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Licenciatura em Engenharia dos Materiais

Development of Flexible Bone Grafts

Dissertação para obtenção do Grau de Mestre em
Engenharia de Materiais

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

May, 2018

“Our greatest weakness lies in giving up.
The most certain way to
succeed
Is always to try just one more
time”

Thomas Edison, 1847 - 1931

Development of Flexible Bone Grafts

Acknowledgements

Antes de mais, gostaria agradecer a oportunidade que me foi confiada de poder realizar a minha Dissertação para obtenção do grau de Mestre em Engenharia de Materiais na empresa Medbone – Medical Devices. Foi um privilégio participar neste projecto proposto pela empresa, assim como acompanhar uma área de trabalho pela qual me interesso e motivo.

Gostaria de agradecer à minha orientadora, Engª. Cláudia Ranito, por me ter recebido na sua empresa de braços abertos, por todo o seu tempo e auxílio prestado neste projecto, pela partilha de conhecimento que me proporcionou e pelo compromisso demonstrado ao longo deste projecto.

Ao Professor Doutor João Paulo Borges, meu orientador da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa neste projecto, queria agradecer toda a sua disponibilidade e ajuda não só na minha dissertação mas ao longo de todo o curso de Engenharia de Materiais, que com toda sua simpatia e boa disposição sempre me auxiliou quando precisei.

A todos os docentes do Departamento de Ciência de Materiais da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, um obrigado por todo o conhecimento que me transmitiram ao longo desta etapa.

A toda a equipa de trabalho da empresa Medbone, João, Filipe, Zé, Nelson, Sérgio, Daniela, Rui, Patrícia e Filipa, um enorme obrigado por toda a vossa simpatia, companheirismo e amizade que levo após esta etapa.

Aos docentes do CENIMAT e CEMOP, um obrigado pela disponibilidade na realização dos testes de caracterização utilizados neste projecto.

A toda minha família, por todo o amor e carinho, por estarem sempre presentes na minha vida e por me terem sempre apoiado em todos os momentos e nas minhas decisões.

À minha Mãe, pelo seu amor e apoio, por todo o carinho, amizade e por me ter incutido sempre o sentido de responsabilidade.

Aos meus avós e tias, por sempre me acompanharem em todas as minhas etapas com todo o seu carinho, amor e amizade.

Á minha namorada, obrigado por estares na minha vida, sempre aqui para me apoiar, motivar, por todo o teu amor, carinho, amizade e compreensão.

A todas as amizades que fiz ao longo deste curso, por todos os momentos que passamos e que ficarão gravados na minha memória.

Abstract

With the aging of world's population, diseases and bone related problems are becoming more frequent. Bone grafts are normally used in the treatment and repair of bone defects. The aim of this project was to develop a flexible bone graft in order to be adjustable to the patient's problem.

The development of this product consisted in combining a natural protein, type I collagen, extracted from bovine tendon, with β-TCP, a calcium phosphate. The composition of this product was 10:90 wt%, respectively. Its production focused in a lyophilization cycle and posteriorly Dehydrothermal crosslinking, using three different treatments: 24, 48 and 72h. The products were characterized by XRD, FTIR, SEM, Archimedes Method, compression tests and TGA-DSC.

All the composites manufactured showed a porous structure, revealing values in the range of 84 to 87%.The β-TCP particles were homogeneously distributed and overlapped in the collagen matrix. The crystalline phases of the samples were studied and the presence of β-TCP was identified. Molecular groups were identified as referring to collagen and β-TCP. The observed amide peaks I, II, III, A and B tend to be less pronounced when the crosslinking exposure time increases, while the phosphate groups were observed with clarity. The products exhibited Young's Modulus values ranging from 180 to 200 kPa. These values tend to increase when the exposure time of crosslinking increases. Mass losses were not considered significant for the crosslinked products, varying between 8 and 11%. However it was shown that the Dehydrothermal crosslinking minimizes mass losses when compared to composites without this treatment.

It is possible to conclude that a flexible bone graft was successfully developed. For that reason, the collagen / β-TCP composite showed a better handling when compared to calcium phosphates bone grafts.

Key-Words: Flexible Bone Graft, Type I Bovine Tendon Collagen, Calcium β-Triphosphate, Biomaterial and Composite

Resumo

Com o envelhecimento da população mundial, problemas ósseos são mais frequentes. Os enxertos ósseos são utilizados em tratamentos ou reparações de defeitos ósseos. O objectivo deste projecto foi desenvolver um enxerto ósseo flexível, de forma a ser ajustável ao problema do paciente.

O desenvolvimento deste produto consistiu em combinar uma proteína natural, colagénio tipo I, extraída de tendão de bovino, com β-TCP, um fosfato de cálcio. A sua composição foi de 10:90wt%, respectivamente. A sua produção incidiu num ciclo de liofilização e posteriormente reticulação térmica, tendo sido estudados três durações: 24, 48 e 72h. Os produtos foram caracterizados por DRX, FTIR, SEM, Método de Arquimedes, ensaios de compressão e TGA-DSC.

Todos os compósitos produzidos neste projecto apresentaram uma estrutura porosa, relevando valores entre 84% e 87%. As partículas de β-TCP encontravam-se distribuídas homogeneamente e sobrepostas na matriz de colagénio. As fases cristalinas foram estudadas, tendo-se identificado a presença de β-TCP em todos os compósitos. Os grupos moleculares foram identificados como referentes ao colagénio e ao β-TCP. Os picos de amida I, II, III, A e B observados tendem a ser menos pronunciados aquando do aumento do tempo de exposição de reticulação, enquanto os grupos fosfatos foram observados com clareza. Os produtos apresentaram valores de módulo de Young compreendidos entre 180 e 200 kPa. Os valores tendem a aumentar quando o tempo de reticulação aumenta. As perdas de massa não foram consideradas significativas para os produtos reticulados, tendo variado entre 8 e 11%. No entanto, foi apresentado que a reticulação térmica minimiza as perdas de massa quando comparado com os compósitos sem este tipo de tratamento.

Conclui-se que foi desenvolvido com sucesso um enxerto ósseo flexível. Desta forma, o compósito de colagénio/β-TCP apresentou melhor manuseamento quando comparado com enxertos ósseos de fosfato de cálcio.

Palavras-Chave: Enxerto Ósseo Flexível, Colagénio Tipo I de Tendão Bovino, β-Trifosfato de cálcio, Biomaterial, Compósito

Contents

Abbreviations

CaP	calcium Phosphates
Ob	osteoblasts
Oc	osteocytes
Ocl	osteoclasts
HAp	hydroxyapatite
β –TCP	beta Tricalcium Phosphate
BCP	biphasic calcium phosphate
ECM	extracellular matrix
Col-CaP	collagen-calcium phosphate composite
DHT	dehydrothermal
SEM	scanning electron microscopy
FTIR	fourier transform infrared spectroscopy
XRD	x-ray diffraction
TGA	thermogravimetric analysis
DSC	differential scanning calorimetry
Col/β-TCP	collagen/β-tricalcium phosphate composite

Symbols

L	liter
mm	millimeters
g	grams
°C	celsius degree
min	minutes
mbar	millibar
h	hour
kV	kilo volts
Å	ångström
mA	milli ampere
Θ	teta angle
°	degree
K/min	kelvin per minute
N	newton
mm/min	millimeter per minute
cm	centimeter
ε	strain
σ	stress
m^2	square meter
N/m^2 or Pa	newton per square meter or pascal
kPa	kilo pascal
MPa	mega pascal

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Motivation

With the aging of world's population, diseases and problems related to bones are becoming more frequent, as well as aesthetic concerns. According to experts, it is projected that, in a few years, 30% of hospitals will be occupied by osteoporosis patients, increasing the need to create and develop bone grafts that promote formation of new bone and improve population's life conditions [1]. For that reason, there is a growing need for this type of products. Nowadays (2018), the bone grafting industry is evaluated in about 2 billion euros, with the American continent holding about 50% of the market, while the rest is divided by the remaining continents. Due to confidentiality agreement, this market study cannot be quoted, however this information is updated and confirmed by the sales department of Medbone® - Medical Devices.

Medbone® - Medical Devices is a Portuguese company founded in 2008, being specialized in producing synthetic bone grafts that will be applied in medical surgery. The company's main objective is to develop and manufacture these products with the highest quality in the market in order to provide the best service to patients, improving their life conditions. At the moment, Medbone is represented, either in national and international market, in areas like dental, orthopaedic and veterinary and has available several products, such as Adbone® TCP, Adbone®BCP, Adbone® VCP and Adoss®TCP. Medbone® - Medical Devices always tries to evolve and follow market's needs and for that reason they work hard in order to develop and commercialize new products and innovations [2].

The bone grafting industry has a wide variety of products available on the market and consequently it can be said that there is a high competition. It is extremely important to know the competition's products and companies.

Bone grafts are normally used in the treatment and repair of bone defects. These bone defects occur in several sizes and shapes and it is not always easy to find the ideal dimension of bone graft to treat the defect. Custom-made bone grafts may not be the answer to this problem because they are a very expensive and not a sustainable solution. So, the ideal answer to this problem consists in producing a flexible bone graft that can easily adjust to the bone defect to create a stable graft and allow bone regeneration. By flexible, it is understood that the product lost stiffness when comparing to calcium phosphate bone grafts.

The main purpose of this project, "Development of Flexible Bone Grafts", was to produce an optimal flexible bone graft, adapting and combining the synthetic bone produced by Medbone® - Medical Devices and an animal protein, type I collagen, extracted from bovine tendon.

1 Introduction

1.1 Bone: Composition and Functions

Bone is a mineralized connective tissue which is mainly composed by an inorganic phase, calcium phosphates (CaP) and an organic phase, collagen [3,4]. It is responsible for locomotion in vertebrates, supports the body, has the capability to regenerate and protects vital organs in order to maintain their proper functioning [3–5]. Bones are constituted by four main different types of cells: osteoblasts (Ob), osteoprogenitor cells, osteocytes (Oc) and osteoclasts (Ocl), each one responsible for different activities [4,5]. Figure 1.1 is a schematic representation of different types of cells that compose natural bone, as well as their function.

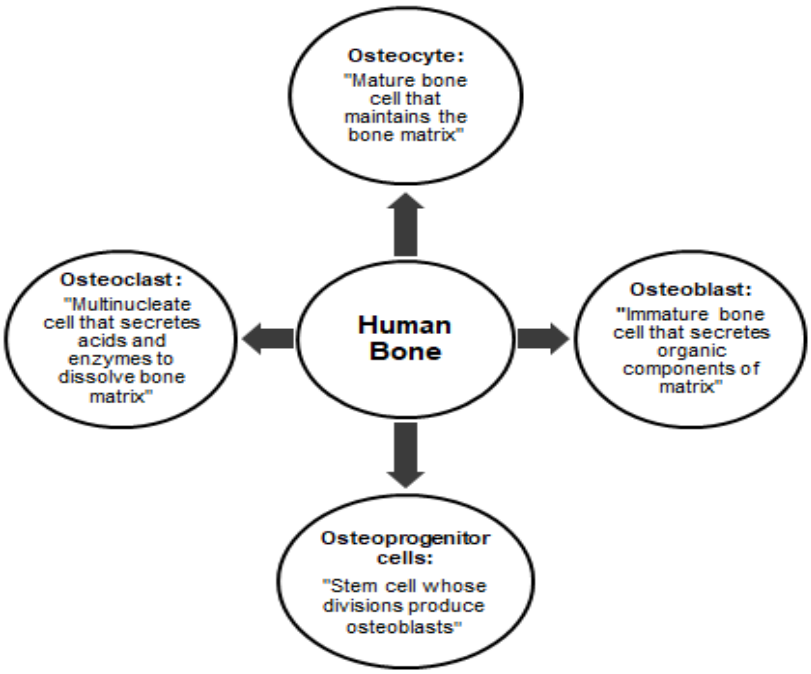

Figure 1.1. Schematic representation of different types of cells that compose the human bone, as well as their function in the body (Adapted from [4]).

1.2 Bone grafting

Bone grafting is a medical procedure that is used to repair or reconstruct bone defects [6]. Bone grafts can be used in fractures with bone loss, resulting from surgery, trauma, infections, congenital malformations and fragmentation. It also may be used in the treatment of bone cysts and tumors [6–8]. It is a very important treatment because it maintains bone contour, reduces infections and acts as an agglomeration of minerals that accelerate the formation of new bone [6–8].

The missing bone is replaced with materials, which can be from natural or artificial (synthetic) origin [8]. There are several materials used as bone grafts that can be applied, such as autografts, allografts, xenografts and synthetic (alloplastic) grafts [7–9]. Medbone only develops synthetic grafts, which were used in this project. Therefore, this type of grafts will be explained in more detail in this chapter.

To produce an optimal bone graft, several conditions and properties are important. This product should be bioactive, biocompatible, osteoconductive and osteoinductive [10]. It should ideally possess mechanical properties similar to the bone, exhibit a high porosity and be reabsorbable [10,11]. Table 1.1 shows several properties that bone grafts should display for medical applications.

Table 1.1. List of properties that bone grafts should display for medical applications. It is important to refer that the ones listed are biological mechanisms [10].

Property	Definition/Function
Bioactivity	"The inherent ability of a material to participate in specific biological reactions or have an effect on living tissues" [10]
Biocompatibility	"The ability of a material to perform with an appropriate host response in a specific application" [10]
Osteoconduction	"Ability to provide a scaffold for the formation of new bone" [10]
Osteoinduction	"The process by which osteogenesis is induced. This term means that primitive, undifferentiated and pluripotent cells are somehow stimulated to develop into the bone-forming cell lineage" [10]
Resorption	"Gradual degradation over time to replace the biomaterial with the natural host tissue" [10]

1.3 Synthetic grafts

Synthetic grafts are considered to be one of the most advanced subjects in the biomaterial science [12]. One of the main challenges in this industry is to create synthetically a graft that can match perfectly human bone tissue. Nowadays, there are several synthetic materials that can be applied in bone tissue, such as metals, polymers and ceramics, being the most commonly one used in scaffolds ceramics, more properly CaP [12–15].

There are several ceramics in the market that allow a total or partial regeneration of the affected area [16–21]. However, this depends on factors such as the properties of the material, size of the bone defect, area of application and the individual himself [6].

1.3.1 Calcium phosphates

CaP constitutes the largest group of biomineral found in the vertebrate animals, being this fact the main reason why these synthetic products are derived from them. They are composed by calcium and phosphate ions with a ratio relation between them [3]. This Ca/P ratio is - responsible for forming different types of products with different properties [1,10]. Parameters like absorption rate and mechanical properties are controlled by this ratio [22]. The higher the calcium-phosphate ratio, the lower the solubility of this product, so it is possible to conclude that the higher this ratio the lower the rate of resorption [22, 23].

These products are bioactive, biocompatible, their structure is similar to the mineral phase of bone and can be manufactured in order to have osteoconductive properties [12]. They are also characterized by their hardness, high compressive strength and high melting point [4]. However, these products present several disadvantages, such as, absence of an organic phase and a long and complex manufacturing procedure [12]. They are also brittle and have poor

shear and tensile properties [4]. These conditions can be explained due to their high porosity, where fractures in the structure begin [10]**.** In table 1.2, there are presented several CaP that are applied in the biomedical industry.

Table 1.2. List of CaP applied in the biomedical industry. They are listed according to name, symbol, chemical formulation and Ca/P atomic ratio [1,10].

Name	Symbol	Formula	Ca/P
Dicalcium phosphate	DCP	$CaHPO_4$	1.00
Dicalcium phosphate dehydrate	DCPD	$CaHPO_4 \cdot 2H_2O$	1.00
Octocalcium phosphate	OCP	$Ca_8H_2(PO_4)_2 \cdot 5H_2O$	1.33
α-Tricalcium phosphate	α-TCP	$\alpha - Ca_3(PO_4)_2$	1.50
β-Tricalcium phosphate	β-TCP	$\beta - Ca_3(PO_4)_2$	1.50
Sintered hydroxyapatite	SHA	$Ca_{10}(PO_4)_6(OH)_2$	1.67
Tetracalcium phosphate	TetCP	$Ca_{10}(PO_4)_2O$	2.00

CaP are applied in medical industry, more properly in the orthopaedics and dental fields, being the most common type hydroxyapatite (HAp), beta-tricalcium phosphate (β-TCP) and biphasic calcium phosphate (BCP) [12–14, 24, 25]. In Table 1.3, it is represented a list of some products, as well as their producers, that are available in the market. The calcium phosphate selected for this project was β-TCP due to Medbone´s market interest. When comparing the resorption rates of HAp, β-TCP and BCP, it is possible to verify that β-TCP presents the highest rate. By saying this, Medbone® - Medical Devices chose this calcium phosphate because the company desired a product with a fast resorption.

Table 1.3. List of products and companies that are available in the market. All the products listed are made from CaP, distributed according composition.

Composition	Products	Producers
HAp	Bone Source [26]	Stryker Orthopedics, USA
	IngeniOs® [27]	Zimmer, USA
	HAP-91 [16]	JHS Biomaterials, BRA
β-TCP	adbone®TCP [17]	Medbone, POR
	Cerasorb [18]	Curasan, GER
	SynthoGraft [19]	Synthograft, USA
BCP	adbone®BCP [21]	Medbone, POR
	MBCP™ [28]	Biomatlante, FRA
	BoneCeramic [29]	Staummann, SWI

1.4 Collagen

Collagen can be defined as an extracellular matrix (ECM) protein that has the capability of maintaining the structural integrity of body organs [30]. It represents about 30% of protein structure of vertebrates, with bones and tendons comprising 90% of collagen and skin comprising 50% [30–32]. Currently, there are twenty-eight types of collagen known to scientists, being collagen type I the most common and it can be found in bones, tendons, skin, eyes and ligaments [30,33]. Collagen is being applied in the biomedical industry due to its excellent biocompatible properties, pore structure and can be extracted from animal source, generally from bovine or porcine [11,34,35]. It is important to refer that this protein is easily degradable and consequently resorbed by the body, provides the cells attachment and exhibits flexibility properties [34,36–38]. However, it demonstrates low mechanical properties. Therefore, collagen is being combined with calcium phosphates in order to produce an optimal and flexible bone graft [34].

1.5 Collagen-calcium phosphates composites

One of the most challenges in biomedical industry is to develop a synthetic bone substitute that can perfectly match the natural bone [36]. Ceramics, such as HAp, β-TCP and BCP can be applied as a bone substitute, being these materials biocompatible and osteocondutive, in matter in fact, they are produced from a similar material to the inorganic phase of natural bone [4, 36, 39]. These biomaterials have a hard and brittle behaviour in compression. However, they do not have the proper properties to be perfectly applied in the body, having a poor shear and tensile properties [4]. On the other hand, collagen is also biocompatible, allows cells attachment, is resorbed by the body and exhibits flexible properties [36]. But like ceramics, these materials do not have the right properties, being collagen weak when suffering compression. For those reasons, the production of a bone substitute that combines inorganic-organic phase is being developed in order to create an optimal product [37,40].

Collagen-calcium phosphates composites (Col-CaP) are considered to be the most similar product to natural bone, due to the fact that natural bone is composed by collagen and CaP [37]. These composites allow the surgeon to have a better shape control, adapting the product to the patient's problem due to the fact that collagen is flexible, increase particle, wall adhesion and spatial adaptation [36]. When combining these inorganic and organic phases, osteogenesis is accelerated and osteoconduction is increased [36]. It provides higher stability and upgrade mechanical properties [36]. However, these composites are more expensive when comparing to only calcium phosphate grafts, being this the main disadvantage.

Summing up, Col-CaP composites have good mechanical properties and biocompatibility, are considered to be flexible and adjustable, promote healing process, improves cells attachment to the biomaterials and the beginning of bone regeneration [14,24,41]. HAp, β-TCP and BCP can be mixed into collagen, developing different products with different compositions [14,42]. The Col-CaP composites products provide an advantage in this industry when

compared to other products [36]. In table 1.4, there is a list of some products and producers available in the market that combines collagen and CaP with different compositions.

Table 1.4. List of collagen-calcium phosphate products that are available in the market. Manufacturers and composition of the product are also represented.

Product's Name	Producers	Composition
Osteon™Collagen [43]	GENOSS, KR	BCP + collagen
Collagraft® [44]	Zimmer, USA	BCP + collagen
CERACELL® Foam [45]	Curasan, GER	β -TCP + collagen
TCP-Plug Collagen Bone Graft Matrix [46]	Bio B2B, TWN	β -TCP + collagen
COL HAP-91 [47]	JHS Biomaterials, BRA	HA + collagen

1.6 Production Techniques for developing collagen/TCP composites

In this project, the lyophilization technique was used to produce the composite and then, a crosslinking treatment was performed.

Lyophilization is a process used to dry a material without changing its biological, chemical and physical properties, which means that, the solvent will be removed from the material without changing its properties [48,49]. All the activities are performed with an equipment called lyophilizer [48]. Therefore, the material is frozen and submitted to vacuum after cooling, where the ice changes directly from a solid to vapor phase without passing through liquid phase [48], [49]. This concept is known as sublimation [48,49]. In the chapter 2, during the production of the product, the lyophilization conditions, as well as, its steps will be properly explained.

Crosslinking is a treatment technique which is applied to improve the mechanical properties of products composed by collagen and biomaterials, in this case collagen and β-TCP [50]. This mechanical properties' improvement happens due to the formation of bonds between collagen network and molecules [50]. Crosslinking can be divided into two different techniques: chemical and nonchemical [50,51]. In this project, it was applied a nonchemical treatment, more specific, Dehydrothermal (DHT) crosslinking. This technique consists in applying a high temperature (over 98ºC) under vacuum conditions, with the purpose to remove water existing in the collagen molecules, leading to a formation of crosslinks between these molecules throughout condensation reactions [51]. It is considered to have advantage over chemical treatments because it does not cause adverse effects and does not modifies the structure of the composite, it creates strong links between the two phases (organic and inorganic) and it increases degradation time [51]. For those reasons, the crosslinking nonchemical via DHT was always the initial idea for this project.

2 Materials, Methods and Characterization Techniques

2.1 Type of Product Developed

The product developed in this project was a collagen/ β-TCP (Col/β-TCP) composite with 10:90 wt% of composition, respectively. The composition's choice is based on the market's study performed in partnership with Medbone® - Medical Devices with potential clients. Therefore, it is important not to limit the functionalities of the synthetic bone by placing large percentages of collagen because it is this bone that will promote bone remodelling. The collagen added to Medbone´s synthetic bone was only intended to confer flexibility to the composite.

2.2 Experimental Steps

This project was divided into two important steps. The first step consisted in the production and development of the Col/β-TCP composite. The second step consisted in the composite's characterization, where the base products (bovine tendon type I collagen and β-TCP), as well as, several experimental products of Medbone and commercial products from different companies, were used to compare information with the results obtained from the flexible bone graft manufactured. The second part of this project will be introduced in Chapter 3; however in this chapter, it will be described the techniques used in product's characterization.

2.3 Experimental Procedure

2.3.1 Synthetic Bone and Collagen' Preparation

The synthetic bone used in this project was produced by Medbone® - Medical Devices and due to confidentiality agreement, the production methods cannot be revealed. This synthetic bone was composed by β-TCP, had a particle size of 0.1-0.5 mm and it is applied in the dental industry. The product's description, as well as its characteristics, can be consulted through the web page of the company and will be placed in this master thesis. The company supplied its bone already developed and for that reason, it is assumed as a raw material in this project.

Next, it is presented Medbone's web page for consulting the product used this project. http://www.medbone.eu/en/products/dental/adbone-tcp.html

The collagen used in this project was type I, extracted from bovine tendon and treated by an external commercial company. This type of collagen was selected because it is considered to be the most common and study in the market, being well accepted for the scientific community.

2.3.2 Production of the Collagen/β-TCP composite

The production and development of Col/β-TCP composite was divided into several steps, which will be further explained:

1. It was prepared a sample of bovine tendon collagen type I with 20g, a sample of β-TCP (0.1-0.5 mm) with 180g and 1L of distilled water at the temperature of 5ºC. It is important

to control the water's temperature and keep it down because the solution's mixing produces heat and this low water's temperature is a method to protect the samples.

2. After being all prepared, mixing was performed with the help of a blender (Waring Commercial), where the distilled water, collagen and β-TCP were combined. First, it was mixed the collagen with the distilled water for about 3 minutes. Then, β-TCP was added to the blender and mixed for about two more minutes. The mixing speed was 10 (maximum speed according to the blender's specifications) and maintained during this time.
3. Simultaneously, as the mixing takes place, the tray where the sample was placed was prepared. This tray plays an important role because the greater its thickness, the lower is its cooling rate and less homogeneous is the final sample. For that reason, the chosen tray was a plastic thin film. It was chosen by Medbone® - Medical Devices and it is considered to be one of the most efficient trays available by the company.
4. The content previously prepared was deposited on the tray. This deposition has to be performed slowly and progressively in order to spread the content in a homogeneous way. There is a part of the content that remains in the bender, so the person performing this experiment should have this in consideration. It is important to take advantage of the vast majority of the content because a big waste could lead to wrong products' compositions.
5. After the deposition, the sample was placed into vacuum equipment (VACUCELL) with the purpose to remove air bubbles that were present in the sample. This process took about 20 minutes and it was performed at 20°C. It is important to refer that when opening the machine to place the sample in the lyophilizer, it always ends up forming new air bubbles. However, it is in a much smaller quantity. There is no way to control this bubble formation during this stage.
6. After completing all the previous stages, lyophilization process started to be prepared. This process was performed using a lyophilizer Telstar LyoBeta. The lyophilizer shelves (that are inside the equipment) were cooled until -40°C was reached. It was needed 15 min to perform this activity. The equipment has a probe that controls the interior temperature of the sample. It is important that this probe has the same temperature as the shelves. However, there is a difference between the temperatures of those two parts, being the shelves close to what is desire (major goal when the Lyophilizer is being prepared). The probe is shown in figure 1 a) of appendix.
7. After this preparation and before its opening to place the sample, the condenser was set at room temperature (20°C) and the vacuum was 2mbar.
8. The sample was then placed in the lyophilizer. When it was opened, a variation of temperature occurred, being this variation felt more in the probe than in the shelves. For that reason and to control the temperature, before starting the cycle, the shelves and probe's temperature were allowed to stabilize and return to the desired temperature, -

40°C. This stabilization took about 1h30 min. It is important to refer that the probe's temperature took more time to reach this temperature than the shelves.

9. After this preparation, lyophilization process was started. The initial temperature of the process (which is given by the probe) was -38.9ºC and the shelves' temperature was -40ºC, being heated until 55ºC during 6 hours and then followed by a secondary drying until 25ºC for 1 hour. The process is monitored by Telstar LyoBeta Software and it is important to have a tight control during the cycle.

Table 2.1. Lyophilization cycle used to produce the composite.

Lyophilization steps	Maximum Temperature (ºC)	Time
Freezing	-40	15 min
Primary drying	55	6h
Secondary drying	25	1h

10. After completing lyophilization, the sample was left at room temperature (around 20°C) in the cleanroom. A sample for analysing its properties before crosslinking was prepared. In figure 1 b), c) and d) of appendix, it is represented the sample's thickness, as well as the sample itself, after completing lyophilization.
11. Crosslinking was performed via DHT by a vacuum oven (VACUCELL) with 3mbar at 110°C during three different duration time: 24, 48 and 72 h. It was prepared three samples to observe the effect of crosslinking in their properties, as well as study the time's influence in the samples' properties.

2.3.3 Collagen/β-TCP composite characterization

After manufacturing the composite, the next step consisted in its characterization. The raw materials used to produce the flexible bone graft were analysed and used to compare with the final products. For that reason, it was prepared two different types of samples: one of the synthetic bone produced by Medbone (β-TCP) with 0.1 - 0.5 mm and one of commercial collagen (Samples A and B, respectively). It is important to note that the ceramic supplied by Medbone® - Medical Devices is considered to be a raw material, although it has undergone treatment to achieve the form that was used in this project. However, due to the agreements of confidentiality with the company, these treatments cannot be mention. It is also important that the ceramic shows a small grain size with the purpose to be easily combined and mixed with collagen matrix.

For a better understanding of this project, several samples were prepared: three samples of lyophilized collagen with different types of trays and with 0.5L of distilled water (Samples C, D and E); one sample of lyophilized collagen with 1L of distilled water (Sample F); one sample of collagen combined with β-TCP with a composition of 40% and 60%, respectively (Sample G); four samples of products developed by different companies (Col-HAp from JHS Biomaterials,

Osteogen P from Impladent LTD, Osteogen S also from Impladent LTD and Matri Bone from Biom'up).

This part was taken into account for this project because, in this way, it is possible to observe the effect of lyophilization in samples of collagen with different amounts of water and different forms of manufacturing, being possible to optimize the lyophilization process. It was prepared three samples of lyophilized collagen with 0.5L of distilled water because these samples were manufactured using different trays (thin, medium and large thickness trays), which affected directly the lyophilization's conditions. The Col/ β-TCP sample with 40:60 wt% was study to compare products with different compositions (10% collagen and 90% β-TCP). The samples from other companies were only analysed by Scanning Electron Microscope (SEM) technique and were used to compare them with the final product.

In table 1 of Appendix, it is summed up all the samples used for characterising the product.

2.4 Characterization techniques

The grafts previously manufactured were characterized with the purpose of analysing and describing their physical, chemical and mechanical properties. For that reason, several tests were performed, such as SEM, XRD (X-Ray Diffraction), FTIR (Fourier Transform Infrared Spectroscopy), porosity measurements, Compression Test and DSC-TGA (Differential Scanning Calorimetry- Thermogravimetric analysis) [51,52].

Next, the techniques used for the products' characterization will be explained.

2.4.1 XRD (X-Ray Diffractometry)

X-Ray Diffractometry is a characterization technique which was used to identify the presence of β-TCP in the produced samples.

The equipment used in this characterization was a PANalytical's X'Pert PRO MRD using a Cu-Kα (wavelength of 1.540598 Å) radiation with a potential acceleration of 45 kV and current beam of 30 mA. The scans were performed with 2θ values from 10º to 90º [53]. The crystalline phases were identified by comparing the peaks positions and intensities, which were obtained from the diffractogram, with the standard files from JCPDS (#09-0169). The equipment used is represented in figure 3 b) of Appendix.

2.4.2 FTIR (Fourier Transform Infrared Spectroscopy)

FTIR is a characterization technique which is used to identify the molecular groups in materials; in this case, it was used to identify the organic and inorganic compounds of collagen, β-TCP and Col/β-TCP samples. It was also used to observe if any modifications occurred when the samples suffer treatment by crosslinking.

FTIR characterization was performed using a FT-IR Nicolet 6700 – Thermo Electron Corporation (CENIMAT/I3N) with a 4 cm^{-1} resolution in a wave number range of 520-4500 cm^{-1}. It was also used to perform this analysis the KBr pellet method [54]. The equipment used is represented in figure 3 a) of Appendix.

2.4.3 SEM (Scanning Electronic Microscope)

Scanning Electronic Microscope is a characterization technique which is used to analyse the surface's morphology, defines the sample´s microstructure composition. In this project, SEM was performed with the purpose to observe the structure of Col/β-TCP composites produced and its components. It was also performed in order to study the optimization of lyophilization cycle as well as products from different companies.

The sample´s microstructure was analysed by a Tabletop Microscope TM3030 Plus +Quantax 70: Scanning Electron Microscopy (Hitachi) with a voltage of 5kV. It is important to refer that the samples were not covered with any type of conductive material, being the samples under low vacuum conditions [55]. The equipment used is represented in figure 2 of Appendix.

2.4.4 Porosity Measurements

The porosity of the samples performs an important function in the bone graft-cells interaction and consequently, it influences the choice of the composite to be applied.

The samples' porosity was measured using the Archimedes method. This is considered to be a fast process to determine the porosity and also less expensive.

In order to perform this process, three samples were prepared: one composite with 24h of crosslinking, another one with 48h and lastly one with 72h of crosslinking. The initial mass of the composites previously prepared was measured (m_{dry}) and after that these were immerse in a recipient with ethanol, being their mass posteriorly measured ($m_{impregnated}$). Lastly, the mass of composites when suspended in the ethanol was measured ($m_{suspended}$) [56].

For this purpose, Open Porosity (P_{open}) and Closed Porosity (P_{closed}) were determined using equation 1 and 2:

$$Open\ Porosity\ (P_{open}) = \frac{V_{open\ p}}{V_t} \times 100\% \qquad \text{Equation (1)}$$

$$Closed\ Porosity\ (P_{closed}) = \frac{V_{closed\ p}}{V_t} \times 100\% \qquad \text{Equation (2)}$$

Where $V_{open\ p}$ represents the volume of the open pores, V_t represents the total volume of the composite and $V_{closed\ p}$ represents the volume of closed pores.

In Appendix, it is explained the equations applied for the calculations of open and closed porosity.

2.4.5 Mechanical Testing - Compressive test

Compressive test was performed with the purpose to study the behaviour of collagen/β-TCP composites when it is suffering a compressive load. This characterization test recorded the evolution of the strain (ε) and stress (σ) variables during the compression test, and these values were used to determine stress-strain curves [57,58]. These curves allowed the quantification of parameters such as the Young's Modulus, collapse stress and the initial extent of densification.

In chapter 3, section 3.5 will be explained how strain and stress variables, as well as the parameters described previously, were calculated. This study was also important in order to compare the effect of crosslinking in the mechanical properties of composites and test several crosslinking conditions, where mechanical properties were compared.

This test was performed using a Rheometrics Scientific MINIMAT Model Firmware 3.1 at room temperature (around 24ºC) with a static load cell of 20N and a loading speed of 1mm/min. They were performed between parallel steel plates. The equipment used is represented in figure 4 of Appendix.

Five samples were prepared (G, H, I, J and L), each one was divided into 10 prismatic different specimens. The specimens had lengths and widths of less than 2.5 and 1.5 cm, respective, due to the maximum dimensions of the equipment's sample holder. Statistical analysis was performed using the Software IBM SPSS Statistics 22 and all the results of Young's Modulus were determined with a 95% interval confidence for the mean.

2.4.6 DSC-TGA (Differential Scanning Calorimetry-Thermogravimetry Analysis)

Differential Scanning Calorimetry is a thermal analysis technique that was used to measure the amount of energy absorbed or released by the sample, when it is heated or cooled, being possible to quantify endothermic and exothermic processes. By other words, this means that this technique will analyse the material's behaviour in terms of heat capacity when the temperature changes.

Thermogravimetry Analysis is a thermal technique that is used for measure the mass loss of the samples over time or temperature.

For the DSC-TGA analysis, a Simultaneas Thermal Analyser STA 449 F3 Jupiter with a heating up to 1000ºC and a ramp of 10 K/min was used [59]. The equipment is represented in figure 3 c) of Appendix.

3 Results and Discussion

As previously stated in chapter 2, samples were characterized via XRD, FTIR, SEM, porosity measurements, compression test and DSC-TGA.

3.1 DRX analysis

The XRD technique was used to identify the presence of β-TCP. In figure 3.1, it is shown the diffractogram of β-TCP sample, produced by Medbone® - Medical Devices. It was compared with the literature using data sheet from JCDP no. 09-0169, corresponding to whitlockite ($Ca_3(PO_4)_2$).

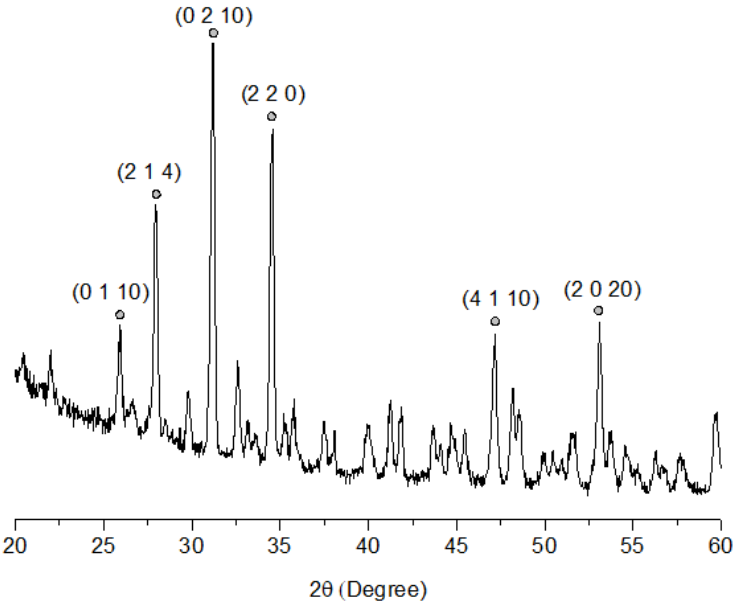

Figure 3.1. Diffractogram of β-TCP sample. The circles represent the characteristic peaks of β-TCP.

Throughout the diffractogram's analysis (figure 3.1), it was possible to identify six characteristic peaks of β-TCP, being these represented in the graphic by a circle. From the spectrum obtained, the characteristic peaks of β-TCP were, respectively, 25.89º (1 0 10), 27.95º (2 1 4), 31.19º (0 2 10), 34.57º (2 2 0), 47.19º (4 0 10) and 53,05º (2 0 20). These obtained values were compared with the JCPDS file #09-0169 and it is easy to perceive that the only peaks observed were the characteristic peaks from β-TCP. Being that said, it is possible to confirm that β-TCP did not suffer any modification during the sintering process. All diffraction peaks were considered to be sharp and well defined, which means that the sample analysed showed a highly crystalline structure. This aspect is compatible with the information provided by Medbone® - Medical Devices, where its product is higher than 98% crystalline.

Figure 3.2 a) represents the diffractogram of Col/β-TCP (40:60 wt%), which was an experimental product manufactured by Medbone® - Medical Devices. This sample was considered to be important in this project because it was used to compare with the final product. This means that this composite gives a view of different composition structures and their influence on the main properties. Figure 3.2 b) shows the diffractogram of Col/ β-TCP (10:90

wt%) without crosslinking. Both samples were compared with the literature using data sheet from JCDP no. 09-0169, corresponding to whitlockite ($Ca_3(PO_4)_2$). In figure 3.3, it is represented the diffractogram of the collagen.

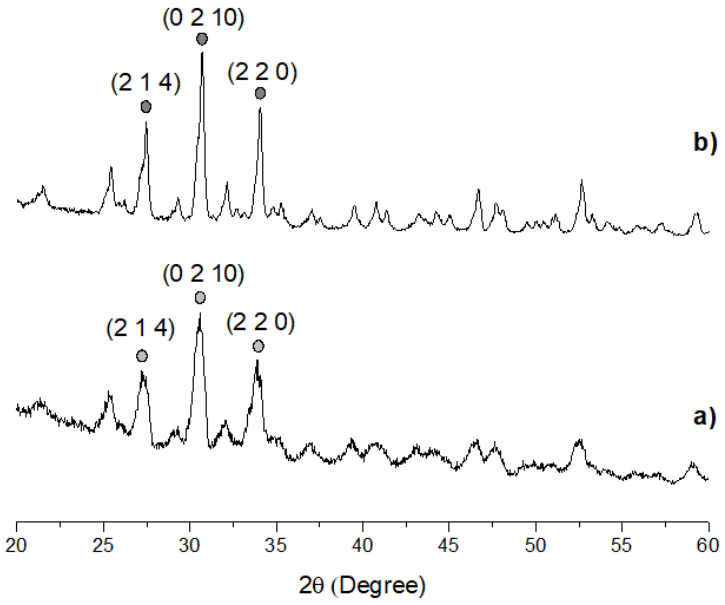

Figure 3.2. Diffractogram of Col/β-TCP samples without crosslinking stage: a) composite with composition of 40:60 wt%; b) composite with composition of 10:90 wt%. The circles represent the characteristic peaks of the composites.

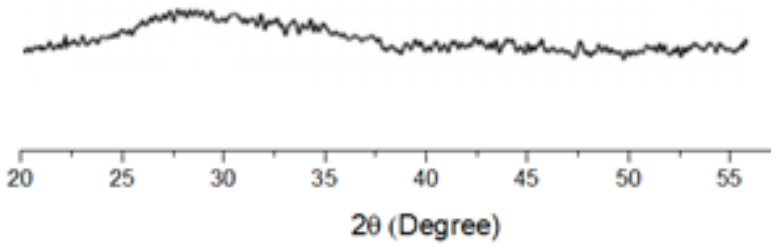

Figure 3.3. Diffractogram of collagen.

The bovine tendon type I collagen used to produce these samples does not have characteristic peaks in the diffractogram because collagen has an amorphous structure, aspect that is possible to verify in figure 3.3. However, the presence of collagen can be observed around 2θ values between 25º and 35º since, analysing figure 3.3, it is in this 2θ region that a variation occurs. In addition, it can also be visually detected in XRD of figure 3.2 due to the fact that, in this range of 2θ, is verified the enlargement of the peaks when comparing with the diffractogram containing only β-TCP.

From the diffractogram obtained in figure 3.2 a), it is possible to identify three major peaks, being these in 27.21º, 30.59º and 33.90º, which are associated to the presence of β-TCP. From the diffractogram obtained in figure 3.2 b), it can be observed three major peaks, being these in 27.48 º, 30.73º and 34.07º, which are also related to the presence of β-TCP. All the previously values are properly identified by a circle.

When comparing these two samples, both without the crosslinking stage, it is likely to say that, according to the figure 3.2, the curve b) has a higher crystallinity. The width at half peak is lower in the Col/β-TCP composite with 10:90 wt% composition, suggesting that this composite is more crystalline. Another factor that allows proving that Col/ β-TCP composite with 10:90 composition has a higher crystalline structure is the intensity of the characteristics peaks, which are clearly higher for this sample when comparing to Col/β-TCP composite with 40:60 wt% composition.

The XRD characterization of the composites with crosslinking was not performed. Since the thermal crosslinking was performed at a temperature of 110ºC, it is considered that this treatment will not cause a phase change in the respective samples. This means that, there is no phase change until the 200°C. In this way, the XRD results for the crosslinking composites would be exactly the same as the XRD results for the composite without crosslinking, previously described. This aspect will be further observed and proved in section 3.6 through DSC-TGA analysis.

3.2 FTIR Analysis

Figure 3.4 shows the Fourier Transform Infrared spectra of β-TCP (a), Collagen (b), Co/β-TCP composite with 40:60 wt% composition without crosslinking treatment (c) and Col/β-TCP composite with 10:90 wt% composition without crosslinking treatment (d).

The spectrum of β-TCP, shown in figure 3.4 a) , is composed by characteristic peaks of phosphates groups, which are located between 900 to 1160 cm^{-1} , more properly, at 1115, 1078, 1112 and 941 cm^{-1}. These peaks can be assigned to vibrational mode of $\upsilon_3PO_4^{3-}$. According to the literature, the peaks represented at 968 and 941 cm^{-1} are considered to be confirmations for the existence of β-TCP in the sample. Groups of $\upsilon_4PO_4^{3-}$ are located at 602 and 577 cm^{-1} in β-TCP sample [65,66].

For collagen sample (figure 3.4 b), it is possible to identify the characteristic band of amide I at around 1626 cm^{-1}, where the C=O stretching of carboxamide functional groups along the polypeptide matrix is observed. The peak at around 1533 cm^{-1} can be identified as amide II band, being related to its N-H bending vibrations and the peak at around 1231 cm^{-1} is related to the C-H stretching of amide III. According to the literature, a collagen characteristic peak can be assign to the broad band with range of 3200 cm^{-1} to 3600 cm^{-1}. By analysing the collagen curve from the infrared spectra (b), this information can be confirmed. This broad range can be assign to the N-H stretching at around 3292 cm^{-1} for amide A and the N-H deformation at around 3075 cm^{-1} for amide B [66–68].

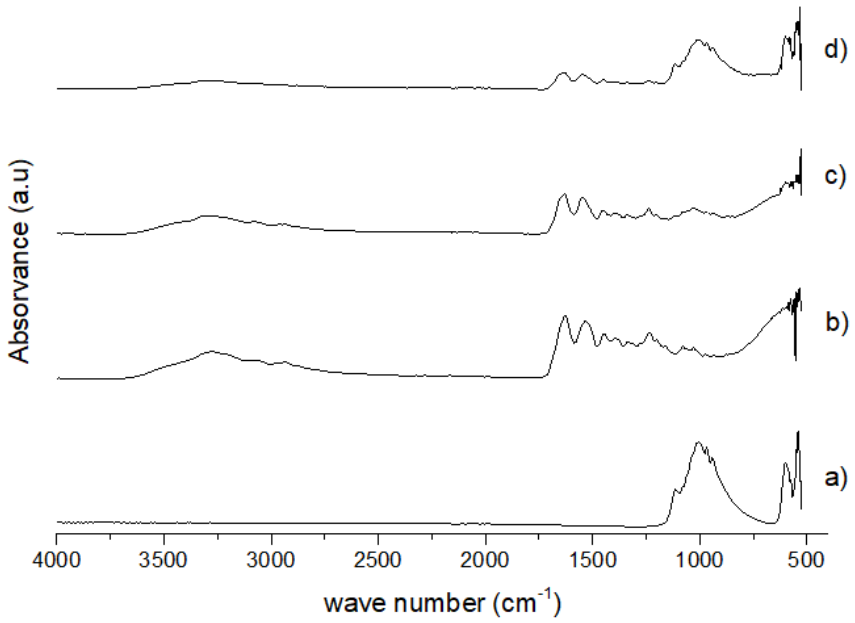

Figure 3.4. Fourier Transform Infrared spectra of: a) β-TCP sample; b) collagen sample; c) sample of Col/β-TCP composite with 40:60 wt% composition without crosslinking treatment; d) sample of Col/β-TCP composite with 10:90 wt% composition without crosslinking treatment.

By analysing the infrared spectra in figure 3.4 c) and d), it is possible to say that both characteristics peaks of collagen and β-TCP are observed in the infrared spectrum of Col/β-TCP composite. However, due to different composite compositions (40:60 wt% and 10:90 wt% of collagen and β-TCP, respectively), it is possible to observe that, by varying the percentage of collagen and β-TCP, their characteristic peaks will be more significant or not in the FTIR spectrums. This means that, by increasing the percentage of β-TCP and consequently decreasing the collagen's percentage in the composite (60% to 90% and 40% to 10%, respectively), the characteristic peaks of β-TCP will be more visible when comparing to the collagen ones.

The results obtained confirm what was said previously, and it can be deduced that the higher the percentage of β-TCP, more visible will be its peaks in the composite.

In Col/ β-TCP composite with 40:60 wt% composition without crosslinking (figure 3.4 c), it can be observed that, in characteristic range of 900 to 1160 cm^{-1} , groups phosphates, which are derived from β-TCP, are still poorly visible. The amide I (1635 cm^{-1}), II (1540 cm^{-1}) , III (1444 cm^{-1}) and Amide A (3292 cm^{-1}) and B (3068 cm^{-1}) functional groups can be clearly observed, however it is not as pronounced and intense as the sample with only collagen, aspect that was supposed to occur. An interesting and peculiar aspect in this sample remotes to the fact that the β-TCP characteristics peaks obtained are not so intense, which means that, being the sample composed by a higher percentage of ceramic, the peaks should be more intense and wider, something that does not happen. Loss of mass of raw material, condition mentioned in chapter 2, may be behind this event. For that reason, it is necessary to have a better control in the process of mixing these two compounds.

In Col/ β-TCP composite with 10:90 wt% composition without crosslinking (figure 3.4 d), the characteristics peaks of β-TCP can be clearly distinguished, where phosphates groups can be

identified at range of 900 to 1160 cm^{-1}. The amide I (1635 cm^{-1}), II (1540 cm^{-1}) and III (1444 cm^{-1}) functional groups can also be visible; however it is not as pronounced as the sample with 40:60 wt% composition. When comparing to the same sample, it also has a decrease in the peaks' intensity. The same aspects occurred to Amine A and B, however it is not possible to determine the peaks because it is assigned as a broad region.

Figure 3.5 shows the infrared spectra of β-TCP sample (a), collagen sample (b), Col/β-TCP composite with 10:90 wt% compositions with 24h, 48h and 72h of crosslinking treatment (c, d and e, respectively). The main purpose of this FTIR characterization was to compare the effect of crosslinking in the characteristic peak.

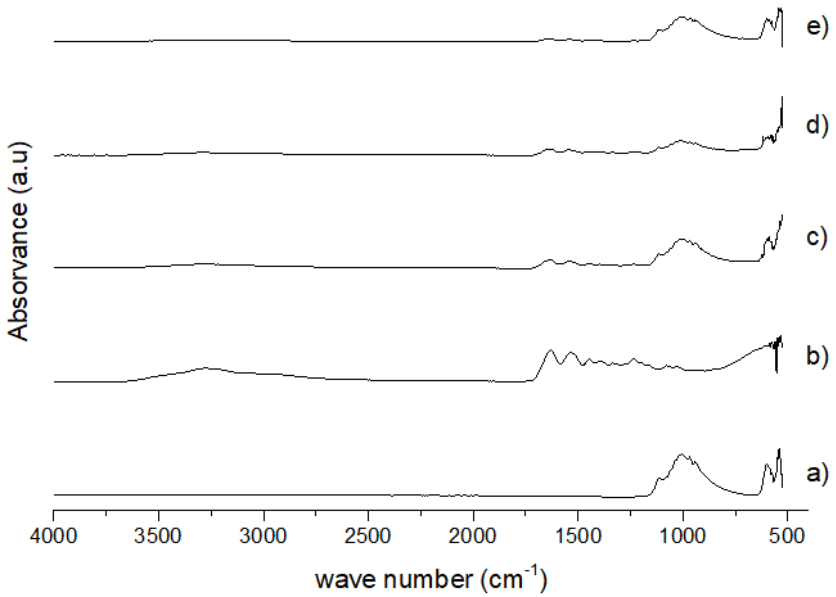

Figure 3.5. Fourier infrared spectra of: a) β-TCP sample ; b) collagen sample; c) Col/ β-TCP composite with 10:90 wt% composition with 24h of crosslinking treatment; d) Col/ β-TCP composite with 10:90 wt% composition with 48h of crosslinking treatment; e) Col/ β-TCP composite with 10:90 wt% composition with 72h of crosslinking treatment.

By analysing the spectrum in figure 3.5, it is possible to verify that there is a pattern within the characteristic peaks of collagen: as the time of crosslinking increases, these peaks become less pronounced and more difficult to identify.

As previously said in Chapter 1 and 2, the crosslinking performed in the samples was via DHT. This type of crosslinking can be controlled by analysing the amide II absorbance peak at 1533 cm^{-1}. The amide II band at this peak is considered to be proportional to the formation of NH_2, which during the formation of crosslinks through condensation reactions, is converted to NH. For that reason, a decrease in the absorbance at peak 1533 cm^{-1} corresponds to an increase in the number of crosslinks. According to the literature [64], if the peak's absorbance at 1533 cm^{-1} is normalized to peak's absorbance at 1450 cm^{-1} (which is not affected by the formation of crosslinks or denaturation), it defines a ratio. The inverse of this ratio is defined as the effective crosslink density, which means that the decreasing of this ratio causes an increasing in the number of crosslinks. This ratio is used to prove that the number of crosslinks

increased as the crosslinking time increased, causing an augmentation of NH, which leads to a less pronounced peak. Through the infrared spectrum obtained in figure 3.5, it is possible to prove to that as the crosslinking time increases, the peak where Amide II band is located is no longer so pronounced. This aspect is also valid for Amide A, B, I and III peaks.

In relation to β-TCP, it is verified that the time of crosslinking did not cause any changes in its characteristic peaks since the temperature used in crosslinking was not enough to cause any changes. Lastly, it is possible to say that both characteristics peaks of collagen and β-TCP are observed in the infrared spectrum of Col/β-TCP composite with crosslinking, however for collagen the peaks turn out to be less pronounced, aspect that was explained above.

3.3 SEM Analysis

In figure 3.6, it is observed the β-TCP and commercial bovine tendon type I collagen used in this project. According to the data supplied by Medbone, the grain size of β-TCP used was comprised between 0.1 and 0.5 mm. This SEM image was taken with the aim to observe the different grain sizes used in the product development. Through the software Image J version 1.51j8, it was possible to confirm that its size varies in the range of values considered above, being these represented in a bar graphic (figure 3.7). It should be noted that the image reflects only a small portion of β-TCP used and only 30 measurements were taken to determine the grain size.

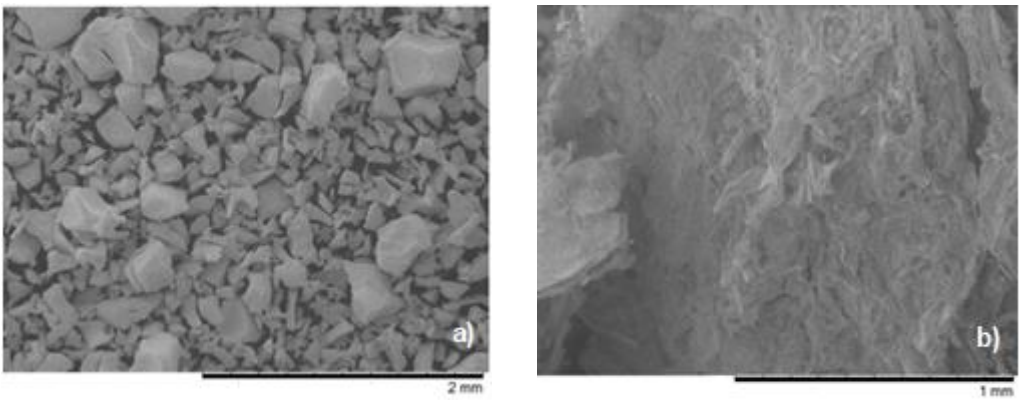

Figure 3.6. SEM images of: a) β-TCP sample, which was produced by Medbone; b) commercial collagen sample, which was extracted and treated by an external company.

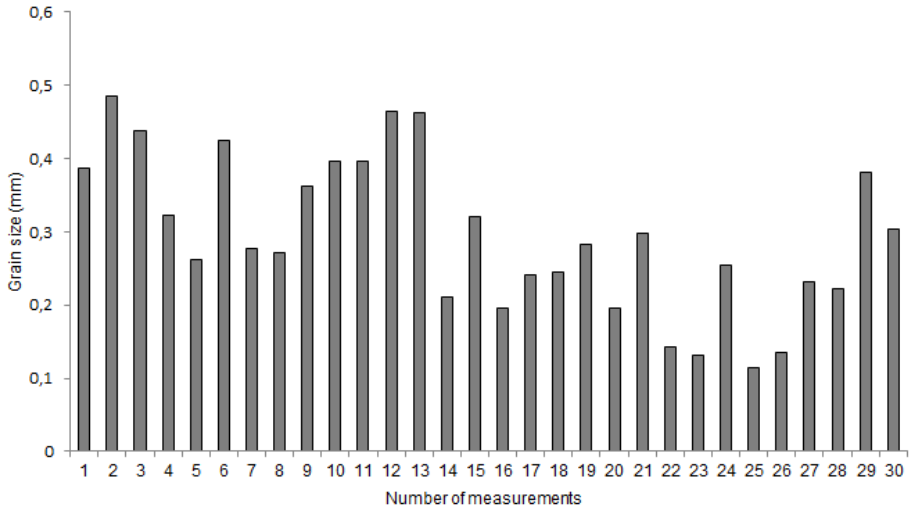

Figure 3.7. Bar graphic representing the grain size of the β-TCP product. All the measurements were between 0.1 and 0.5 mm, information proven by Medbone®- Medical Devices.

The collagen, in its natural form, tends to bond, forming dense structures, where it becomes difficult to add calcium phosphates, in this case β-TCP particles [56]. As the collagen used in this project was a commercial type, extracted by an external company, it is known by anticipation that a chemical treatment and respective lyophilization cycle had been performed. The collagen's structure derives from its method of treatment and processing. These conditions caused the collagen fibers to unwind and spaces between them were created. For that reason, the collagen fibers developed into a porous structure, being able to receive other compounds (β-TCP) [56]. By analysing figure 3.6 b) it is possible to observe a porous structure of collagen, an aspect that coincides with the manufacturer's information.

In figure 3.8, there are represented several Medbone's experimental products: a) shows a collagen lyophilized scaffold that was produced with 0.5 L of distilled water using a thin tray; b) shows a collagen lyophilized scaffold with 0.5 L of distilled water using a medium thickness tray and different lyophilization conditions; c) shows a collagen lyophilized scaffold with 1L of distilled water using a large thickness tray and different lyophilization conditions.

The porosity is considered to be a very important condition for bone tissue formation, allowing migration and proliferation of osteoblasts and other cells. It also improves mechanical stability between the synthetic bone and the natural one [65]. This porosity is achieved, in this project, by lyophilization. For that reason, it is critical to create an optimal cycle, which is achieved by optimizing several conditions that are reported in the literature [51,61,73]. According to Medbone® - Medical Devices previously studies, the type of tray used in lyophilization cycle may also influence the structure of the product. As mentioned in Chapter 2, section 2.3.2, point 4, the chosen tray can induce changes in the samples' homogeneity.

These products were studied with the aim to obtain the right cycle of lyophilization and to ensure the best tray choice to be applied for the accomplishment of this activity. The amount of distilled water used (0.5, 0.5 and 1L, respectively) only influences the final thickness of the product. It is important to refer that these samples were only composed by collagen. The

lyophilization conditions cannot be revealed in order to protect the major interests of the company.

In figure 3.8 a), it is possible to observe that the sample is composed by a highly porous homogeneous structure, where the collagen network is well defined and developed. Figure 3.8 b) shows a porous structure, however it presents lower standers when comparing to the figure a). Its homogeneity was also not reached, suggesting that the lyophilization cycle and tray's choice was not the right one. Lastly, the same aspects of figure 3.6 b) occurred in figure 3.8 c).

With the use of these SEM images, it was possible to overcome the problems occurred with the lyophilization and assume the right tray to perform this stage.

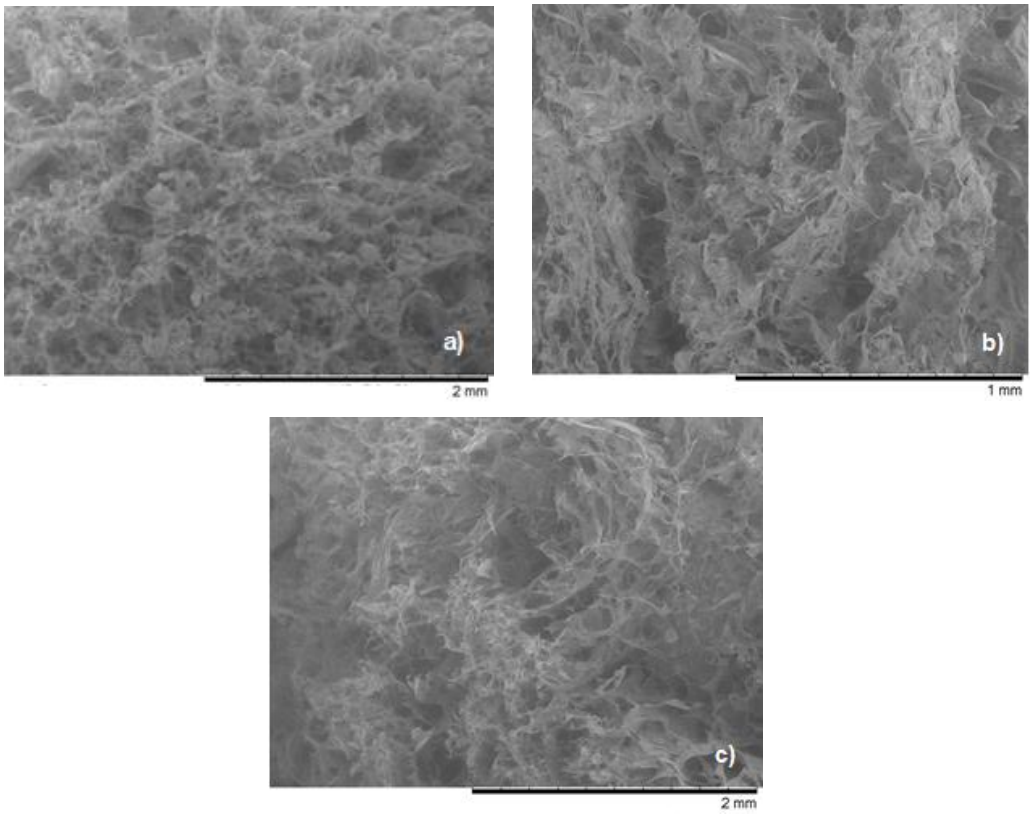

Figure 3.8. SEM images of several Medbone's experiments, which were used to obtain the right cycle of lyophilozation. All these experiments were collagen lyophilized scaffolds produced using different conditions: a) 0.5 L of distilled water using a thin thickness tray; b) 0.5 L of distilled water using a medium thickness tray; c) 1 L of distilled water using a large thickness tray.

Figure 3.9 represents the composite with Col/β-TCP with 40:60 wt% composition (a) and Col/β-TCP with 10:90 wt% composition (b), both without crosslinking stage.´

These composites were observed in order to morphologically compare the effect of different amounts of β-TCP particles. By analysing the figure 3.9 a) and b), it is possible to infer that there were differences in the ceramic distribution among the collagen. This aspect is inevitably associated with the fact that the composite shown in figure b) presents a larger amount of β-TCP particles. Therefore, an increasing in the composite of the amount of β-TCP promotes the overlap in the collagen matrix [56]. The composite in figure a) also shows the collagen matrix more visible than figure b). Both composites seemed to have well incorporated β-TCP particles in the collagen matrix; however this aspect is, apparently, slightly higher for the composite with

higher amount of β-TCP (figure b). This situation could be associated to less efficient manufacturing process, more properly in the mixing process and lyophilization cycle (referred in chapter 2, section 2.3.2). Both figures were taken from the central region of the samples.

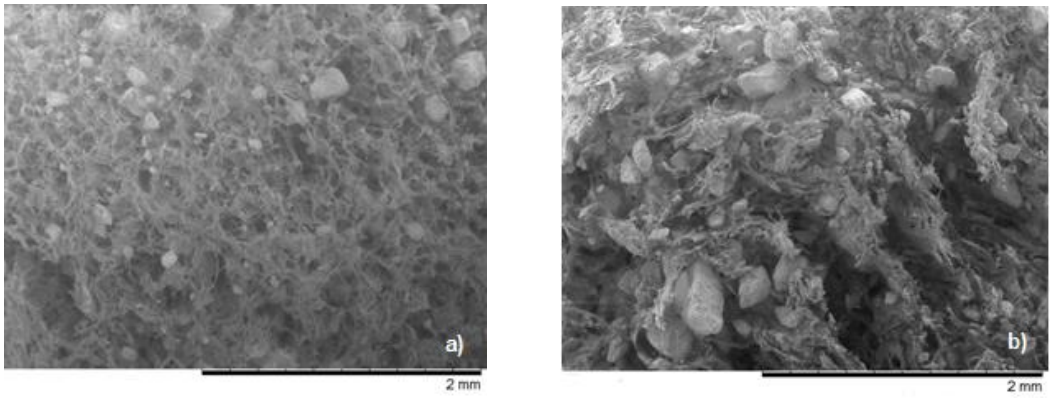

Figure 3.9. SEM image of the Col/β-TCP composites with different compositions and without crosslinking treatment: a) 40:60 wt%; b) 10:90 wt%.

Next, the composites' morphologies resulted from crosslinking stage of 24h, 48h and 72h are, respectively, shown in figure 3.10 a), b) and c). The main difference between these composites was their exposure time to crosslinking via DHT, which causes an increase in stiffness of collagen matrix. In section 3.5, this aspect will be verified by the determination of Young's Modulus. All the composites have a porous structure, where the β-TCP particles seemed to be well integrated and overlapped in the collagen matrix.

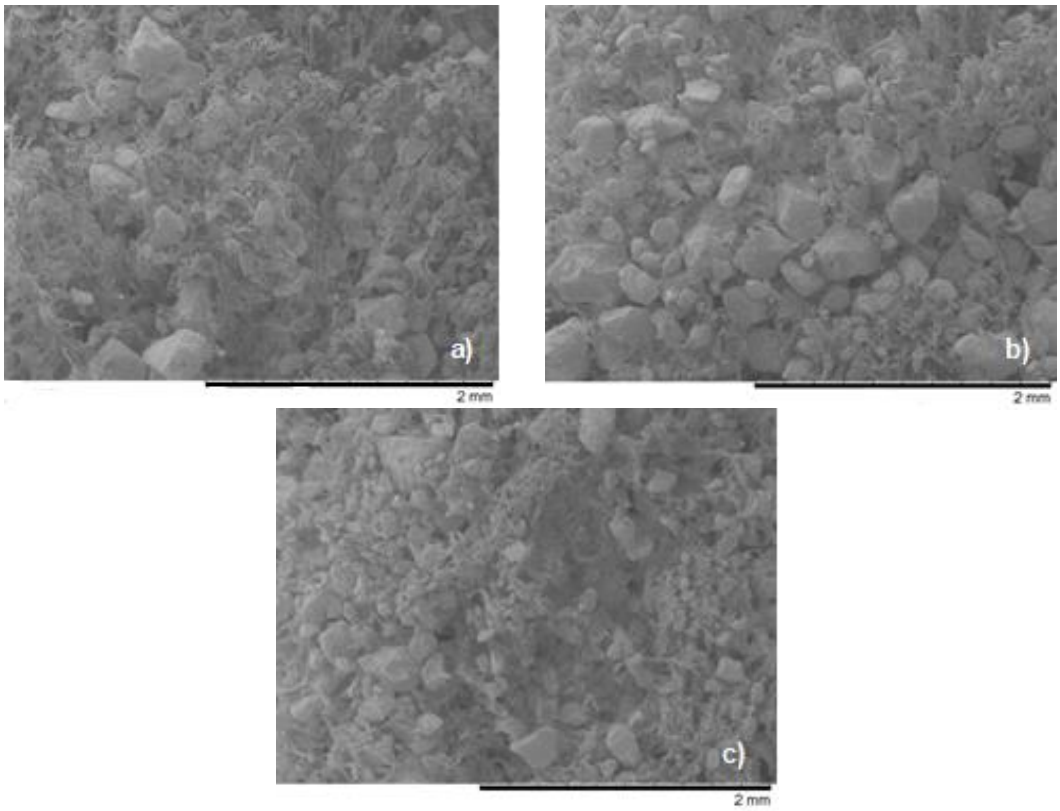

Figure 3.10. SEM images of the composites with the same composition (10:90 wt%), however with different crosslinking times: a) Col/β-TCP 24h of crosslinking treatment; b) Col/β-TCP with 48h of crosslinking treatment; c) Col/β-TCP with 72h of crosslinking treatment.

In figure 3.11, there is presented four SEM images of products manufactured by different companies. Respecting the order from a) to d), the products of different companies are Col-HAp (JHS Biomaterials), Osteogen P (Implandent LTD), Matri™ Bone (Biom'up) and Osteogen S (Impladent LTD).

Col-HAp is formed by HAp and collagen. This product is composed by a porous structure, where de HAp particles were well distributed and absorbed in the collagen matrix. Osteogen S (b) is only composed by collagen, reason why it is not observed any ceramic compound. It is also possible to infer that the product is highly porous. Matri™ Bone (c) is a combination of BCP and collagen. This product shows that the ceramic component was well distributed along the collagen matrix, being possible to observe different sizes of particles. This product was also composed by a highly porous structure, aspect that is possible to observe in figure 3.11 c). Osteogen S (d) is a combination of calcium apatite crystals and type I Achilles bovine tendon collagen. By SEM observation, the ceramic particles were considered to have different sizes and were well distributed along collagen matrix.

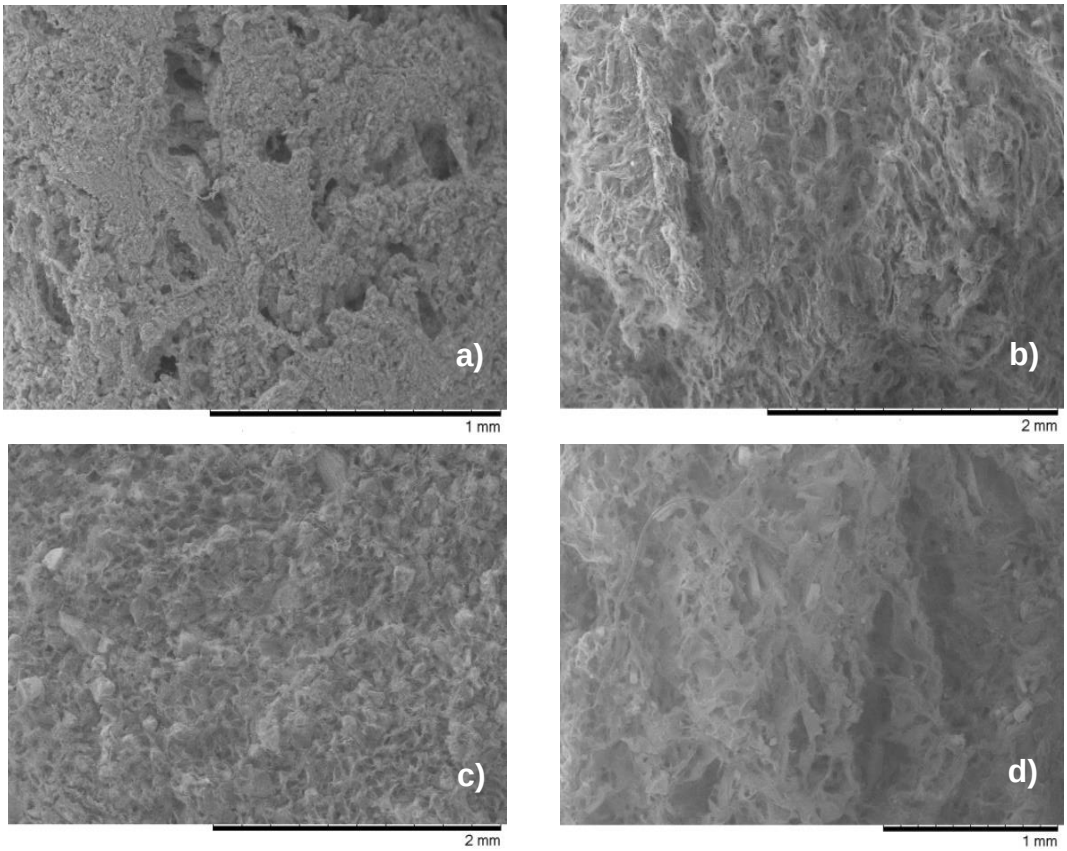

Figure 3.11. SEM images of products manufactured by different companies: a) Col-HAp (JHS Biomaterial b) Osteogen P (Impladent LTD); c) Matri Bone (Biom'up); d) Osteogen S (Impladent LTD).

Summing up, one feature that all the commercial products had in common is the fact that they were composed by a porous structure, an important condition for the use of these products in the biomedical industry.

3.4 Determination of the samples' porosity

As mentioned and described in Chapter 2, the chosen method to measure porosity was the Archimedes method. It was extremely important to perform this measurement in order to determine the samples' porosity. Only one porosity measurement per sample was performed by Archimedes method due to material and financial constraints, since the raw materials used in this project are considered expensive products. Table 3.1 shows the mass measurements of the composites performed by the Archimedes method. These values were used to determine the open and closed porosity of the samples.

Table 3.1. Mass measurements of the composites performed by Archimedes method. These values were used to determine the open and closed porosity of the samples.

Composites	Dry Mass (g)	Suspense Mass (g)	Impregnated Mass (g)
Col/β-TCP 10:90 wt% and 24h of DHT crosslinking	0.240	0.162	0.732
Col/β-TCP 10:90 wt% and 48h of DHT crosslinking	0.176	0.122	0.534
Col/β-TCP 10:90 wt% and 72h of DHT crosslinking	0.253	0.173	0.661

After the measurement of the respective masses, equations 1 and 2 were used for the determination of open and closed porosity. For respective calculations, the equations 11 to 18 were used, being represented in Appendix. The results obtained for the crosslinked composites are presented in table 3.2.

Table 3.2. Values of open and closed porosity for Col/β-TCP composition with 10:90 wt% composition with crosslinking time of 24h, 48h, 72h, using the Archimedes method.

Composites	Open Porosity (%)	Closed Porosity (%)
Col/β-TCP 10:90% composite with 24h of crosslinking	86	1.4
Col/β-TCP 10:90% composite with 48h of crosslinking	87	0.6
Col/β-TCP 10:90% composite with 72h of crosslinking	84	1.2

According to the literature, it is important that the composites, in order to be applied in the dentistry field, hold high porosities, since this characteristic enhances bone tissue and cell ingrowth after being applied in the patient [10,72]. The porosity of the composites is achieved [51] and controlled by lyophilization cycle, so for that reason it is extremely important to optimize its parameters. Different lyophilization conditions could lead to different porosity in the composites.

The results obtained by the Archimedes method revealed composites with high porosities ranging from 84% to 87. These values were considered to be valid for application in the medical industry. It is important to infer that the values difference between the composites were not considered significant and the explanation found for such situation is that these samples were removed from different regions of the lyophilizer tray. The values of porosity obtained by the Archimedes Method do not correspond to the values of porosity obtained by SEM images (figure 3.10). For that reason, it would be interesting, in future studies, to make different cuts and planes in the samples to verify this characteristic of high porosity by SEM images.

3.5 Mechanical Testing

With the equipment described in Chapter 2, load vs displacement curves were recorded. In order to obtain stress-strain curves, these parameters were calculated from load-displacement relations [61,74]. For that reason, stress is giving by dividing the load (in N) by the cross-section area (in m2), as shown in equation 4 and strain is giving by dividing the displacement's variation (in mm) with the distance between plates of the equipment (in mm), as shown in equation 5.

$$\sigma = \frac{F}{A} \qquad \text{Equation (4)}$$

Where σ represents Stress (in N/m^2, Pa), F is the load applied under compressive test (in N) and A is the specimen's cross-section area (in m^2).

$$\varepsilon = \frac{\Delta l}{l_0} \qquad \text{Equation (5)}$$

Where ε represents Strain, Δl is the displacements variation (in mm) and l_0 is the distance between plates of the equipment (in mm).

These formulas were used to normalize the sample's geometry and to make the sample's measurements independent; this means that, all the calculations are no longer dependent on the geometry and dimensions of the sample. This makes it possible to use specimens with different sizes for the same sample without causing any type of errors in calculations.

Figure 3.12 shows a characteristic Stress-Strain curve for Col/β-TCP composite, which was obtained through the compression test of the composite with 10:90 wt% composition and 72h of crosslinking treatment. It is important to note that all curves showed similar shapes, and this one was chosen for representing the obtained results.

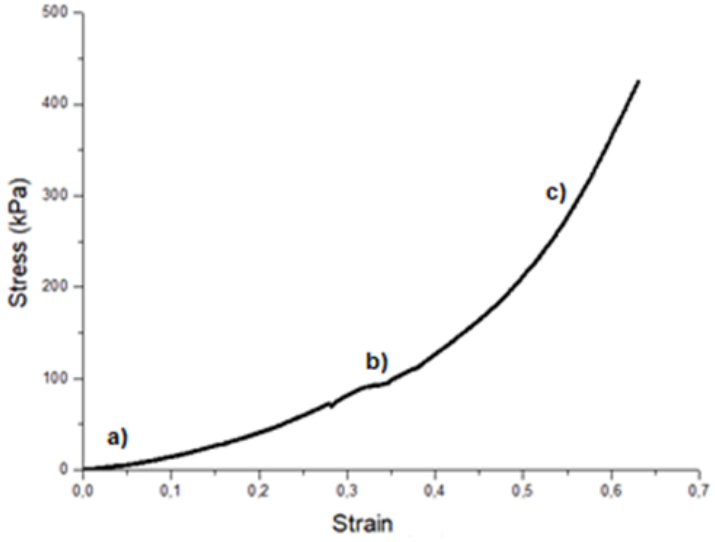

Figure 3.12. Characteristic Strain-Stress curve for Col/β-TCP composite. The curve chosen was obtained through the compression test of the composite with 10:90 wt% composition and 72h of crosslinking treatment.

By analysing the example curve shown in figure 3.12, it is possible to infer that the composite was constituted by a region a) related to an elastic region, followed by a collapse plateau (b) and consequent densification (c) [58]. It was possible to determine the Young's modulus. The Young's Modulus is defined by the ratio between the applied stress and the strain suffered by the material when its mechanical behaviour is linear [70]. It was calculated via linear regression of the initial linear regime of the stress–strain curve [74,77].

On the other hand, the collapse stress occurs when the pores begin to break, which means that, the composite's walls start to bond, causing a loss in the sample's porosity. From collapse stress value, the composite's densification occurs, in other words, the composite enter into a plastic regime where the product's initial structure begins to modify. However, the interpretation of the behaviour of these curves was not that straightforward. In most of the mechanical results, the collapse plateau is not clearly pronounced. There is only a change of slopes after elastic region and before the composite's densification is reached. For that reason, this change of slope is considered to be the collapse plateau, although it cannot be observed with perspicuity. Another important aspect is that in several specimens this slope's change did not clearly occur and consequently the plateau could not be defined. Therefore, the collapse stress could not be clearly assessed and its value was not calculated.

In order to prove that the Stress-Strain curves continue to have an elastic and plastic behaviour, not only elastic, the initial dimensions of the specimens' composites (before performing the compression test) and their final dimensions (after performing the compression test) were measured. Two options were analysed: if the specimens inside a group sample had the same dimensions, it meant that the specimen behaved as a fully elastic material, which means that, after being submitted to compression, it would be able to recover all dimensions. On the other hand, if the specimens' dimensions inside a group sample were different, it can be said that it has plastic deformation. After this procedure, it was concluded that all samples had an elastic behaviour followed by a plastic one.

In table 3.3, the Young's Modulus of the composites is presented.

Table 3.3. List of Young's Modulus values from the composites produced in this project. Results obtained for products without crosslinking and with crosslinking treatment already performed are presented with the purpose to compare the differences between them.

Sample	Young's Modulus (kPa)
Col/β-TCP 40:60% composite without crosslinking stage	71 ± 22
Col/β-TCP 10:90% composite without crosslinking stage	93 ± 44
Col/β-TCP 10:90% composite with 24h of crosslinking	180 ± 21
Col/β-TCP 10:90% composite with 48h of crosslinking stage	182 ± 17
Col/β-TCP 10:90% composite with 72h of crosslinking stage	200 ±12

Before starting to analyse the results, it is very important to reinforce that the products (collagen and β-TCP) used in this project are not referenced in the literature because they are commercial products.

By comparing the values for the five different types of composites (Table 3.3), it is possible to say that the one with the highest value of Young's Modulus is the Col/β-TCP 10:90% composite with 72h of crosslinking stage. From data obtained in table 3.3, it is also possible to observe that the dispersion of Young's Modulus values in samples with crosslinking and higher percentage of β-TCP is decreasing when the duration time of this treatment is increased. This means that, the dispersion of the obtained results is lower for higher crosslinking times, which is probably due to the enhancement of the mechanical properties of collagen due to the increase in crosslinking density.

The presence of β-TCP in the collagen matrix enhances the Young's Modulus when submitted to compression, aspect that can be proven by the obtained data from table 3.3. This also had been confirmed in the literature, where the increasing of the percentage of β-TCP in the composite, enhances the elastic modulus, having therefore a more rigid structure [76,78].

Crosslinking treatment is responsible for improving several composite's mechanical properties, such as, Young's Modulus. As previously said in chapter 1 and 2, the crosslinking performed was via DHT, a process that involves heating the sample under vacuum conditions for a certain time. Crosslinking DHT is used to remove all residual components and to bound covalent crosslinks with the collagen matrix and maintains the open pore structure. As reported in the literature, crosslinks are considered to be bonds in the amino acids' chains that composed the collagen molecules. These bonds increase the stiffness of collagen fibers preventing them from slipping between themselves when submitted to stress [64].

In this project, three samples with different times of crosslinking and the same temperature (110ºC) and pressure (3 mbar) conditions were mechanical analysed. As previously reported in other studies, it has been proved that an increasing on DHT crosslinking' temperature and time, increases the mechanical properties of collagen fibers and consequently the composite's mechanical properties are also increased [69,79]. The values obtained experimentally are in agreement with the literature, which means that crosslinking via DHT directly influences the determination of the Young's Modulus and its dispersion, having greater values of modulus and lower values of dispersion when time conditions are increased [72].

The main goal of this projected was to develop a flexible bone graft and for that reason compression tests were performed with the purpose of verifying if the composite acquired flexibility. By flexibility it is understood that the composite lost stiffness when comparing to calcium phosphate bone grafts and has the ability to conform to surgeon's clinical desire. Beyond the decreasing of stiffness, the composite should possess sufficient conditions to perform the desired bone regeneration. In figure 3.13, it is possible to observe that the produced composite has the flexible characteristic.

Figure 3.13. Demonstration of the flexible characteristic of the produced composites.

As discussed in Chapter 2, statistical analysis (Software IBM SPSS Statistics 22) was used to confirm if the registered values of the Young's Modulus were considered to be significantly different, which means that, the variable time in the crosslinking treatment was statistically investigated. Two comparisons were performed using ANOVA and LSD (Least Significant Difference) test: analysing the differences between composites with and without crosslinking treatment and analysing the differences between composites with different crosslinking times. Therefore, it was confirmed that there was a statistically significant difference between composites with and without crosslinking treatment via DHT, which suggests the application of this type of treatment in samples. On the other hand, it was confirmed that there were no statistically significant differences between composites with 24h, 48h and 72h. This analysis suggests that the different times chosen for the crosslinking were not statistically significant, which implies that any time could be applied in the composites in order to obtain a valid Young's Modulus for dental application. Thereby, it can be inferred that the composite with 24h of crosslinking was the one that gathers a greater efficiency in the manufacturing process, presenting faster productions and consequently a lower cost for the company.

A very important aspect that should be analysed is the variability of results, in other words, the dispersion obtained in the Young's modulus values for different composites. From table 3.3, it is possible to observe a decrease in the dispersion of values caused by the increase of crosslinking time. This aspect assumes an important character for business context, which lower values of dispersion lead to greater repeatability of the results, guaranteeing a more controlled production. The minimum dispersion is reached for composites with crosslinking time of 72h.

Being this said, the choice of what time should be used in the crosslinking treatment for the future commercial product is in charge of Medbone® - Medical Devices. Previously, two arguments were presented to decide what product should be chosen. Summing up, the composite with 24h of crosslinking represents higher efficiency in the manufacturing process (faster and lower costs for the company). On the other hand, the composite with 72h of crosslinking presented a higher value of Young's Modulus and a minimum results' dispersion, where it is possible to have a greater control at manufacturing level; however it represents a higher cost at production for the company.

The values of Young's Modulus , based in the literature, for collagen scaffolds are comprised in a range of 0.5 to 1 kPa [64], while for trabecular mandibular bone is comprised in range of 3.5 to 907 MPa [73]. The Young's Modulus of the natural bone varies depending on which type of bone is being study. For that reason, the trabecular bone was chosen because it is a porous bone and presents more similar properties to the product developed in this project. The choice of the mandibular bone was based on the fact that the dental industry represents a major interest for the company Medbone® - Medical Devices. The values of Young's Modulus for the chosen products here developed in this project were 180 ± 21 kPa for the composite with 24h of crosslinking and 200 ± 12 kPa for the composite with 72h of crosslinking.

Developing a product that replaces the human natural bone and presents an equal Young's Modulus is complex and a difficult process. Although the Young's Modulus for the composites here developed were lower when comparing to the value of trabecular mandibular bone, they were higher when comparing to the value of collagen alone scaffolds. Therefore, as the purpose of the product was to acquire flexibility, it is possible to conclude that this characteristic was achieved, being these obtained Young's Modulus valid for this purpose (aspect that can be observed in figure 3.13).

3.6 Theoretical Young Modulus Prediction

In order to compare if the obtained results are compatible with the models described in the literature, Young's Modulus was theoretically predicted using the Ashby and Gibson model. Through the porosity results obtained in section 3.4, it is possible to verify that the composites produced had values of open porosity between 84 and 87% and values of closed porosity in the range of 0.6 and 1.4%. Consequently, when comparing these two values of porosity, the open one is clearly higher than the closed one, reason why closed porosity could be disregarded.

For this reason, the Ashby and Gibson model was applied taking into account open cell foams [77,81]:

$$\frac{E}{E_0} = C\left(\frac{\rho}{\rho_s}\right)^n \qquad \text{Equation (6)}$$

Where E is the predicted Young's Modulus, E_0 is the Young's Modulus for the dense composite, C and n are geometric constant of proportionality for open cell foams and ρ/ρs is the relative density (ρ - Density of foam and ρ_s - Density of cell-wall material) [77,81].

In this project, as studied in section 3.4, the porosity of the composites did not vary, being in fact a consequence of the lyophilization process. For that reason, the Ashby and Gibson model was adapted to predict the theoretical Young's Modulus. The applied model for open cell foams was the same as equation 6, however the parameters determination was slightly different:

$$\frac{E}{E_0} = C(1-p)^n \qquad \text{Equation (7)}$$

Where, 1 – *p* represents the ratio of not porous area.

$$E_0 = \phi_{v,col} \times E_{col} + \phi_{v,\beta-TCP} \times E_{\beta-TCP} \qquad \text{Equation (8)}$$

$$\phi_{V,A} = \frac{V_A}{V_A + V_B} \qquad \text{Equation (9)}$$

$$\rho = \frac{m}{v} \qquad \text{Equation (10)}$$

Where, $\phi_{v,col}$ represents the volumetric fraction of collagen, $\phi_{v,\beta-TCP}$ is the volumetric fraction of β-TCP, E_{col} represents the Young's Modulus of a collagen dense film, $E_{\beta-TCP}$ is the Young's Modulus of a β-TCP film dense, m represents mass and v represents volume.

It is important to refer that the value for the Young Modulus of collagen and β-TCP's dense film depends on several factors such as method and treatment conditions applied and products used. The volumetric fraction was calculated from the density formulation (equation 10), from which the volume was determined and then applied to equation 9 [74]. After that, equation 8 was used to calculate the E_0 parameter [75].

Using the values obtained in section 3.4 for the samples' porosity, it is possible to determine the (1 – p) parameter, in order to predict the theoretical Young Modulus.

Table 3.4, presents the theoretical values of Young's Modulus for crosslinking composites. These values were only estimated for composites where crosslinking was performed because this treatment causes an increase in stiffness in the porous structure of collagen, being its properties less affected and influenced by local variations.

Table 3.4. Values of the theoretical Young Modulus for Col/β-TCP samples with 10:90 wt% composition and crosslinking stage of 24h, 48h and 72h.

Samples	Theoretical Young's Modulus Values (kPa)
Col/β-TCP 10:90% composite with 24h of crosslinking	[436.25 ; 438,39]
Col/β-TCP 10:90% composite with 48h of crosslinking stage	[449.83 ; 452.04]
Col/β-TCP 10:90% composite with 72h of crosslinking stage	[662.72 ; 665.96]

As it is possible to analyse by the data of table 3.4, the theoretical values present the same order of magnitude than the values obtained experimentally (kPa). On the other hand, it is also possible to confirm that the values for theoretical Young Modulus increased when the crosslinking time increased. This aspect is in agreement with the values obtained in this project, suggesting that the adapted Ashby and Gibson model applied is viable.

3.7 DSC-TGA Analysis

The DSC-TGA analysis was performed in order to study the thermal behavior of the composites and raw materials used to produced them. The thermograms are represented in figure 3.11, where a) is referent to β-TCP, manufactured by Medbone® - Medical Devices, b) is referent to commercial collagen, c) is referent to composite with 10:90 wt% composition without crosslinking treatment and d) is referent to composite with 10:90 wt% composition and 24h of crosslinking. It is important to refer that it was only shown the thermogram of one of the composites with crosslinling treatment because the other two (composites with crosslinking treatment 48h and 72h) were considered to be similar to the composite with 24h of crosslinking.

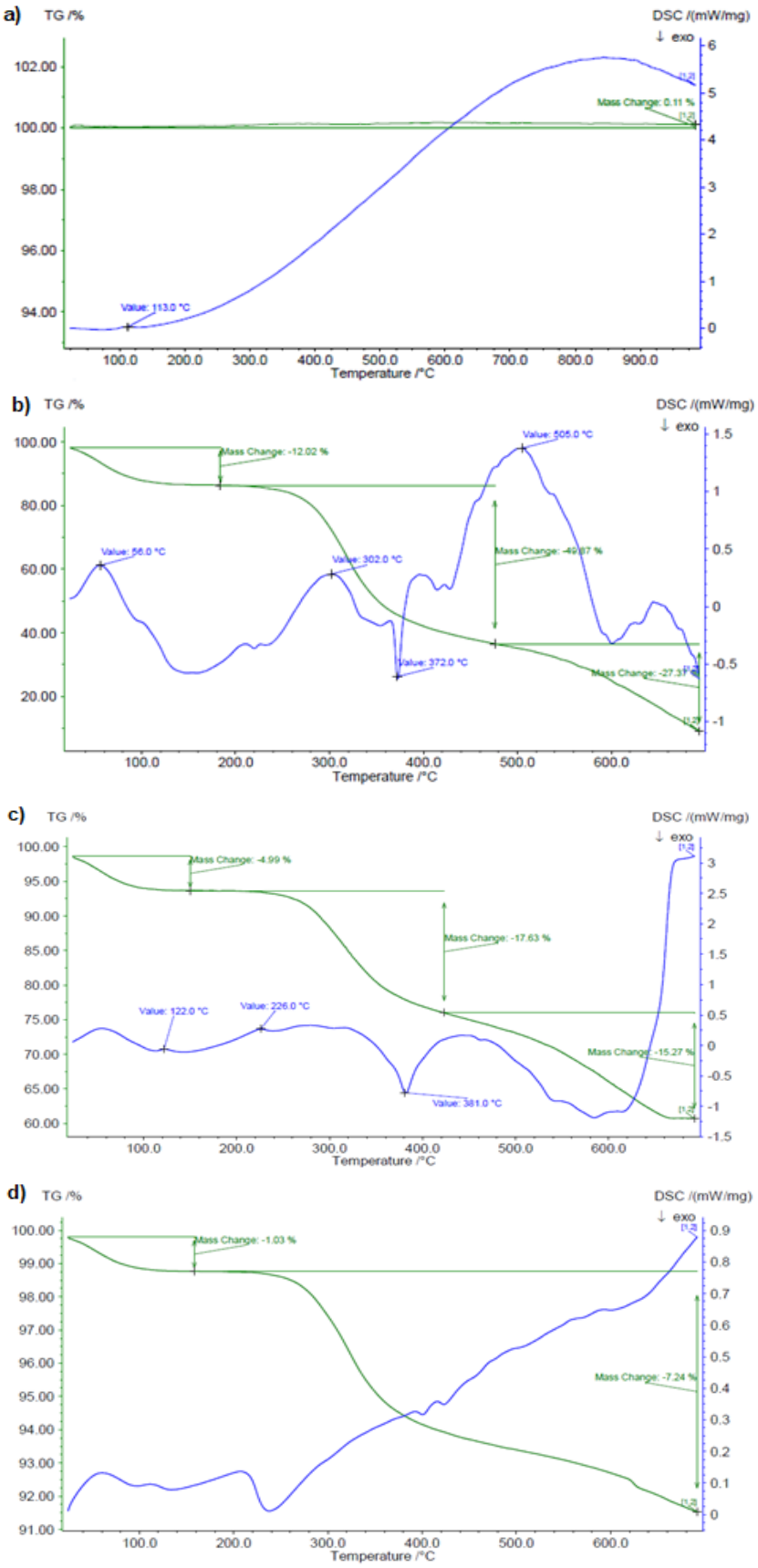

Figure 3.14. Thermograms of the samples: a) β-TCP, manufactured by Medbone® - Medical Devices, b) commercial collagen, produced by an external company, c) composite with 10:90 wt% composition without crosslinking treatment, d) composite with 10:90 wt% composition and 24h of crosslinking.

Table 3.5 shows the temperature were thermal transitions occurred for the samples.

According to the literature and table 3.5, for this type of products (collagen, β-TCP and collagen/β-TCP composite) it is possible to assume that, in this project, there are three thermal transitions. In the first transition (temperature 1), between 50 to 200 ºC, occurred dehydration of samples B, H, I, J and L, where the majority of the water molecules were removed from its surface [52]. Regarding the second transition, a loss of hydrogen bonds occurred in samples B, H and J, which are responsible for the collagen denaturation, was assign to temperatures between 200 and 240ºC. The degradation of collagen occurred between 240 and 480 ºC. Lastly, the third transition represents the combustion of collagen fibers and its residues and it is assigned to temperatures 480 to 700ºC for samples B, H and J [52]. It is also important to refer that for samples I and L the third transition was not visible, being these two constituted by only two thermal transitions.

Table 3.5. Temperature of thermal transitions for the samples. Temperature 1 is referred to the first thermal transition, temperature 2 is related to the second one and the temperature 3 is assigned to the third one. Samples were designated as: A) β-TCP, manufactured by Medbone® - Medical Devices; B - commercial collagen, produced by an external company; H – Col/β-TCP composite with 10:90 wt% composition without crosslinking treatment; I - Col/β-TCP composite with 10:90 wt% composition and 24h of crosslinking; J - Col/β-TCP composite with 10:90 wt% composition and 48h of crosslinking; L - Col/β-TCP composite with 10:90 wt% composition with 72h of crosslinking.

Samples	Temperature 1 (°C)	Temperature 2 (°C)	Temperature 3 (°C)
A	-	-	-
B	50 - 180	180 - 480	480 -700
H	50 - 160	160 - 420	420 - 700
I	50 - 180	180 - 620	620 – 700
J	50 - 180	180 - 420	420 - 700
L	50 - 200	200 - 700	-

Table 3.6 shows the samples mass losses, where it is possible to observe the percentage of mass that was lost according to thermal transitions and their sum (Total value of mass loss).

Table 3.6. Samples mass losses, where it is possible to observe the percentage of mass that was lost according to thermal transitions and their sum (Total value of mass losses). Samples were designated as: A - β-TCP, manufactured by Medbone® - Medical Devices; B - commercial collagen, produced by an external company; H - Col/β-TCP composite with 10:90 wt% composition without crosslinking treatment; I - Col/β-TCP composite with 10:90 wt% composition and 24h of crosslinking; J - Col/β-TCP composite with 10:90 wt% composition and 48h of crosslinking; L - Col/β-TCP composite with 10:90 wt% composition with 72h of crosslinking.

Samples	1st Mass Loss (%)	2nd Mass Loss (%)	3rd Mass Loss (%)	Total (%)
A	-	-	-	-
B	12.02	49.87	27.37	89.26
H	4.99	17.63	15.27	37.89
I	1.03	7.24	-	8.27
J	1.32	5.27	4.24	10.83
L	0.95	7.44	-	8.39

Lastly, table 3.7 shows the endothermic and exothermic peaks of the samples that were analysed.

Table 3.7. Endothermic and exothermic peaks of the samples: A - β-TCP, manufactured by Medbone® - Medical Devices; B - commercial collagen, produced by an external company; H - Col/β-TCP composite with 10:90 wt% composition without crosslinking treatment; I - Col/β-TCP composite with 10:90 wt% composition and 24h of crosslinking; J - Col/β-TCP composite with 10:90 wt% composition and 48h of crosslinking; L - Col/β-TCP composite with 10:90 wt% composition with 72h of crosslinking.

Samples	Endothermic Peaks (°C)	Exothermic Peaks (°C)
A	-	-
B	140 372	505
H	110 381	122 226 460
I	130 240	120 220
J	130 394	110 220
L	140 405	110 220

The values for β-TCP were not quantified because, in the temperature range used for the DSC-TGA studies, no thermal transitions were observed for this product.

By the analysis of table 3.5 and 3.6, it is possible to observe that the products which had a smaller mass were the composites with 10:90 wt% where the crosslinking treatment had been performed. It was not possible to observe any pattern among their differences, so for that reason, it is assumed that these differences are not significant.

An important aspect to consider is the fact that no changes were recorded in the composites until the temperature of 200 °C. Only dehydration of the structure occurred, phenomenon which did not cause changes in the composites.

4 Conclusions and Future Perspectives

The aim of this project was developing a flexible bone graft, which can be applied in the dental industry. Lyophilization technique and crosslinking treatment via DHT were used to manufacture the product. Several techniques, such as XRD, FTIR, SEM, porosity measurements, mechanical testing and DSC-TGA were used to describe and characterize the developed products and their main compounds.

Through the characterization techniques applied in this project, it is possible to conclude that it was successfully developed a collagen/β-TCP composite. The choice of composite to represent the Medbone® - Medical Devices in the market requires a thoughtful and critical analysis on the subject.

All the composites manufactured in this project showed a porous structure, information that can be supported by the porosity obtained results through Archimedes method, which revealed values of porosity in the range of 84 to 87%. The β-TCP particles also seemed to be well integrated and overlapped in the collagen matrix. In this study, the XRD analysis for the composites with the crosslinking treatment was not performed. This aspect was due to the fact that, according to the DSC-TGA results, no changes occurred in the materials below temperature of 200°C. For that reason and because crosslinking via DHT was performed at 110ºC, it was confirmed that crosslinking treatment should not induce any modification in the presented phases, and its results would be exactly the same as the XRD results for the composite with 10:90 wt% composition and without crosslinking. Therefore, the presence of β-TCP was identified for all composites (with and without crosslinking treatment with 10:90 wt% composition). The molecular groups of crosslinked composites were characterized, where it is possible to identify the groups matching to collagen and β-TCP. Mass losses were not considered to be significant for the composites with crosslinking treatment. At the level of mechanical tests, statistical analysis confirmed that all composites with crosslinking treatment (collagen/βTCP 10:90 wt% with 24h, 48h and 72h, respectively) did not present statistically significant differences for the values of Young's Modulus, which suggests that any of the composites could be applied. Therefore, the composite with 24h of crosslinking treatment is a valid choice because it presents a greater efficiency in the production process. However, it should be considered another important factor (also at the level of mechanical tests), the dispersion of the obtained results. In a business context, it is important to reach the highest repeatability of the results, which will guarantee a better product's control. The composite that reached this condition was the one with 72h of crosslinking treatment. Being this said, there were presented two options: one that guarantees a great efficiency in the production process (composite with 24h of crosslinking treatment) and another one which assumes greater Young's Modulus repeatability (composite with 72h of crosslinking treatment). Both composites had good suitable mechanical properties for application in the dental industry, where the determined experimental Young's Modulus was between 181 and 200 kPa. The products here referred had a better handling when compared to only calcium phosphates grafts, registering the flexible

characteristic. Therefore, the products can be adapted to the desire position to treat the bone defect.

The selection of which product will be released and sell in the market is in charge of Medbone® - Medical Devices.

One condition that should be taken into account is that crosslinking was performed varying only the exposure time. However, another possible way to evaluate the behaviour of the composites would be to use different conditions, this means that, samples should be crosslinked with different exposure times, different temperatures and different pressures. This would be a positive aspect in order to obtain more data that would be used for comparing these conditions and optimize parameters. This was not performed in this project due to logistics issues.

Finally, another aspect that would be interesting to study would be composites' behaviour after sterilization and therefore perform cytotoxicity and bioactivity tests. These tests would be used to prove the viability and guarantee safe used of the product in dental field. These procedures were not performed in this project due to logistical issues.

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Appendix

Next, there are presented several figures that were referenced in chapter 2 and 3.

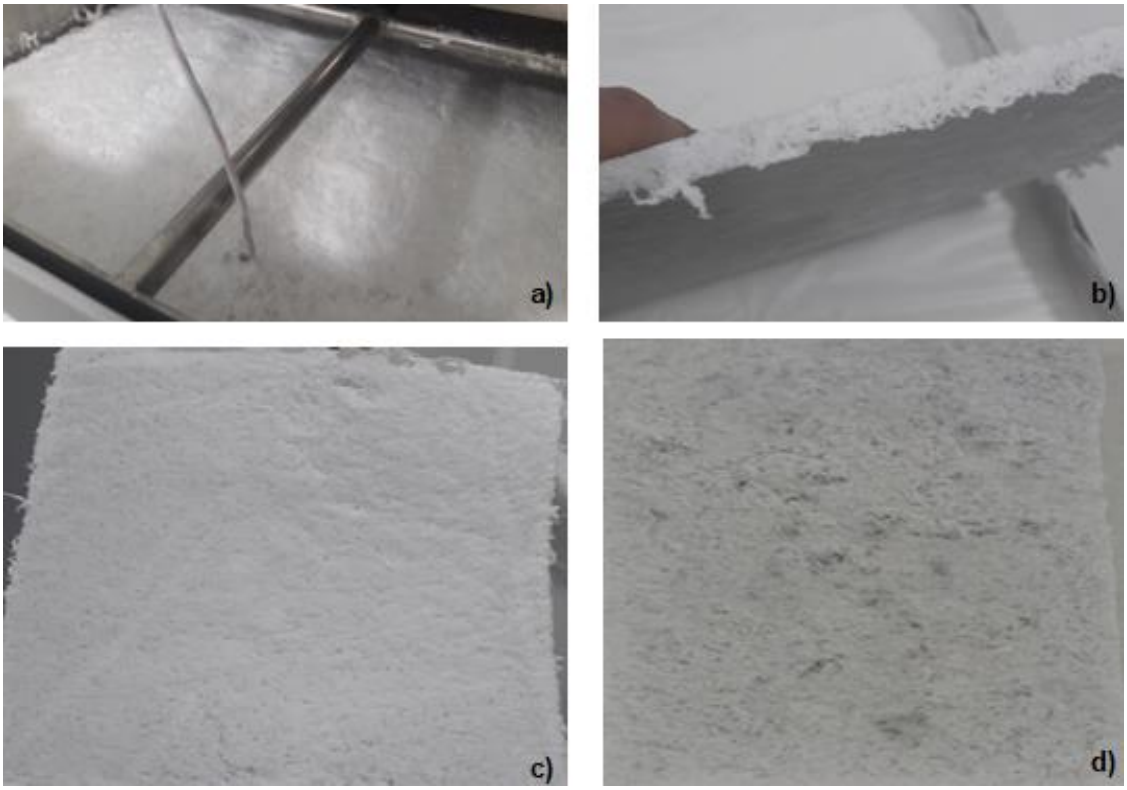

Figure 1. Col/β-TCP composite with 10:90 wt% without crosslinking: a) lyophilizer's probe immersed in the mixture of the composite before and during lyophilization process; b) representation of the sample's thickness; c), d) Sample representation after the lyophilzation process is completed.

In table 1, it is summed up all the samples used in this project.

Table 1. List of the samples used in this project.

Samples	Composition
A	β-TCP with 0.1–0.5 mm produced by Medbone®-Medical Devices.
B	Commercial collagen, extracted from collagen type I bovine tendon.
C	Lyophilized collagen with thin thickness tray and with 0.5L of distilled water.
D	Lyophilized collagen with medium thickness tray and with 0.5L of distilled water.
E	Lyophilized collagen with large thickness tray and with 0.5L of distilled water.
F	Lyophilized collagen with 1L of distilled water.
G	Composite with 40:60 wt% and without crosslinking treatment.
H	Composite with 10:90 wt% and without crosslinking treatment.
I	Composite with 10:90 wt% and 24h of crosslinking treatment.
J	Composite with 10:90 wt% and 48h of crosslinking treatment.
L	Composite with 10:90 wt% and 72h of crosslinking treatment.
Commercial 1	Col-HAp, manufactured by JHS Biomaterials.
Commercial 2	Osteogen P, manufactured by Impladent LDT.
Commercial 3	Osteogen S, manufactured by Impladent LDT.
Commercial 4	Matri Bone, manufactured by Biom'up.

Next, it is represented all the equipment used for the characterization of the products.

Figure 2. SEM equipment used in this project (a). The samples were placed in the sample holder (b) and then placed inside the equipment, represented by a black rectangle. The samples' arrangements were made randomly.

Figure 3. Equipment used in characterization of this project. It is represented the equipment from FTIR (a), XRD (b) and DSC-TGA (c).

In Chapter 2, section 4.4, the equations for the calculation of open and closed porosity are presented in equation 1 and 2 (P_{open} and P_{closed}, respectively). Next, it will be discuss how these equations were obtained. In this project, it is possible to distinguish open porosity from closed porosity, within the developed products. Open porosity is the volume fraction that can be occupied by a fluid. On the other hand, closed porosity represents the fraction of volume in which the fluid cannot penetrate. Firstly, it is necessary to define the total volume of the sample (V_t), presented in equation 11. These are composed by the volume of the solid (V_s), total volume of open pores ($V_{open\ p}$) and total volume of closed pores ($V_{closed\ p}$). It is important to decompose this equation in order to determine its variables.

$$Open\ Porosity\ (P_{open}) = \frac{V_{open\ p}}{V_t} \times 100\% \qquad \text{Equation (1)}$$

$$Closed\ Porosity\ (P_{closed}) = \frac{V_{closed\ p}}{V_t} \times 100\% \qquad \text{Equation (2)}$$

$$V_t = V_s + V_{open\ p} + V_{closed\ p} \qquad \text{Equation (11)}$$

Where P_{open} represents the open porosity, P_{closed} represents closed porosity, V_t represents total volume of the composite, V_s represents the volume of solid, $V_{open\ p}$ is the volume of open pores and $V_{closed\ p}$ is the volume of closed pores.

The volume of solid in the composites (V_s) was determined by equation 12. Through the density (ρ) and the dry mass (m_{dry} - which was measured before the Archimedes method was performed), it is possible to calculate the volume of solid.

$$\rho = \frac{m_{dry}}{V_s} \Leftrightarrow V_s = \frac{m_{dry}}{\rho} \qquad \text{Equation (12)}$$

Where ρ is density of the composite, m_{dry} is the initial mass of the composites before Archimedes method was performed and V_s is the volume of solid.

Equation 13 presents the apparent volume (V_{app}) , which is considered to be the sum of the volume of solid (V_s) and the volume of closed pores ($V_{closed\ p}$).

Consequently, it is possible to replace this relation in the equation of total volume, being presented in the equation 14. The next step was to calculate the volume of open pores ($V_{open\ p}$), apparent volume (V_{app}) and total volume (V_t), presented in equations 15, 16 and 17.

The volume of closed pores ($V_{closed\ p}$) was calculated by subtraction apparent volume and of volume of solid, presented in equation 18.

$$V_{app} = V_s + V_{closed\ p} \quad \text{Equation (13)}$$

$$V_t = V_{app} + V_{open\ p} \quad \text{Equation (14)}$$

$$V_{open\ p} = \frac{m_{impregnated} - m_{dry}}{\rho_{ethanol}} \quad \text{Equation (15)}$$

$$V_{app} = \frac{m_{dry} - m_{suspense}}{\rho_{ethanol}} \quad \text{Equation (16)}$$

$$V_t = \frac{m_{impregnated} - m_{suspense}}{\rho_{ethanol}} \quad \text{Equation (17)}$$

$$V_{closed\ p} = V_a - V_s \quad \text{Equation (18)}$$

Where V_{app} is the apparent volume, V_s is the volume of solid, $V_{closed\ p}$ represents the volume of closed pores, $V_{open\ p}$ represents the volume of open pores, $m_{impregnated}$ is the mass of the composites after being immersed in a recipient with ethanol, $m_{suspense}$ is the mass of the composite when suspended in ethanol and $\rho_{ethanol}$ represents the density of ethanol. With these values, it is possible to determine the percentage of open and closed porosity (P_{open} and P_{closed}, respectively) of the composites, using equations 1 and 2 available in chapter 2, section 4.4.

Next, it is represented the equipment used for perfoming the mechanical tests.

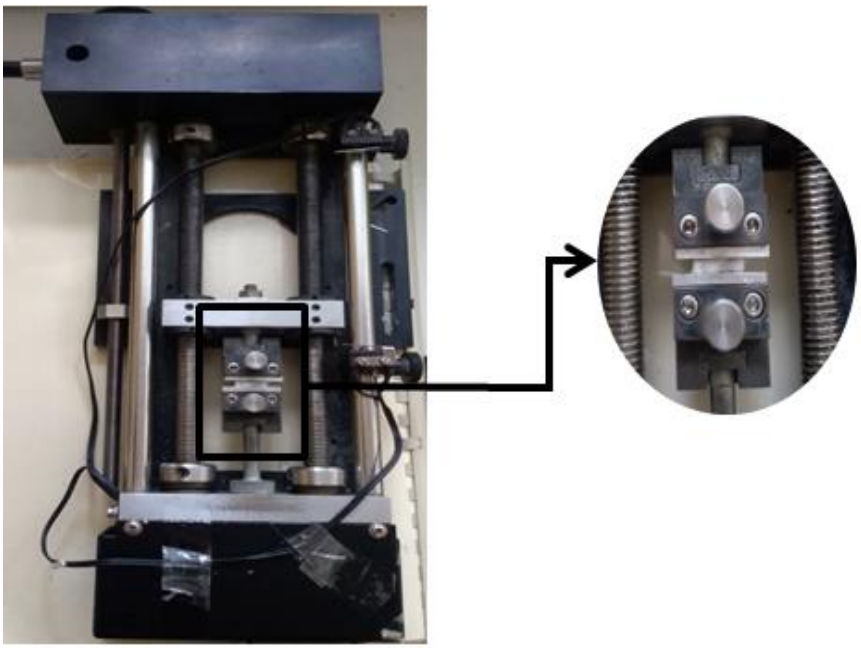

Figure 4. Compression machine used in this project. There is also represented a zoom in the region where compression forces were applied in the sample's specimen.