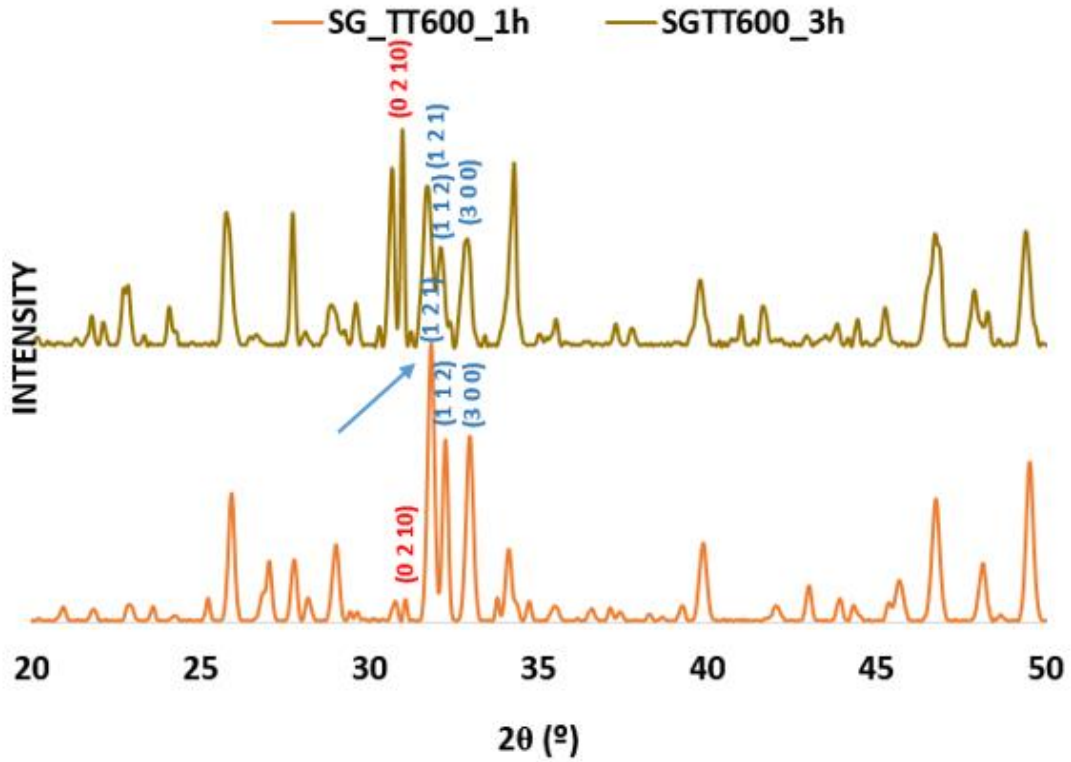

Figure 10: DRX spectrum of samples treated with different time durations.

In the *Table 8*, the RIR of B-TCP are presented. Samples treated during 3 hours had a B-TCP ratio bigger than those treated during 1 hour. Therefore, as **time**

duration of thermal treatment is reduced, more Hap phase and less B-TCP is obtained.

RIR of hydroxyapatite samples	
Code sample	RIR (%)
HA_SG_PA007_**600ºC_3h**	58.6
HA_SG_PA007_**600ºC_1h**	8.9

Table 8: RIR of samples treated at different time durations.

Therefore, the heat treatment selected will be with the following conditions: **1 hour and 600 degrees**. These treatment conditions are the optimal due to samples showed major hydroxyapatite phase.

7.4.2. DRX of substituted hydroxyapatite

A characterization by DRX has been performed on the samples of substituted hydroxyapatite with the aim of quantifying possible changes in the crystalline phase and degree of crystallinity caused by the addition of Magnesium, Zinc and Strontium ions.

In *Table 9*, RIR and degree of crystallinity parameters have been compared between sol-gel Hap and commercial Hap. As it can be observed, commercial Hap shows a lower phase ratio of B-TCP and a major crystallinity degree than the sample obtained by sol-gel method.

Comparison between Commercial Hap and Hap obtained with sol-gel		
Sample	RIR(%)	Xc(%)
Commercial Hap	0,286	98,4
HA_SG_PA007_**600ºC_1h**	8,86	77,56

Table 9: Comparison of RIR and degree of crystallinity between commercial hydroxyapatite and sol-gel

Firstly, as it displayed in *Figure 11*, a DRX of HA_SG_SrMgZn_2,5%_600ºC_1h sample has been compared with the HA_SG_PA007_600ºC_1h. After the cationic substitution, there is an increase of the diffraction peak located in the plane (3 0 0), which is highlighted in **red** in the figure. The increase of this peak is caused by the stabilization of beta tricalcium phosphate in substituted hydroxyapatite.

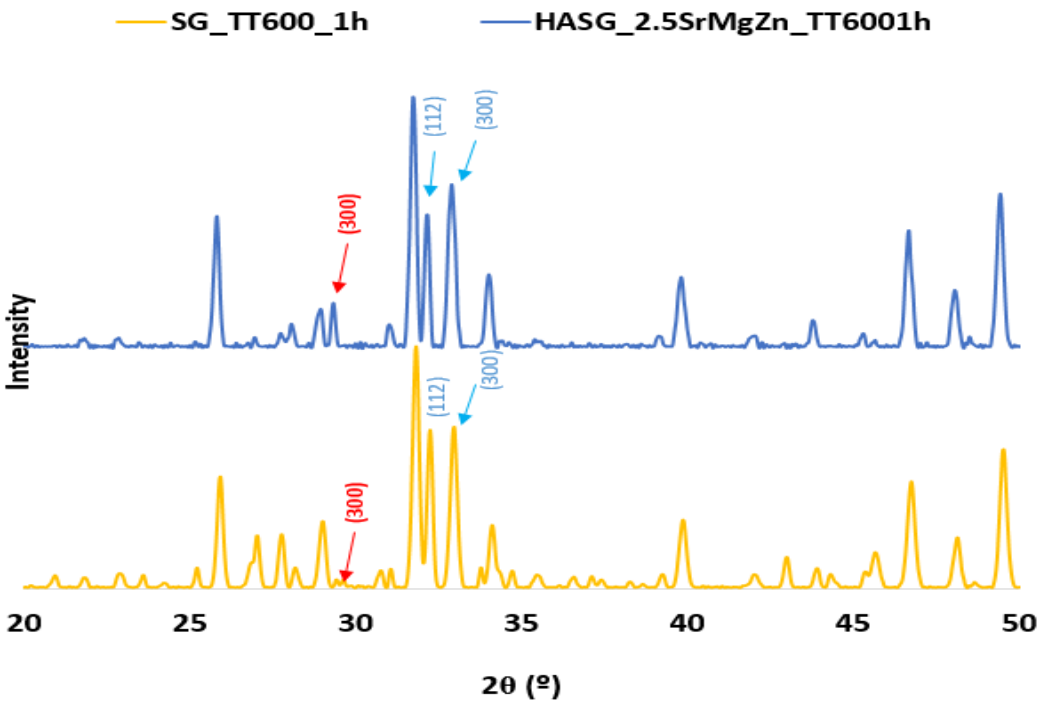

Figure 11: DRX spectrum of sol-gel tri-substituted hydroxyapatite.

In *Table 10*, the degree crystallinity before and after the ionic substitution is displayed. The cationic substitution on sol-gel samples decrease the crystallinity of Hap.

Degree crystallinity before and after ionic substitution of sol-gel samples	
Code sample	Degree of crystallinity Xc (%)
HA_SG_PA007_600ºC_1h	77.6
HA_SG_Sr_Mg_Zn_2,5%_600ºC_º_1h	50.5

Table 10: Comparisoin of degree crystalinity before and after the ionic substitution of sol-gel samples.

Moreover, as it is shown in *Figure 12*, spectrums of commercial Hap and substituted hydroxyapatite made by ball milling method are compared. In substituted hydroxyapatite spectrum also appears the peak of the plane (3 0 0), (highlighted in **red**). Moreover, as it can be seen in figure x, the substitution of Magnesium, Zinc and Strontium ions has **increased the width of the peaks**. Zinc substitution can increase the line width of peaks and reduce the intensities, especially in region 30-35 degrees.

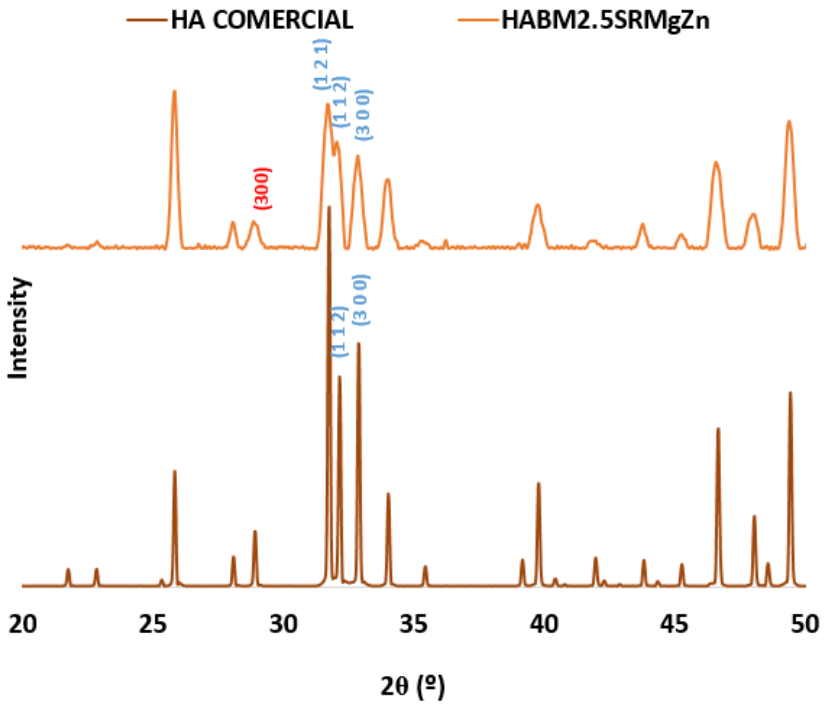

Figure 12: DRX spectrum of ball-milling tri-substitued hydroxyapatite

As it can be observed in table, the crystallinity degree ball-milling substituted Hap has been measured. The **cationic substitution caused a decrease in the crystallinity degree**.

Degree crystallinity before and after ionic substitution of ball-milling	
Code sample	Degree of crystallinity Xc (%)
Commerical Hap	98.4
HA_BM_Sr_Mg_Zn_2,5%	64.8

Table 11: Comparison of degree of crystallinity before and after substitution in ball-milling samples.

7.4.3. IR spectroscopy of substituted hydroxyapatite

Infrared spectroscopy was used to compare the functional groups of commercial hydroxyapatite and the sample obtained by sol-gel and thermally treated at 600ºC for 1h. **HA_SG_PA007_600ºC_1h**.

In the *Figure 13*, the IR spectrum of commercial hydroxyapatite and sol-gel synthetized hydroxyapatite treated with thermal treatment of 600ºC and 1 hour is displayed. The first indication for the formation of Hap is the form of a broad FTIR band centred at 1000 and 1100 $cm^{-1,}$, which is highlighted with a **black** rectangle. Also, the signal from the **hydroxyl bending** (highlighted in **purple**) band is

normally shown at 3570 cm^{-1}, which supports the presence of Hap [47]. Moreover, the bands formed at 1092 cm^{-1} -1034 cm^{-1} and at 565-601 cm^{-1} correspond different symmetric **PO stretching vibrations of the PO_4^{3-}** (phosphate ions, which are highlighted in **red**)[44]. Therefore, it can be confirmed the presence of Hap in our samples.

It is important to notice that the hydroxyapatite produced by sol-gel method has a characteristic band from **inorganic carbonate ion,** (highlighted in **blue**), which is located around 1465-1415 cm^{-1} is present in the FT-IR spectra of the Hap-sol gel sample but is not founded in the commercial Hap [47].

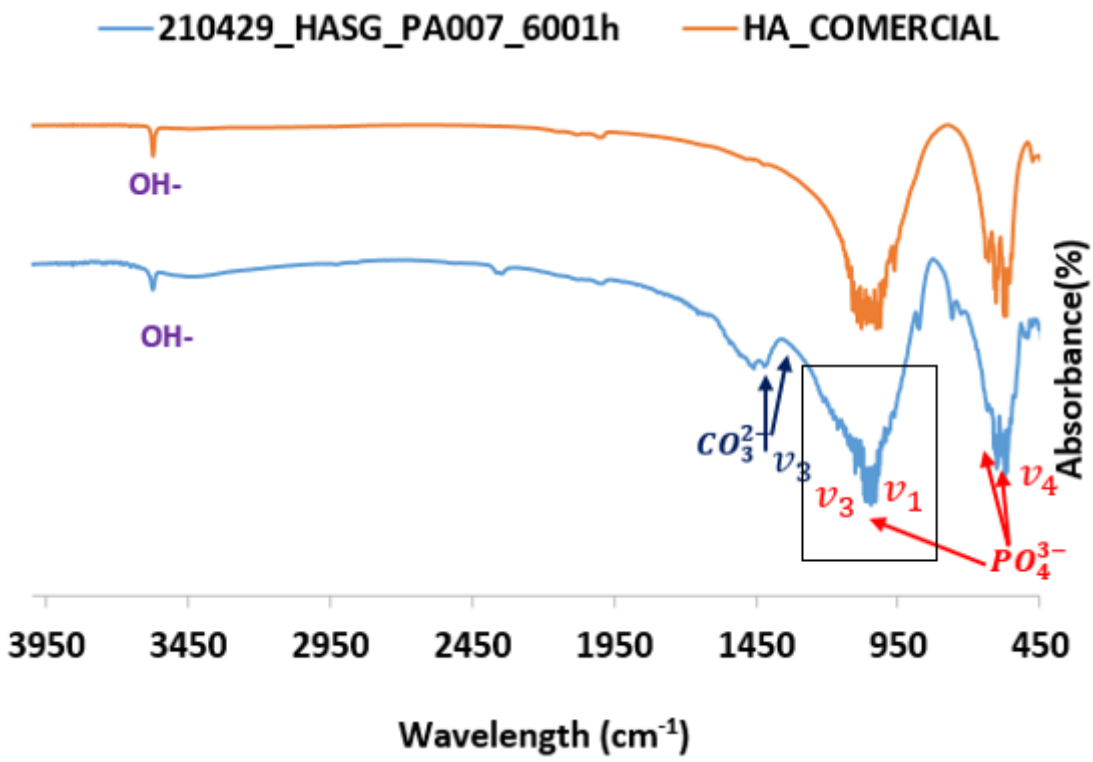

Figure 13: IR spectrums of commercial Hap and hydroxyapatite sol-gel.

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Furthermore, an IR of tri-substituted hydroxyapatite obtained by sol-gel method and ball-milling method has been conducted in order to asses and study the effects of ion substitution.

As it can be seen in *Figure 14*, the multi-substituted sol-gel hydroxyapatite has a peak which corresponds to **P-O bond stretching** of tri-calcium phosphate (highlighted in **purple**). The formation of this peak is related to the stabilization of tri-calcium phase induced by cationic substitution.

This band which corresponds to **inorganic carbonate**, which is highlighted in **blue**, which is located around 1465-1415 cm^{-1} does not appear neither in commercial hydroxyapatite and ball-milling substituted hydroxyapatite.

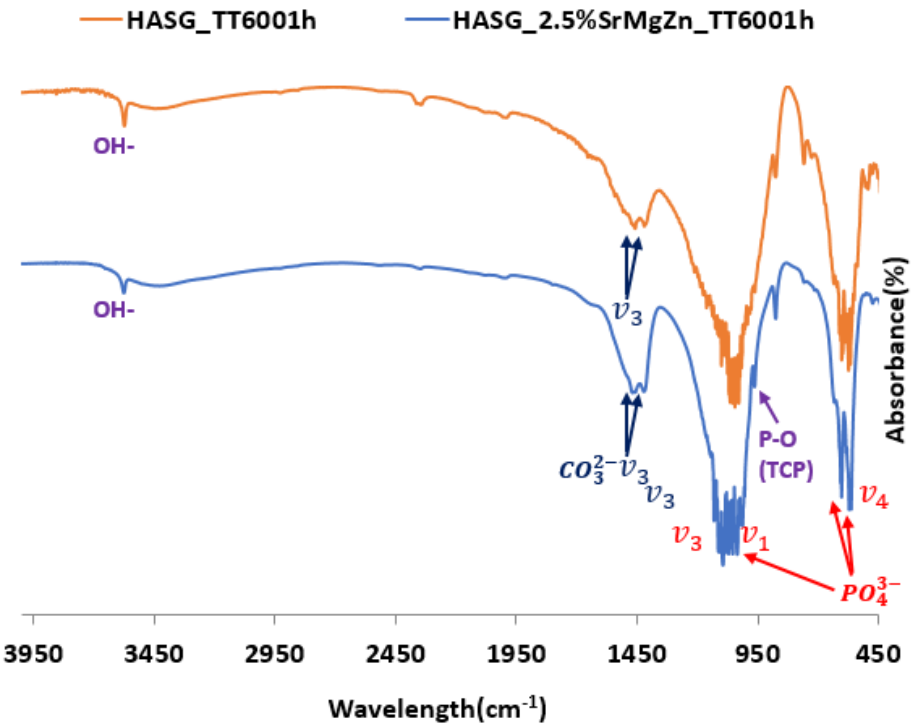


Figure 14: IR spectrum of sol-gel sample

The IR spectrum of ball-milling tri-substituted hydroxyapatite is displayed in *Figure 15*. The main difference between HA_SG_PA001_Sr_Mg_Zn_2,5% is that no carbonate has appeared.

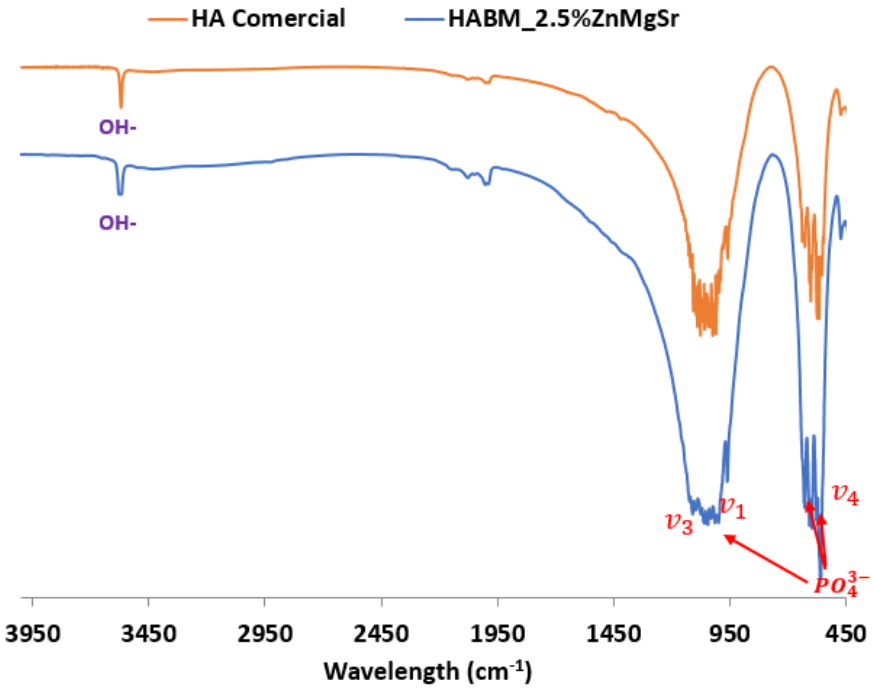


Figure 15: IR of ball-milling multi-substituted sample

In both spectrums, the cationic substitutions can be identified due to slight changes in signals.

The incorporation of strontium has an effect on the phosphate bands, which are shifted from higher to lower wavenumbers[48]. **V_3** phosphate peaks were slightly moved to 1092 cm^{-1} and 1034 cm^{-1} to 1076 cm^{-1} and 1028 cm^{-1} respectively. This confirms the incorporation of strontium ions in the sample [48].

A magnesium substitution can be confirmed due to the decrease of hydroxyl vibration intensity peaks. Mg incorporation caused changes in **PO_4^{3-}** absorption band in the form of broadening and splitting of this group [49].Therefore, extra peaks ascribed to vibration modes of phosphate characteristic of Mg-B-TCP were detected at 945, 974, 988, 1117, and 1152 cm^{-1} [49].

Finally, zinc incorporation has also changed the spectrum. Zn-substituted Hap have lower **OH^- stretching** vibrational mode. The incorporation of zinc caused that OH stretching band has slightly shifted from lover to higher wavenumber [50].

The effects of cationic substitution have been reflected in infrared spectrums of HA_SG_SrMgZn_2,5%_600ºC1h and HA_BM_SrMgZn_2,5%, which are displayed in figures 14 and 15. Firstly, the **cationic substitution decreases the intensity of peaks of OH^-**. As it can be observed in both spectra's, the peak which corresponds to OH^- has decreased. In *Table 12*, it can be observed in more detail the reduction in the intensity of this peak.

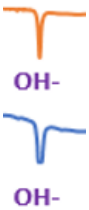
Reduction of intensity of OH peak	
Sol-gel	Ball-milling
	

Table 12: Reduction of hydroxyl peak.

7.4.4. SEM results

A SEM image and colour mapping has been performed of HA_SG_Sr_Mg_Zn_PA001_2,5% and HA_BM_SrMgZn_2,5%. In *Table 13*, the colour maps and the main results are displayed.

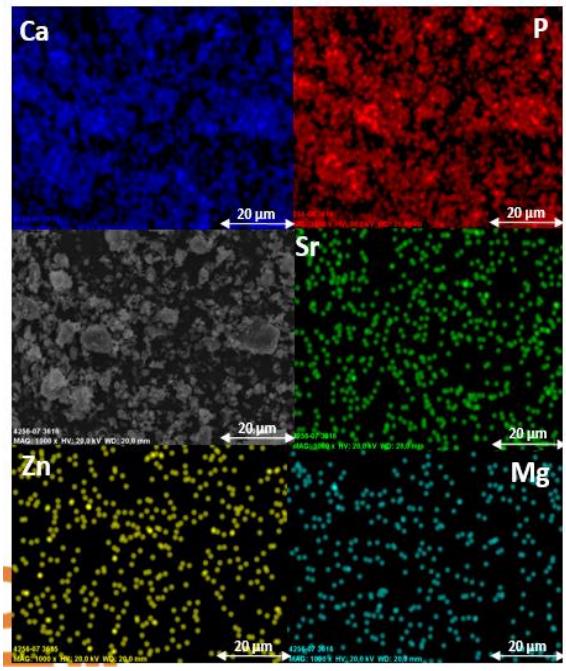
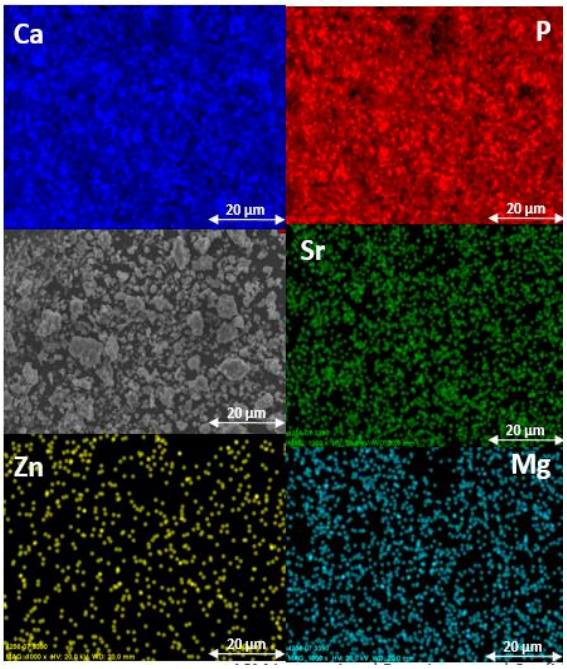
SEM Images	
Sol-gel sample	**Ball-milling sample**
	
Both methods achieve a **homogeneous distribution** of the substituted elements. Moreover, SEM images show **non-spherical particles**, **particles have a polygonal morphology.**	

Table 13: SEM images of ball-milling and solgel samples after the ionic subtitution.

7.4.5. Laser scattering results

In *Figure 16*, the particle size of HA_SG_Sr_Mg_Zn_2,5% sample is displayed in function of the volume distribution. It can be observed that the sample has a very large particle size variation and is conformed mainly by particles in microns. The mean particle size of the sol-gel sample is approximately **50µm.**

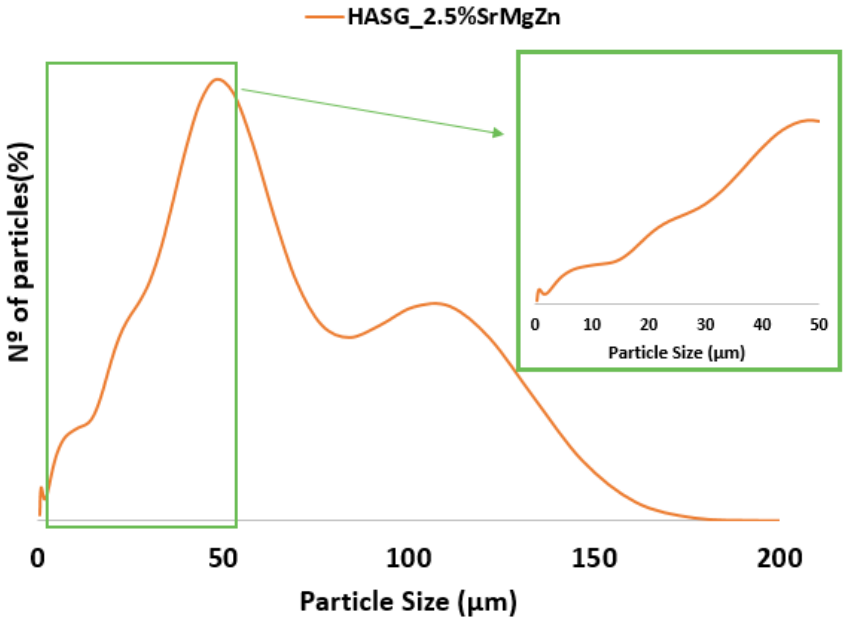


Figure 16: Particle size vs volume distribution of HA_SG_Sr_Mg_Zn_2,5%_600ºC_1h

Moreover, the particle size of ball-milling hydroxyapatite before and after the substitution is presented in *Figure 17*. The commercial Hap (presented in blue line) show a homogeneous particle size around 4 µm**.** The ball-milling process produces a particle size reduction and the samples after ball-milling present a particle mean size of **0.5µm.**

However, the particle size needed to coat titanium alloy substrates with the Hap must be between 40 and 60 µm. Therefore, an agglomeration of the milled powder was conducted in order to increase the particle size. Powders were mixed with polyvinyl alcohol (PVA), which is a water-soluble synthetic polymer. For 5 g of powder, 1mL of PVA was used. Furthermore, the mixture was then dried in an over at 70ºC overnight. Finally, was heated at 300ºC for 1 hour in order to eliminate the polymer. As it can be seen in Figure 17, particle size after agglomeration process slightly increased (grey line).

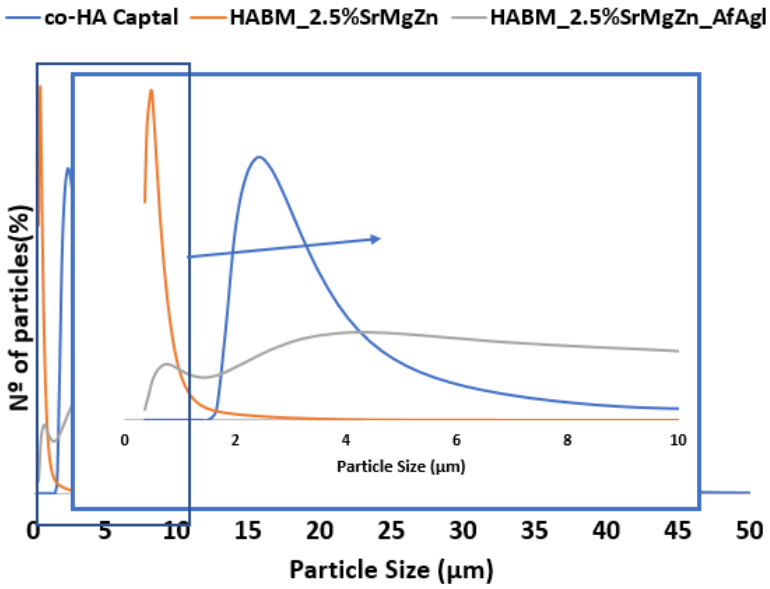


Figure 17: Particle size vs. volume distribution of HA_BM_SrMgZn_2,5% before and after agglomeration process.

To sum up, there is **a huge difference in particle size between hydroxyapatite** synthetized by **sol-gel methodology** and the sample obtained by **ball-milling**. Both powder samples need an additional step to achieve a correct particle size range to be used in plasma spraying process.

7.4.6. Cytotoxicity results

The cytotoxicity of HA_SG_Sr_Mg_Zn_PA001_2,5%_600ºC_1h has been studied and compared to the cytotoxicity of hydroxyapatite produced by sol-gel method (HA_SG_PA007_600ºC_1h). In *Figure 18*, the absorbance of MTS of each sample is displayed. The reduction in the metabolic activity of human osteoblastic cells is 18%.

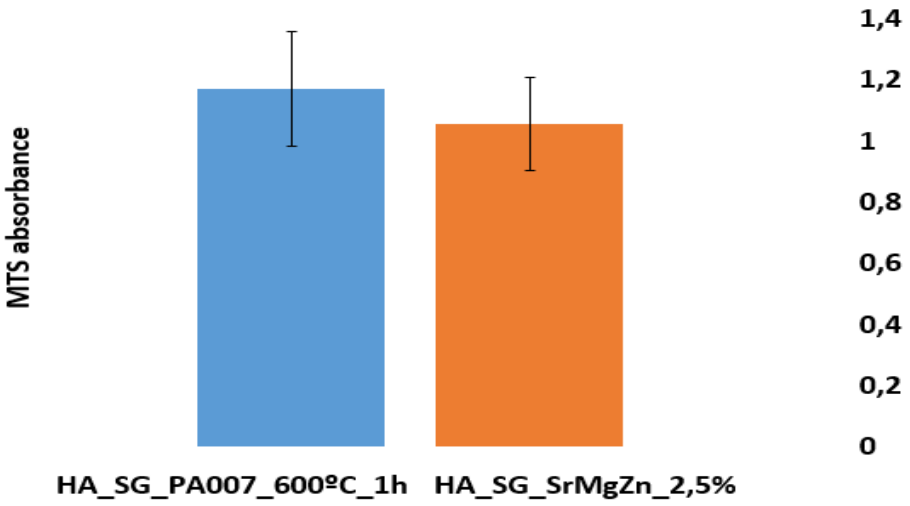


Figure 18: MTS Absorbance of hydroxyapatite produced by sol-gel methodology.

Moreover, the cytotoxicity of ball milling substituted hydroxyapatite has also been assessed. As it can be seen in the *Figure 19*, values of MTS absorbance are

nearly the same compared to the commercial Hap. In this case, the reduction of absorbance is 10%.

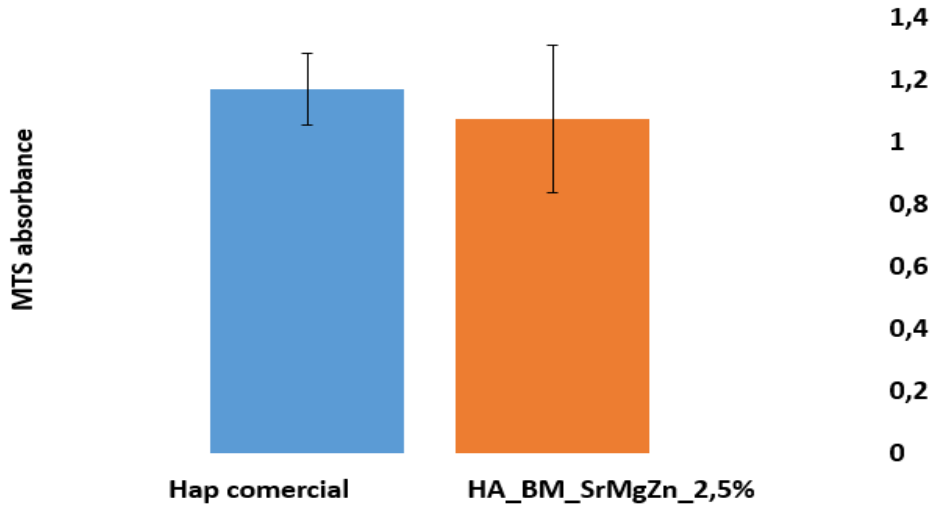


Figure 19: MTS absorbance of hydroxyapatite produced by ball-milling

Both hydroxyapatite samples (HA_SG_SrMgZn_2,5% and HA_BM_SrMgZn_2,5%) do not show cytotoxicity against primary human osteoblasts cells. The absorbance of MTS was **not reduced by more than 30%**, therefore, the powders are not consider as cytotoxic.

7.4.7. Plasma spray results

The roughness of titanium alloy surfaces was increased. Surface roughness before shot peeing was of 260nm. After the surface treatment, the 1,1μm of roughness was achieved.

However, hydroxyapatite could not be sprayed correctly to the surfaces of titanium alloys. This problem is due to the fact that the obtained powders did not flow properly, and consequently, a uniform layer cannot be obtained. In *Figure 20*, it can observe the difference between a proper Hap coating and the coating obtained.

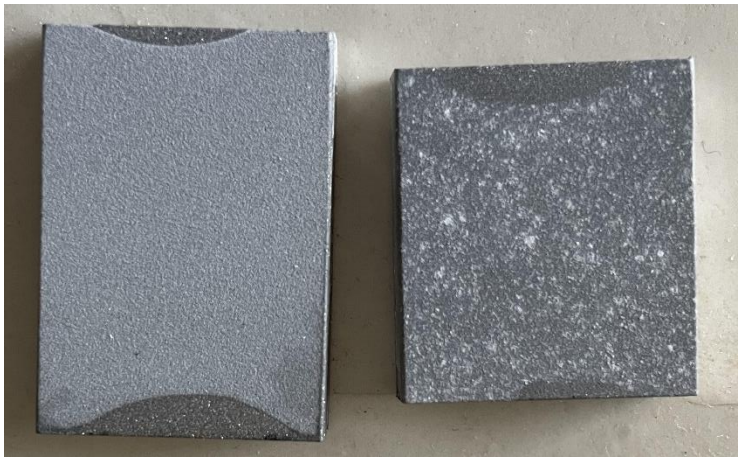


Figure 20: In the left, a correct Hap coating. In the right, the Hap coating obtained.

The plasma spray technician commented that the **lack of fluidity could be caused by the small size of the powder particles.** Even an agglomeration process has been conducted, powders did not flow properly, and consequently, a proper Hap coating on titanium alloy have not been achieved.

8. EXECUTION SCHEDULE

In every project, the management of times and activities is essential in order to get successful results. In this section, and EDT will be displayed, as well as defining the numerous activities, its time and degree of complexity and also make PERT and GANT diagram.

8.1. EDT DIAGRAMM

An EDT diagram has been done in order to have a general idea and structure of the project. In the following figure, an EDT diagram can be observed. This bachelor thesis, has been divided in to five steps: Start of the project, Experimental procedure, Experimental analysis, Results and Project delivery.

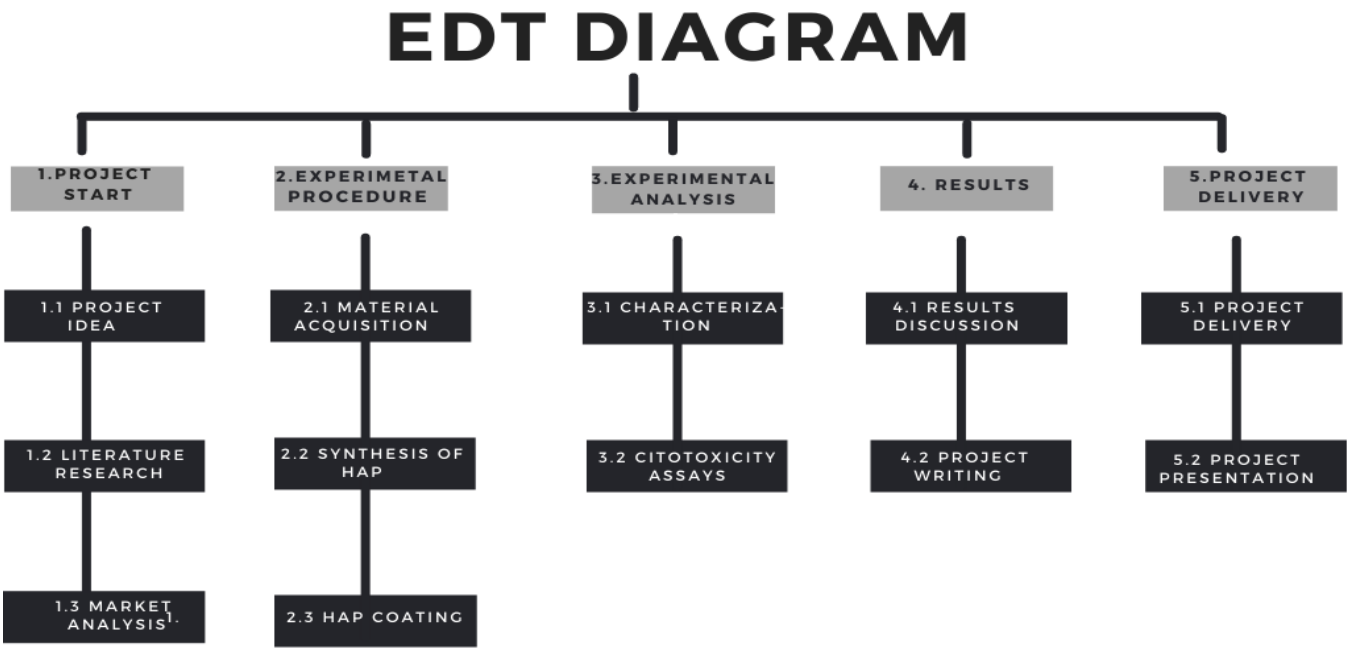


Figure 21: EDT diagram of the project, which expose the main activities performed.

Below, in the EDT dictionary, each block and each activity will be briefly explained.

Reference	1.1	**EDT Title**	Project idea
Description	The activity consists of choosing the project topic among different possible theses.		
Responsible	Alex Carrasco		
Duration	3 days		

Reference	1.2	**EDT Title**	Literature research
Description	Read and collect information about the structure, properties, applications and synthesis methods of hydroxyapatite		

Responsible	Alex Carrasco
Duration	16 days

Reference	1.3	**EDT Title**	Market analysis
Description	The market situation of hydroxyapatite has been analysed. The total cost of the project has been assessed as well.		
Responsible	Alex Carrasco		
Duration	1 day		

Reference	2.1	**EDT Title**	Material acquisition
Description	All the required materials and regents were acquired		
Responsible	Javier Fernandez and Raisha García		
Duration	3 days		

Reference	2.2	**EDT Title**	Synthesis of Hap
Description	The substituted hydroxyapatite was synthetized by ball-milling and sol-gel methods		
Responsible	Alex Carrasco		
Duration	120 days		

Reference	2.3	**EDT Title**	Hap-coating
Description	Titanium alloy substrates were coated with the Hap obtained through plasma spray.		
Responsible	Victor (CPT technician)		
Duration	4 days		

Reference	3.1	**EDT Title**	Characterization of Hap
Description	The synthesized hydroxyapatite was characterized with different methods such as DRX, IR, among others.		
Responsible	Alex Carrasco and Raisha García		
Duration	10 days		

Reference	3.2	**EDT Title**	Cytotoxicity assays

Description	The cytotoxicity of the synthesized powders against osteoblast human cells was studied.
Responsible	Alex Carrasco
Duration	6 days

Reference	4.1	**EDT Title**	Results discussion
Description	The results obtained were discussed and interpreted.		
Responsible	Alex Carrasco		
Duration	10 days		

Reference	4.2	**EDT Title**	Project writing
Description	All the different processes followed in this Bachelor Thesis has been written down.		
Responsible	Alex Carrasco		
Duration	25 days		

Reference	5.1	**EDT Title**	Project delivery
Description	The project has been reviewed and delivered through the Campus Virtual at the corresponding deadline.		
Responsible	Alex Carrasco		
Duration	1 day		

Reference	5.2	**EDT Title**	Project presentation
Description	A project presentation has been prepared in order to expose the main parts of the thesis.		
Responsible	Alex Carrasco		
Duration	5 days		

8.2. Pert diagram

In this section a PERT diagram will be displayed. Firstly, a dependence matrix is needed to perform a PERT diagram.

EDT Reference	CODE	Precedence	Optimistic time	Pessimistic time	Probable time	Mean times
1.1	A	-	2	5	3	3,2
1.2	B	A	15	21	16	16,7
1.3	C	A	1	4	1	1,5
2.1	D	B,C	2	5	3	3,2
2.2	E	D	140	160	150	150
2.3	F	E	3	6	4	4,2
3.1	G	F	7	20	10	11,2
3.2	H	G	5	7	6	6
4.1	I	H	7	12	10	10
4.2	J	I	22	30	25	25,4
5.1	K	J	1	2	1	1,2
5.2	L	K	7	12	5	6,5

Table 14: Dependence matrix of the project, indicating the time needed for each activity.

Moreover, a PERT diagram has been done due to is a powerful tool to analyse any project task and estimate the amount of time required to complete each activity in the project. With this information, it is possible to estimate the minimum time needed to complete the whole project.

Once the diagram is completed, the critical path estimation will be executed. The critical path is defined as “the sequence of scheduled activities that determines the duration of the project”. In *Figure 22*, it can be observed the critical path, which is highlighted in red and corresponds to: ***A-C-D-E-F-G-H-I-J-K-L***

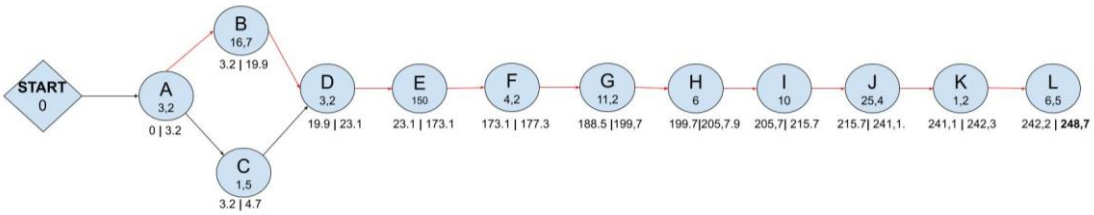


Figure 22: PERT diagram of the bachelor thesis

The project will last a total of **248.7 days** approximately.

8.3. Gant chart diagram

This section will detail the time evolution of the work. A Gantt chart diagram have been done due to is a useful way of showing activities or events of any project displayed against time.

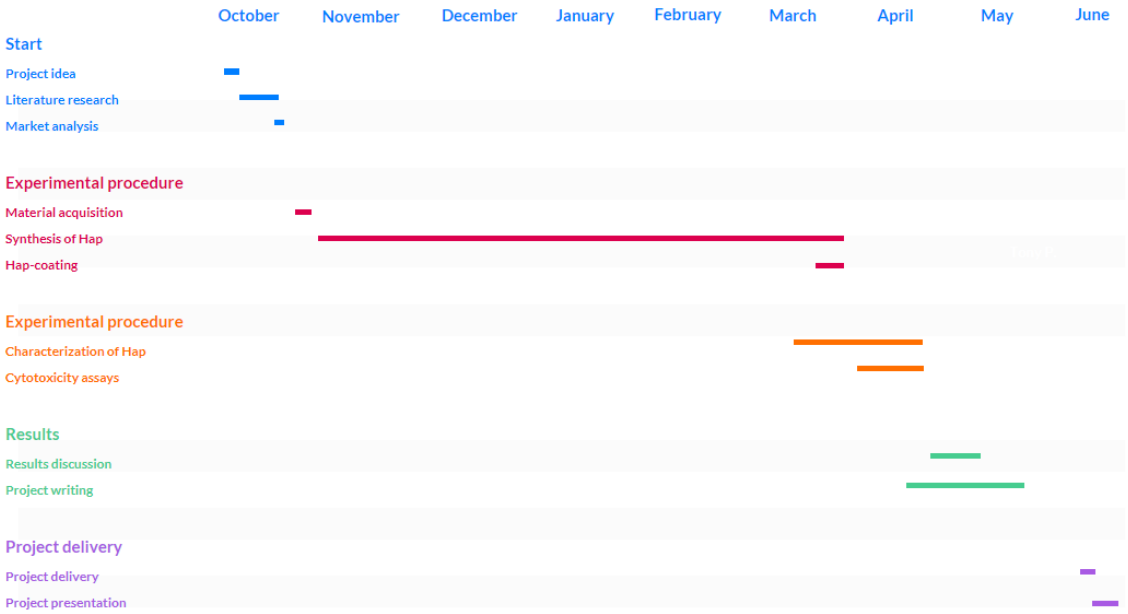


Figure 23: Gantt chart diagram of the different tasks of the project.

The project started in October 2020, where the project idea was proposed. A few days were needed to decide the topic of the project. Since then, 16 days were foreseen for searching information and read articles and papers. Then, a market analysis was performed in order to know the situation in which the project was framed.

At the beginning of November of 2020, the experimental part of the project began. First, all the material necessary to synthesize the hydroxyapatite was acquired. Then, the synthesis of the hydroxyapatite began. This process took 150 days. It started in mid-November and ended in March. The process was long because many samples were synthesized. As the best samples were synthesized, a characterization of each sample was performed. The synthesized hydroxyapatite was used for coating titanium substrates. Several days were spent attempting to coat the surface with the synthesized material. Such characterizations began as the best samples were synthesized. In addition, cytotoxic assays began in early April. As the results came in, they were interpreted and the project was written up.

Finally, in June, the project was reviewed and delivered, and it took five days to prepare the presentation and defence.

9. Technical pre-feasibility

SWOT analysis is a strategic planning technique used to identify the strengths, its weaknesses, its main opportunities and its threats. Such analysis has been done with the aim of identify the internal and external factors which may interfere in the project performance.

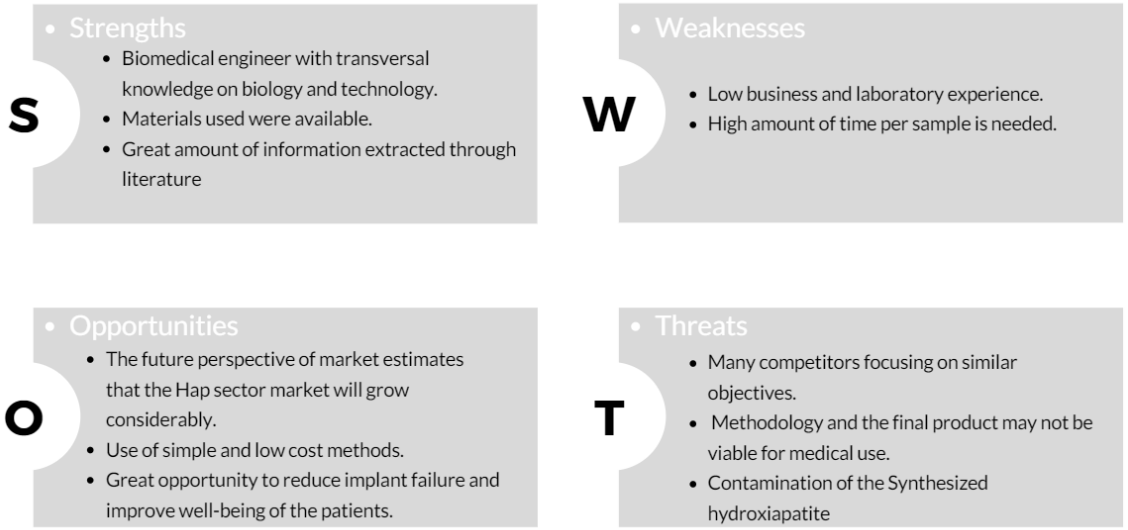


Figure 24: SWOT analysis which balances the main strengths, weaknesses, opportunities and threats of the project

The main strength of this project is that the author is a biomedical engineer, and therefore has a transversal knowledge on biology and technology. In addition, the most prominent opportunities are the use of simple and inexpensive hydroxyapatite manufacturing methods. On the other hand, the greatest weakness of this thesis is the lack of experience of the author in a laboratory. Finally, the biggest threat to this project is the great competition that exists in the market.

10. ECONOMIC FESEABILITY

In this section the economic feasibility of the project will be examined and study in order to determine the overall project budget. This study, normally consist on a cost-benefit analysis of the project, and then determine whether it is possible to implement it. However, in this section it is only going to be studied the total cost of the activities developed. As the aim of this section is to determine the whole expenses of the project, they will be divided in 3: Labour costs (or human costs), the material needed and used-equipment costs.

10.1. Labour costs

Labour costs refer to the sum of all wages paid to employees. In this section, the expenses associated with the salaries of the people involved in the work will be assessed.

Firstly, the number of participants has to be determined. The person which has performed the thesis is a biomedical engineer. In Spain, the average salary of a biomedical engineer is 40000€ per year, and therefore, the engineer gains approximately € 20 / hour. 171 days were worked in the laboratory, and with an average of 4 hours worked per day, 686 hours in total were destined by the biomedical engineer in the laboratory.

Moreover, as the biomedical engineer does not have experience on laboratory practice and use of equipment, a laboratory supervisor is needed in order to guide and contribute partially in the investigation. A laboratory supervisor earns 40€/hour. Since the supervisor guides the engineer and advises and carries out certain activities of more complexity, it will be counted that the hours worked have been less. Approximately a total of 55 hours has been worked by the supervisor.

Role	Worked hours	Price per hour	Total (€)
Biomedical engineer	686	20	13720
Laboratory supervisor	55	40	2200
Total			**15920 €**

Table 15: Labour costs of the project

The total human cost is ***15920€.***

10.2 Manufacturing cost of hydroxyapatite

This work focuses on synthesizing hydroxyapatite substituted by two methods.

Even though it is important to compare both methods in terms of characterization and cell viability, it is also important to compare in terms of costs. In *Table 16*, the total cost per sample obtained by sol-gel method will be displayed. The number of samples obtained by this method are 7.

Sol-gel method costs per sample			
Material/Equipment	Quantity used	Price	Total(€)
Calcium nitrate	21.36 g	126€/kg	2.7
Triethyl phosphite	10 ml	60.8€/L	0.6
Absolute ethanol	50 ml	23.8€/L	1.2
MgCl	0.0995 g	144€/kg	0.014
SrCl	0.177 g	134€/kg	0.02
ZnCl	0.162 g	128€/kg	0.02
Oven	12h	0.13€/h	1.56
Muffle furnance	1h	0.195/h	0.195
			6.2 €/sample
Number of conditions		7	**43.2€**

Table 16: Costs of the sol-gel method process.

Moreover, as shown in *Table 17*, the total cost of producing hydroxyapatite by ball-milling methodology is displayed. Six samples were synthetized:

Ball-milling method costs			
Material/Equipment	Quantity	Price	Total(€)
Commercial Hap	25 g	800€/kg	20€
MgO	0.033	1712€/kg	0.05€
SrO	0.087	1670€/kg	0.14€
ZnO	0.067	1868€/kg	0.13
Zirconia	25.2 g	53€/kg	1.4
Agglomeration process	1	2€/process	2
Milling Machine	6h	0.4€/h	2.4
			26.04€/sample

Number of conditions	6	**156.2 €**

Table 17: Costs of the ball-milling process.

10.3. Characterization costs

The characterization process has also been considered in the overall project budget.

The methods of characterization employed are SEM Microscope, Infrared Spectroscopy, X-Ray diffraction and laser diffraction in order to measure powder size. Finally, a cytotoxicity assay has also been conducted. The number of samples which were characterized are four.

Characterization costs			
Material/Equipment	Hours	Price/hour (€/hr)	Total(€)
DRX	2h/sample	35	70
IR	10min/sample	30	5
SEM	20min/sample	15	5
Laser scattering	1h/sample	25	25
Cytotoxicity	1h	500	500
			605
Number of conditions		4	**2420€**

Table 18: Characterization costs of the project.

10.4. Total costs

In table 16, the overall budget of the bachelor thesis is specified:

Total costs	
Category	Total (€)
Labour costs	15920
Sol-gel costs	40.5
Ball-milling costs	156.2
Characterization	2420
	18536€

Table 19: Overall budget of the project.

The total cost of the project is 18536€.

11. NORMATIVE AND LEGAL ASPECTS

The experimental procedure, which includes all the process of synthesising and characterizing the hydroxyapatite, as well as the cytotoxicity assays has followed the normative and legal aspects described in Materials Science and Physical Chemistry Department at the University of Barcelona.

Researchers and teachers working on the laboratory explained and defined a set of rules and obligations in order to protect the safety of all the members and of the laboratory worked. These standards are included in the laboratory's good practice manual. Wearing a gown and having the eyes protected with goggles, leaving the space clean, not pouring toxic products in inappropriate places, are an example of the obligations that must be followed to be able to conduct a correct and safe laboratory practice.

Moreover, as the final product is expected to be commercialized as medical device, it is important to consider a rigorous and strict regulation.

11.1. Medical device classification

As medical devices are products with its aim is to save or help people's life, a heavy regulation is needed. Its regulation is primarily concerned on enabling patient access to high quality, safe and effective medical devices, and avoiding access to products that are unsafe[51].

The FDA establishes a medical device classification system with the aim of placing each device in a category according to its duration contact with the patient, the part of the body affected and the degree of invasiveness. This classification is established with the aim of providing a reasonable assurance of safety. The FDA, categorized medical devices into the regulatory classes: Class I, II and III. As the level of risk increases, a more safety control will be needed in order to commercialize the medical device [51], [52].

As it is displayed in the *Table 20*, Class I-type devices are low risk and they are only subjected to general controls such as tests of sterility. Furthermore, Class II devices, require more specific controls such as additional labelling requirements. Finally, class III products, such as defibrillators, require clinical studies evaluating its effectiveness and safety. Those studies are called pre-market approval application [52].

Hydroxyapatite coatings are not a medical device itself, however, it will form part of a medical device, in this case, the implant. Therefore, as the coating of hydroxyapatite is an intrinsic part of the implant, it will be evaluated and classified as class II or class III, depending on the ending use of the implant. If is a dental implant, it will be considered as class II, however, knew or hip implants are considered a class III.

Class	Risk	Examples	Controls
I	Low	Hospital Beds	General controls
II	Medium	Absorbable sutures	General and special controls
III	High	Implantable pacemaker, implanted prosthetics	General and special controls, Pre- market authorization

Table 20: Different classes of medical devices classified according to its risk.

11.2. Regulations

11.2.1 Good Laboratory Practice

As it is explained before, as the major part of the bachelor thesis has been performed in the laboratory, a good laboratory practice has been followed. The good laboratory practice is concerned with the organisational process and the conditions under which non-clinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported. [53] This good laboratory practice has several principles. One of the main principals is to ensure that all the processes and machines are correctly and regularly checked. Moreover, all the accuracy of the machines must be ensured in order to have a good quality of results. However, the most important part to consider in this Bachelor thesis is all the set of protocols that must be followed when working on the laboratory. Some examples will be described

For example, when the powders were milled, a specialized mask is required due to when the powder is been milled, a huge amount of nanoparticles are released into the air, which can affect the respiratory system. Therefore, it is very important to ensure a proper ventilation and avoid grinding with people around.

Furthermore, when some toxic or volatiles reagents, such as triethyl phosphite, are handled, it is necessary to use fume cupboards.

Finally, as the bachelor thesis includes cells assays and therefore, manipulation of biological material, some specific requirements must be followed.

Cell cultures are the result of the "in vitro" growth of cells obtained from organisms multicellular. The Spanish Royal Decree, defines, in Article 2 of Royal Decree 664/1997, cell cultures as biological agents. Cell cultures always must be handled in a class II biosafety cabinet. If any culture medium is spilled, is very important to clean it properly[54].

11.2.1 Hydroxyapatite and implant regulation

In Spain, the regulation of medical devices is specified in the Royal Decree 822/93. The final product must follow several ISO norms:

-**ISO 10993**, a set of harmonized standards that address the biological evaluation of medical devices [52]. The purpose of this ISO norm is to test the biocompatibility of the device. Testing the biocompatibility of the implants is essential due to the final product is an implant device which will be in contact with tissues and bones. Several test, such as cytotoxicity or hemocompatibility, must be performed before introducing the device to human body.

-**ISO 14971**: a set of harmonized standards that address the risk management of medical devices, including software and in vitro diagnostic medical devices. This norm intents to assist *manufacturers* of *medical devices* to identify the *hazards* associated with the *medical device*, to estimate and evaluate the associated *risks*, to control these *risks*, and to monitor the effectiveness of the controls [53].

-**ISO 13485**: represents all the necessary requirements for the quality system of medical devices.

Moreover, as the major part of the performance of the project will take part on the laboratory, several ISO norms must be considered, like ISO 15189, that is exclusively designed for medical laboratories or ISO 15189 specifies particular requirements for quality and competence of medical laboratories

Lastly, in order to sell any product freely in any part of the European Economic Area, the **CE marking** is needed. It is a certification mark that indicated conformity with health, safety and environmental protections standards of the EEA (European Economic Area). In order to get this quality marking, some steps must be followed. The assessment will be approved by an external entity. The European Union has its directive regarding active implantable medical devices, DIRECTIVE 90/385/EEC. Once the device has been granted a CE mark in one Member state, it can be marketed in all the other European Member States without further controls and no further evaluations.

12. CONCLUSIONS AND FUTURE TRENDS

This research aimed to synthetize Sr-Mg-Zn substituted hydroxyapatite powders with sol-gel and ball milled process. As main of this project:

- Based on the characterization results, **both techniques are suitable methods for obtaining of Sr, Mg and Zn- substituted hydroxyapatite powders with a homogeneous composition**.

- DRX spectrums showed that **the optimal thermal treatment** selected for sol-gel powder samples in order to obtain an appropriate hydroxyapatite phase was **600ºC for 1 hour**.

- DRX characterization proved that **ionic substitution reduces the crystallinity** of the samples in both ball-milling and sol-gel process.

- Laser scattering results reflected that **ball-milled samples showed smaller size** particle **than** hydroxyapatite obtained by **sol-gel** method.

- **Substituted hydroxyapatite obtained by sol-gel methodology and ball-milling techniques do not show toxicity** against Primary Human Osteoblasts following the methodology described by ISO 10993-5:2009.

- The **fluidity of ball-milled powder** was **not suitable** for a correct deposition, even after steps of agglomeration process.

Due to lack of time, many different experiments and studies have been left for the future. And as future recommendations:

- A deep study and characterization of substituted hydroxyapatite samples with different molar ion concentration is needed in order to asses which percentage of concentration exhibits the optimal properties to the samples.

- Is necessary to optimize the final particle size modification for sol-gel and ball-milled powders due to is the main cause of the plasma spray results failure. It is important optimize the agglomeration process to increase the particle size of ball-milled powder.

- Is study whether the incorporation of different ions, such as silicon or iron substitution provide interesting properties to hydroxyapatite.

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