UNIVERSIDADE DE LISBOA
INSTITUTO SUPERIOR TÉCNICO

Towards High Strength 3D Chitosan-Based Implants for Biomedical Applications

Nuno Guitian Pinheiro Fernandes Oliveira

Supervisor: Doctor Maria Alexandra Sousa Rodrigues
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Thesis approved in public session to obtain the PhD Degree in

Leaders for Technical Industries

Jury final classification: Pass with Merit

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Abstract

Over the last few decades, biomaterials have been extensively developed and applied in medical devices. Among these materials, bioabsorbable polymers have attracted special attention for orthopedic applications where a transient existence of an implant can provide better results, when compared with permanent implants. Chitosan, a natural biopolymer, has generated enormous interest due to its various advantages such as biocompatibility, biodegradability and osteoconductive properties.

This thesis aims to introduce a novel process for the production of 3D dense chitosan-based products as potential medical devices for the future generations of bioabsorbable implants. 3D dense chitosan-based specimens, with different sizes, shapes and properties, were successfully prepared, showing high performance towards different machining techniques. Gel permeation chromatography and nuclear magnetic resonance spectroscopy studies were conducted and showed that the production process does not affect the initial parameters of the polymer. A full physical characterization, namely morphological and mechanical tests, was conducted in order to demonstrate that the process can yield 3D dense specimens with different stiffness and strength.

Experiments were conducted to assess the influence of chitosan's molecular weight and the addition of glycerol on 3D chitosan-based specimens physical properties combined with cytotoxicity and with *in vitro* degradation studies. From the results it was possible to conclude that specimens were neither cytotoxic towards the cells, nor released cytotoxic substances in the culture medium. Preliminary experiments using human mesenchymal stem cells to study cell adhesion and proliferation were also conducted, showing promising results. As far as biodegradability is concerned, the results pointed out that glycerol had not only a high impact in improving the initial mechanical properties of specimens, but it also contributed to increase specimens stability throughout the enzymatic degradation experiment.

Finally, after identifying some of the potential applications for the innovative technology presented in this thesis, as well as a deep analysis of the market and the medical industry for the specific application that was identified and studied, it was concluded that the potentiality of the technology and the product that can be developed is enormous, suggesting that chitosan-based implants for spinal fusion applications, for instance, can be an appealing application.

Keywords: Chitosan, Biopolymers, Production Process, Bioabsorbable Implants, Orthopedic Applications, Mechanical Properties, Cytotoxicity, Enzymatic Degradation, Go-to-Market Strategy

Resumo

Nas últimas duas décadas múltiplos biomateriais têm sido desenvolvidos e amplamente utilizados em dispositivos médicos. Entre estes materiais, destacam-se os polímeros reabsorvíveis usados no fabrico de implantes para aplicações ortopédicas. O quitosano, um biopolímero de origem natural, tem gerado cada vez mais interesse para aplicações médicas devido à sua biocompatibilidade, biodegradabilidade, osteocondutividade, entre outras propriedades.

Esta tese tem como principal objectivo apresentar um novo processo de fabrico de produtos tridimensionais e densos à base de quitosano, o qual foi desenvolvido no âmbito do projecto de doutoramento, com a finalidade de produzir potenciais implantes reabsorvíveis à base de quitosano. As amostras resultantes do processo foram sujeitas a uma completa caracterização físico-química a fim de estudar e melhorar as suas propriedades. Para além dos estudos cromatográficos e espectroscópicos que demonstraram que o processo produtivo não altera as características químicas iniciais do polímero, foram realizados estudos morfológicos e ensaios mecânicos às diversas amostras produzidas, cujos resultados vieram confirmar a possibilidade de produzir amostras com diferentes módulos de rigidez e resistências mecânicas, usando o processo de fabrico desenvolvido.

O impacto do peso molecular do polímero (quitosano) e da adição de um plastificante (glicerol) nas propriedades físicas e biológicas de amostras resultantes do processo desenvolvido também foi avaliado. Os resultados comprovaram que as amostras produzidas, independentemente do peso molecular do quitosano utilizado ou da adição de glicerol, não são citotóxicas. As experiências desenvolvidas com o intuito de avaliar a degradação enzimática destas amostras mostraram que a adição de glicerol não só permitiu melhorar as propriedades mecânicas iniciais das amostras, como também contribui-o para a estabilidade física das mesmas ao longo das experiências.

O processo de fabricação dos produtos tridimensionais à base de quitosano para serem utilizados como dispositivos médicos foi avaliado neste projecto, concluindo-se que implantes à base deste biopolímero que promovam a fusão intervertebral, por exemplo, podem ser uma aplicação bastante interessante na área dos dispositivos médicos.

Palavras-chave: Quitosano, Biopolímeros, Processo de Fabrico, Implantes Reabsorvíveis, Aplicações Ortopédicas, Propriedades Mecânicas, Citotoxicidade, Degradação Enzimática, Estratégia de Mercado

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Abbreviations in use

ACL	Anterior Cruciate Ligament
ATP	Adenosine Triphosphate
BM	Bone Marrow
CAGR	Compound Annual Growth Rate
CMF	Craniomaxillofacial
CNC	Computer Numerical Control
CT	Computed Tomography
DA	Degree of Acetylation
DD	Deacetylation Degree
DP	Degree of Polymerization
EEA	European Economic Area
EU	European Union
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FEM	Finite Element Method
FTIR	Fourier Transform Infrared
GA	Glutaraldehyde
GPC	Gel Permeation Chromatography
HA	Hydroxyapatite
IMDM	Iscove's Modified Dulbecco's Medium
IP	Intellectual Property
LTI	Leaders for Technical Industries
MSC	Mesenchymal Stem Cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
M_w	Molecular Weight
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffered Saline
PBSA	Poly (Butylenes Succinate Adipate)
PBTA	Poly (Butylene Terephthalate Adipate)
PCL	Poly (Caprolactone)
PDO/PDS	Poly(p-Dioxanone)
PEG	Polyethylene Glycol
PGA	Poly (Glycolic Acid)

PLA	Poly (Lactic Acid)
PMA	Phosphomolybdic Acid
PS	Polystyrene
PVP	Polyvinylpyrrolidone
rhBMP	Recombinant Human Bone Morphogenic Protein
SD	Standard Deviation
SME	Small and Medium Enterprise
SR	Swelling Ratio
TCP	Tricalcium Phosphate
T_g	Glass Transition Temperature
T_m	Melting Point
TMC	Poly Trimethylene Carbonate
US	United States
USA	United States of America
WL	Weight Loss

1. Introduction

Polysaccharides, such as chitin and its derivative chitosan, hyaluronan and alginates, are polymers composed of monosaccharide units joined together by glycosidic linkages, a type of ether bonds [1]. Their use as biomaterials has become much more common as new biological functions are identified for these materials. Thus, the large number of polysaccharides with different chemical structures and physical properties constitutes a large source of materials for more and more applications in the future, particularly in the domain of tissue engineering, drug vehicles, controlled release of drugs, among other applications [1]. Also, the range of materials that can be investigated has been increasing due to new methods that have been developed for processing polysaccharides in different forms (e.g. beads, fibers, films, shaped objects, solutions, gels). Their biocompatibility, biodegradability, processability, and bioactivity make polysaccharides very promising natural biomaterials [2]. In addition to polysaccharides of human origin – hyaluronan (or hyaluronic acid), chondroitin sulfate, heparin, keratin and dermatan – there are a number of molecules from other sources that have shown potential as degradable polymeric biomaterials. Among these materials, whose relevance is becoming increasingly evident, are chitin and its main derivative, chitosan.

Although chitin was isolated in 1811 for the first time from mushrooms by Braconnot [3], about 30 years earlier than cellulose, it remained an unused biomass resource for a long time, in sharp contrast to cellulose [4]. The interest in this abundant biopolymer has increased enormously in recent years and research activity focus in chitin is now surprisingly high worldwide, both in academia and industry, as evidenced by the rapid increase in the numbers of relevant research papers and patents. Since polysaccharides have peculiar structures and properties, significantly different from those of synthetic polymers, they are considered promising biopolymers for developing desirable advanced functions. Chitin, but mainly chitosan exhibits unique physicochemical properties, as it is a biocompatible, antibacterial, biodegradable and environmentally friendly polyelectrolyte. Therefore, a big variety of both medical and nonmedical applications have been explored during the last decades, including water treatment, chromatography, additives for cosmetics, textile treatment for antimicrobial activity, novel fibers for textiles, photographic papers, biodegradable films, biomedical devices, and microcapsule implants for controlled release in drug delivery [4–9].

Chitosan has generated enormous interest for biomedical applications due to its various advantages such as: easy availability, positive charge, biocompatibility, biodegradability and antimicrobial activity. It has been reported that chitosan can be a promising material as a temporary mechanical supporter for bone fractures since chitin's role in the exoskeleton of crustaceans is analogous to that of collagen in bone [10–12]. However, unlike the majority of the synthetic polymers that present thermoplastic properties and therefore can be heated, softened, formed and then cooled to retain their shape, three-dimensional (3D) chitosan-based objects cannot be shaped using conventional plastics processing techniques, as it undergoes

thermal degradation at 270 ºC prior to melting [13–15]. Therefore, although chitosan has been proving to be one of the most promising biopolymers for orthopedic applications, there are just few results for this kind of applications.

This thesis aims to introduce a novel process for the production of 3D dense chitosan-based specimens as a potential material to be used in the future generations of bioabsorbable implants, taking advantage of its attractive properties.

1.1. Motivation of the thesis

Although bioabsorbable implants have been employed in a variety of applications, they have several disadvantages, being their low mechanical properties, high cost and undesired biological responses the most frequently mentioned [16]. A desirable material for bioabsorbable implants should provide enough initial mechanical strength, induce or promote new bone formation by osteogenic cells and possess some bioactivity by being osteoinductive. The absorbable fixation should ideally lose strength in concert with healing as well as lose all mass as quickly as possible thereafter [10,17]. Therefore, there is the need to improve the degradation profile of the degradable polymers currently used for osteosynthesis in order to match more closely the bone healing process [13]. Another drawback of such polymer implants is that their acidic degradation (e.g., lactic acid) have an effect on the microenvironment pH and may cause local inflammation [11]. In conclusion, three key challenges that need to be addressed by new bioabsorbable polymers were identified: optimization of degradation rates to better match the healing processes of each tissue; development of materials with higher strength or stiffness for greater load bearing applications; and improved biocompatibility of breakdown products and long-term *in vivo* performance [18].

Bearing in mind the attractive properties of chitosan and the existing needs as far as bioabsorbable implants are concerned, the purpose of this thesis is to present a novel 3D dense chitosan-based products production process, characterize these products and assess their attractiveness for future chitosan-based absorbable implants.

1.2. Objectives of the thesis

The main objective of this project envisages studying the potentiality of shaping chitosan into high strength 3D solid specimens for medical applications. In order to achieve the main objective of this thesis, four main challenges were identified and set as goals:

- Develop a standard manufacturing technique for the production of high strength 3D chitosan-based specimens;

- Produce different chitosan-based specimens by changing some of the standard manufacturing process parameters;
- Characterize chitosan-based specimens;
- Study the competitiveness of the chitosan-based implants.

1.3. Structure of the thesis

This thesis is organized in the following chapters:

- Chapter 2 and 3 are dedicated to the state of the art and the problem definition: taking into account the interesting properties of chitosan and the existing polymer-based bioabsorbable implants drawbacks, future chitosan-based implants seem to be an appealing solution. Thus, chapter 2 provides a bibliographic revision on chitosan preparation, as well as on its main properties and applications, whereas chapter 3 provides context information on the existing bioabsorbable implants. These chapters provide evidence in that there is the need to develop 3D dense chitosan-based implants for biomedical applications.
- In chapter 4, a novel production process of 3D dense chitosan-based products is presented. 3D dense chitosan specimens, with different sizes, shapes and properties, were successfully obtained, being the production process of these specimens and its main steps rigorously described, and the results of the physicochemical characterization presented. Different machining techniques were tested; gel permeation chromatography and nuclear magnetic resonance spectroscopy studies were conducted; physical, morphological and mechanical tests were also performed to prove that the process can yield 3D dense specimens with appealing stiffness and strength.
- Chapter 5 is mainly dedicated to the mechanical characterization of 3D dense chitosan-based specimens. Several specimens were produced according to the developed process described in chapter 4. However, several changes to this production process were intentionally performed, as well as the addition of other materials (blending chitosan with other biomaterials), in order to assess their influence on the mechanical properties of the specimens.
- The aim of chapter 6 is to evaluate the influence of chitosan's molecular weight (M_w) and the addition of one plasticizer (glycerol) on 3D dense chitosan-based products' biomechanical properties. Therefore, several specimens were produced and an *in vitro* study was performed in order to mainly assess the cytotoxicity of these specimens and their physical behavior throughout the enzymatic degradation experiments carried out for different periods of time.

- In chapter 7, an assessment of the potential of the developed innovative production process of 3D solid and dense chitosan-based products for biomedical applications, was performed. Therefore, it starts with a brief explanation of the technology, highlighting its main features. Then, several potential applications and their markets were identified and assessed. After choosing a primary application and market, its potential as well as its uncertainties and risks were identified. A business model suggesting how to materialize the value from the application was sketched. After that, a brief description of the market as well as the identification of the main competitors and their distinctive features was made. The supply chain analysis and the go-to-market strategy were the following steps. In the end, a final recommendation based on the assessment of the information was prepared.
- Chapter 8 presents the main conclusions of this thesis and discusses further research required to improve 3D dense chitosan-based products.

This thesis was written as segmented and irrespective chapters to allow the reader an independently review and assessment of each chapter. The information regarding the materials and methods used was intentionally repeated throughout the chapters in order to facilitate the reading and avoid going back to previous chapters.

2. Chitosan: A Functional Biopolymer for Biomedical Applications

Chitosan is a copolymer commonly prepared from chitin, which is the second most abundant natural polymer in nature (after cellulose) being arthropod shells the most common and easily accessible sources. Other possible sources of chitin consist of krill, clams, oysters, insects, and fungi and it has been estimated that more than 10 million tons of chitin are biosynthesized each year [19,20]. On the other hand, chitosan only occurs naturally in some fungi (e.g. Mucoraceae) and therefore, it is usually prepared by hydrolysis of *N*-acetyl groups of chitin, as described in section 2.1. Since this reaction is rarely conducted to full completion, chitosan polymeric chain is generally presented as a copolymeric structure comprised of *D*-glucosamine (D residues) along with *N*-acetylglucosamine residues (A residues). The molar fraction of *N*-acetyl groups in chitosan is expressed as a degree of *N*-acetylation (DA) or fraction of acetylation. However, the molar fraction of *D*-glucosamine, deacetylation degree (DD), is the most frequently used [4,5,21]. In contrast to chitin, the presence of free amine groups ($-NH_2$) along the chitosan chain allows this macromolecule to dissolve in diluted aqueous acidic solvents, due to the protonation of these groups, rendering the corresponding chitosan salt in solution ($-NH_3^+$) [5,19]. This is one of the main advantages of chitosan, when compared with chitin, facilitating its handling for a wide range of applications, ranging from fungicidal and water treatment to cosmetics, pharmaceutical and biomedical applications.

The aim of this chapter is to present a review on chitosan, by presenting its methods of preparation and characterization, its properties and main applications.

2.1. Methods of preparation and characterization of chitosan

Chitin and chitosan (figure 2.1) are described as a family of linear polysaccharides consisting of varying amounts of β (1→4) linked residues of *N*-acetyl-2 amino-2-deoxy-D-glucose (*N*-acetyl-glucosamine) and 2-amino-2-deoxy-D-glucose (*N*-glucosamine) units [22]. Some authors consider that when the number of *N*-acetyl-glucosamine units is higher than 50%, the biopolymer is termed chitin. Conversely, when the number of *N*-acetyl-glucosamine units is lower, the term chitosan is used [4,6,19]. In order to obtain chitosan, a partial deacetylation of chitin in the solid state under alkaline conditions (concentrated NaOH) or by enzymatic hydrolysis in the presence of a chitin deacetylase is performed. Due to the semicrystalline morphology of chitin, chitosans obtained by a solid-state heterogeneous reaction have an uneven distribution of acetyl groups along the chains [19].

Although chitin is present within numerous taxonomic groups, commercial chitins are usually isolated from marine crustaceans, mainly because a large amount of waste is available

as a by-product of food processing [4,6,23]. Crustacean shells usually consist of 30-40% proteins, 30-50% calcium carbonate, 20-30% chitin and also contain pigments of a lipidic nature such as carotenoids. These proportions vary with species and with season and in this case, *α*-chitin is produced [23]. On the other hand, squid pens are used to produce *β*-chitin, which is associated with a higher protein content but a lower carbonate concentration.

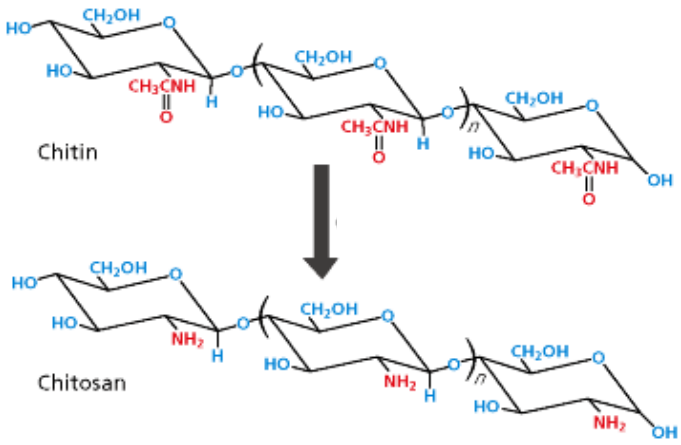


Figure 2.1. Chemical structure of chitin and chitosan [24].

The preparation method of chitosan (figure 2.2) is a factor that affects the sample characteristics, such as molecular weight (M_w), deacetylation degree (DD), among others, depending on the process conditions (e.g. processing time and temperature). Commercial chitins are usually extracted by acid treatment to dissolve the calcium carbonate – demineralization – followed by alkaline extraction to dissolve the proteins – deproteinization – and commonly by a depigmentation step to obtain a colorless product mainly by removing the astaxantine [23,25,26]. Posteriorly, chitosan is prepared by hydrolysis of acetamide groups of chitin – deacetylation – which is normally conducted by severe alkaline hydrolysis treatment. Thermal treatments of chitin under strong aqueous alkali are usually needed to give partially deacetylated chitin (DD higher than 50%), regarded as chitosan. Common conditions to deacetylate chitin avoiding high degradation involve using heterogeneous conditions with NaOH 50%-75% (w/v) and a temperature of 110ºC. The type of crustacean and the chitin isolation process are also factors that affect chitosan quality [26].

There is also evidence that in certain bacteria and fungi, enzymatic deacetylation can take place [27]. Deacetylases have been isolated from various types of fungi, however, the activity of these deacetylases is severely limited by the insolubility of the chitin substrate. There have been some attempts to use amorphous chitin of high DA as a substrate for the deacetylase enzyme, however no acid-soluble chitosan could be isolated and characterized [28]. The lack of solubility of *chitinous* substrates with high DA in aqueous solvents still represents a practical limitation for the preparation of chitosan using the chitin deacetylase system, a process which so far has been just successfully achieved *in vivo* [29].

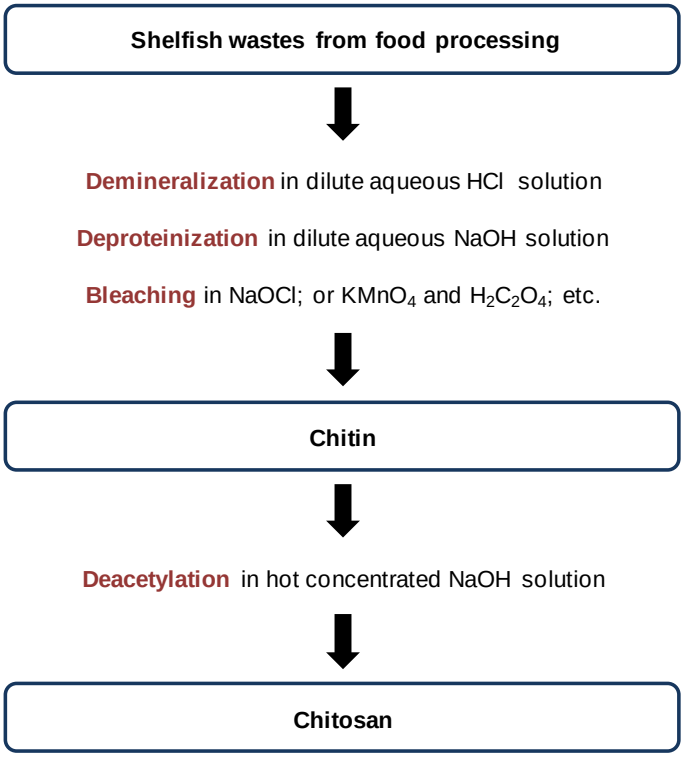


Figure 2.2. Scheme representing the most common way to prepare chitosan.

Chitin and chitosan characteristics have a great effect on their properties and hence on their possible applications. In fact, not every chitin or chitosan sample can be used for the same applications and that explains why a complete characterization of the samples is usually conducted.

Chitosan, as well as chitin, is semicrystalline and shows polymorphism depending on its physical state. Depending on the origin of the polymer and its treatment during extraction from the raw sources, the residual crystallinity may vary considerably. Crystallinity is maximal for both chitin (i.e. 0% deacetylated) and fully deacetylated chitosan (i.e. 100%). Moreover, several factors during production, including high temperature, concentration of alkali, reaction time, previous treatment of the chitin, particle size, chitin concentration, dissolved oxygen concentration and shear stress may influence the characteristic of chitosan [19,23].

The main parameters affecting the polymer properties are DD, M_w, polydispersity and crystallinity [30]. In general, commercially available chitosans are heterogeneous and the DD ranges from 60 to 90%. In addition, the molecular weights of these commercial materials typically range from 50 to 2000 kDa [25]. For applications related to human consumption such as food and medical applications, the purity (ash content), the moisture and the content of heavy metals, endotoxins and proteins must be determined as well [31]. It has been reported that the DD is one of the most important chemical characteristics, which can influence the performance of chitosan in many of its applications, as described later in this chapter [32].

Besides this, the influence of M_w on the aqueous solutions viscosity plays a significant role in the biochemical and biopharmacological outcome of chitosan. It is important to note that due to its low solubility, the M_w of chitin is not easily determined.

Various methods have been reported for the determination of chitin and chitosan characteristics [33,34]. Since different results are obtained when using methods based on different principles, it is important to indicate the characterization method. Nowadays, even the best characterized chitosans available in the market (figure 2.3) are usually described only with regard to their average degree of (de)acetylation and their average degree of polymerization (DP), their ash content and the absence of contaminating bacteria, in some cases also indicating the polydispersity index. The control of these physicochemical characteristics is often critical due to effects on mechanical and chemical properties when chitosan is processed into films, powders, fibers, among other structures.

Figure 2.3. Commercially available chitosan [24]

2.1.1. Composition and deacetylation degree of chitosan

The identity of chitosan and chitosan salts can be established by several methods, including Fourier Transform Infrared (*FTIR*) Spectroscopy [35]. Almost all organic chemical compounds absorb infrared radiation at frequencies characteristic for the functional groups in the compound. Thus, a *FTIR* spectrum shows absorption bands relating to bond stretching and bending and can therefore serve as a unique fingerprint of a specific compound. From the spectrum, the deacetylation degree of chitosan can be calculated [33–35].

Variations in the composition or the sequential structure, or both, may cause differences in the performance of a chitosan in a particular end use and this information may be determined by nuclear magnetic resonance (NMR) spectroscopy [36]. The degree of deacetylation of chitosan can be established using this technique as well, according to the method described by Hirai *et al* [36].

2.1.2. Molecular weight of chitosan

Intrinsic viscosity and gel permeation chromatography (GPC) are the two most commonly used methods for determining the molecular weight of polymers [37]. Molecular mass (molecular weight) of a chitosan defines certain performance characteristics such as viscosity. Depending on the sensitivity of a particular end use to these variations, determination of molecular mass, directly or indirectly, is usually necessary. Therefore, the M_w can be determined by employing the Mark-Houwink-Sakurada equation [33,34,38]:

$$[\eta] = KM^a \quad (2.1)$$

Where η is the intrinsic viscosity; K is a constant for a given solute-solvent system; M is the viscosity derived average molecular weight; and a is an empirical constant describing the conformation of the polymer. The intrinsic viscosity can be determined by measuring the relative viscosity in a Ubbelohde capillary viscometer.

On the other hand, GPC is the most powerful technique for characterizing the polydispersity of polymers. However, it is also a relative method and needs molecular weight standards (e.g. pullulan standards) for the universal calibration of the GPC system to obtain the correlation between elution volume and molecular weight. Standards and samples are usually solubilized in acetic acid/sodium acetate buffer solutions [37,39,40].

Depending on the final use and the required performance control, other characterization assays can additionally be performed, including the polydispersity; the viscosity; dry matter content; ash content; amount of insoluble impurities; endotoxin content; protein content; heavy metal content; among others [29]. The safety of chitosan in biomedical and pharmaceutical applications should be established according to current guidelines such as the ISO 10993 series and the ASTM F748 [34].

2.2. Properties of chitosan

As previously mentioned, chitin and chitosan are currently receiving a great deal of interest as regards medical and pharmaceutical applications because they have interesting properties that make them suitable for use in the biomedical field, such as biocompatibility, biodegradability and non toxicity [41]. Therefore, problems associated with the stimulation of chronic inflammatory reaction and toxicity by synthetic polymers are largely suppressed or eliminated by using natural polymers. Moreover, other properties such as analgesic, antitumor, haemostatic, hypocholesterolemic, antimicrobian, and antioxidant properties have been also studied and reported [6–8].

There are some parameters affecting the polymer properties being the deacetylation degree the one with a higher effect, since the majority of the biological properties are related to

the cationic behavior of chitosan [32]. However, in some cases, the molecular weight has a predominant role. For instance, chitosans with low M_w exhibited greater biological activities than chitosans with high M_w [42]. On the other hand, DD is an important parameter affecting solubility, chemical reactivity, and biodegradability [23].

Another attractive characteristic of natural polymers, such as chitosan, is their ability to be degraded by naturally occurring enzymes, implying that this kind of polymer-based implants will be degraded and metabolized by physiological mechanisms [43]. Although both chitin and chitosan are absent from mammals, they can be degraded *in vivo* by several proteases such as lysozyme, papain, pepsin, among others [4,43,44]. For example lysozyme, an ubiquitous enzyme in the body, hydrolyze the glycosidic bond between neighbor *N*-acetylglucosamine residues [45]. The degradation depends on the degree of acetylation and also varies by crosslinking [46]. The degradation kinetics seems to be inversely related to the degree of crystallinity, which is controlled mainly by the deacetylation degree. Therefore, this enzyme has more degradation activity on chitin than on chitosan because chitin has more *N*-acetyl glucosamine residues, with highly deacetylated chitosans able to last several months *in vivo* without significant chain degradation [47,48]. The understanding and control of the degradation rate of chitin and chitosan-based devices is of great interest since degradation is essential in many small and large molecule release applications and in functional tissue regeneration applications. Ideally, the rate of scaffold degradation should mirror the rate of new tissue formation or be adequate for the controlled release of bioactive molecules. Thus, it is important to understand and control both the mechanism and the rate by which each material is degraded. The degradation rate also affects the biocompatibility since very fast rates of degradation can produce an accumulation of the amino sugars and generate an inflammatory response. Therefore, chitosan samples with low DD may induce an acute inflammatory response while chitosan samples with high DD only induce a minimal response due to the low degradation rate [49].

It has been reported that chitosan, as well as sulphated chitosan oligomers, presents anticoagulant activity [5]. The anticoagulant activity of chitosan seems to be related to its positive charge, when in its salt state, since red blood cells' membranes are negatively charged and therefore chitin is less effective than chitosan.

There are other important properties that have been studied and reported such as cholesterol reduction by chitosan; the antimicrobial activity of chitin, chitosan, and their derivatives against different groups of microorganisms, such as bacteria, yeast and fungi; an antitumor activity of chitosan; analgesic effects; among others [19,50]. Because of the properties that were just mentioned, several potential uses have been proposed, as presented in the following section.

2.3. Applications of chitosan

Since chitin and mainly chitosan are versatile polymers, they have been employed in several different applications (e.g. food, cosmetics, biomedical, pharmaceutical). Among them, the biomedical applications are of particular interest due to the biological properties mentioned above, such as its high biocompatibility, biodegradability, among others.

2.3.1. Wound dressing applications of chitosan

Chitosan has been used for many applications and it is already approved by the U.S. Food and Drug Administration (FDA) for wound dressings [51]. Chitin and chitosan activate immunocytes and inflammatory cells such macrophage, fibroblasts and angioendothelial cells [52]. These effects are related to higher DD of the samples and thus, chitin presents a weaker effect than chitosan. Chitosan oligomers have also exhibited wound-healing properties. It is suggested that their wound-healing properties are due to their ability to stimulate fibroblast production by affecting the fibroblast growth factor. Subsequent collagen production further facilitates the formation of connective tissue [4–6,53]. Therefore, several chitosan-based membranes that accelerate the healing process and perform as a biodegradable template have been investigated and developed for the purpose of wound covering on account its importance in the treatment of individuals who have suffered extensive losses of skin (e.g. in burns), prevention of post surgical adhesions and cosmetic surgery [54].

HidroKi® is a hydrogel, developed by Altakitin S.A. [24], composed of chitosan (figure 2.4). It is intended, as a primary dressing, to cover wounds or burns dehydrated and without exudates, keeping a moistened environment and allowing the autolytic debridement of necrotic tissue. The dressing also protects the wound against abrasion, friction, desiccation and external contaminations.

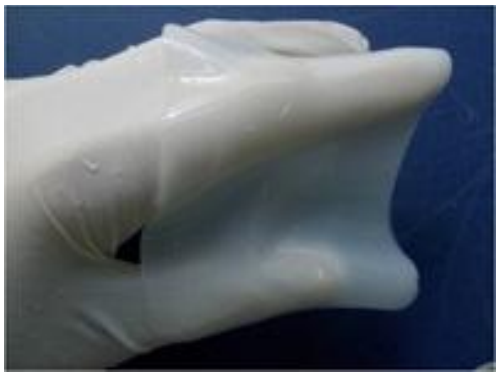


Figure 2.4. Chitosan-based primary dressing to cover wounds or burns [55].

2.3.2. Chitosan-based drug delivery systems

Another important application of chitosan has been the development of drug delivery systems such as nanoparticles, hydrogels, microspheres/microcapsules, films and tablets [56]. Controlled-release dosage forms enhance the safety, efficacy and reliability of drug therapy. They regulate the drug release rate and reduce the frequency of drug administration to encourage patients to comply with dosing instructions. As a result of its cationic character, chitosan is able to react with polyanions, such as DNA, giving rise to polyelectrolyte complexes [57]. These properties, together with the safe toxicity profile, make chitosan an exciting and promising material for pharmaceutical applications including nasal, ocular, oral and transdermal drug delivery [4–6,58].

The use of chitosan as non-viral vector for gene delivery offers several advantages compared to viral vectors, since chitosan does not produce endogenous recombination, oncogenic effects or immunological reactions [44,59].

2.3.3. Chitosan for tissue engineering applications

Recent studies in regenerative tissue engineering suggest the use of scaffolds to support and organize damaged tissue because three-dimensional matrices provide a more favorable ambient for cellular behavior [60,61]. Fiber-based scaffolds were among the first templates proposed for tissue engineering [62]. Three-dimensional scaffolds are built for the accommodation and guidance of the cells growth and the regeneration of three-dimensional tissues with proper structures and functions. The scaffolds are fabricated in proper size and shape, cells are seeded on scaffolds, which are harvested from the patient and implanted in the wound sites. Cells migrate and proliferate in all directions using the three-dimensional scaffold architecture to populate all regions of the constructs. The growth rate of neotissues is generally affected by the properties of scaffolds including their structures, composition, architecture and biocompatibility of the biomaterial; the adhesion, migration and proliferation of cells and the external stimuli including growth factors, nutrients and other bioactive agents that modulate the functions of cells [47]. Up-to-date, there are many different ways to manufacture porous scaffolds: porogen leaching; emulsion freeze-drying; expansion in high temperature gas; 3-D printing; phase separation techniques; thermal phase separation [2,63].

A large variety of absorbable biomaterials have been investigated for tissue engineering. Biodegradable polymers in the shape of woven meshes, non-woven fleeces or foams made out of polyglycolic or polylactid acid and naturally derived polymers such as collagen, hyaluronic acid, starch, or chitosans are currently used (figure 2.5) [64]. Most of these scaffolds are suitable for tissue engineering, however, the characteristics of the engineered tissues vary greatly according to the material used. Due to their low immunogenic activity, controlled biodegradability, porous structure, high affinity to *in vivo* macromolecules, and so on, chitosan-based scaffolds are promising for the design of tissue engineering applications namely, skin,

bone, cartilage, liver, nerve and blood vessel scaffolds [4,6,21,62,65–67]. Furthermore, chitosan and its derivatives have also been reported to stimulate fibroblast proliferation, activate macrophages, and enhance collagen synthesis. Moreover, it was also suggested that they promote bone formation [68]. However, a serious drawback of chitosan is its poor processability due to its molecular structure. Chitosan has strong inter-molecular hydrogen bonding, rigid *D*-glucosamine structures, and high crystallinity [69]. A common solution to address these drawbacks is random deacetylation, which results in reduced molecular weight and crystallinity [70].

Current tissue engineering strategies are focused on the restoration of pathologically altered tissue architecture by transplantation of cells in combination with supportive scaffolds and biomolecules. In recent years, considerable attention has been given to chitosan-based materials and their applications in the field of orthopedic tissue engineering [44]. Interesting characteristics that make chitosan suitable for this purpose are a minimal foreign body reaction, an intrinsic antibacterial nature, and the ability to be molded in various geometries and forms such as porous structures, suitable for cell ingrowth and osteoconduction [44]. Ideal scaffolding materials for use in hard tissue engineering must satisfy certain requirements. The materials, and their degradation products, must be noncytotoxic and allow production of biocompatible structures adapted to the tissue to be regenerated. The materials should be biodegradable with an adjustable degradation rate that should match closely the rate of tissue regeneration. The scaffold must also possess mechanical properties to match those of the tissue at the site of implantation, adequate to support morphogenesis of the neotissue and also to allow for manipulation of the device. It should provide appropriate surface chemistry to facilitate cell attachment, proliferation, and differentiation. Moreover, it should also possess the appropriate pore size and interconnected pore network to facilitate extracellular matrix production and tissue ingrowth, enable vascularization to develop, improve oxygen and nutrients supply, and metabolite removal [53,66].

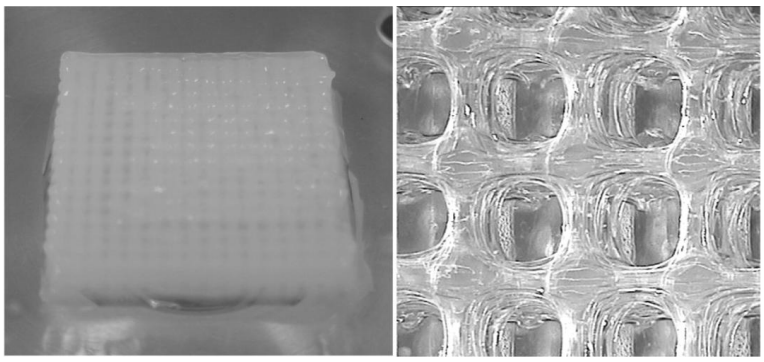


Figure 2.5. Chitosan scaffolds for tissue engineering applications [64].

2.3.4. Chitosan-based bone substitutes

Composite materials are now playing predominant role as scaffolds in bone tissue engineering. Chitosan has numerous advantageous properties for orthopedic applications, as previously described, which make it ideal as a bone graft substituent. Chitosan scaffolds are flexible and their mechanical properties are inferior to those of normal bone, as it is unable to support load bearing bone implants. Furthermore, chitosan scaffolds alone cannot imitate all the properties of natural bone. The substantial development of composite materials with chitosan mimics all the properties of bone. As proven before, calcium phosphate materials are osteoconductive and mimic the inorganic portion of natural bone, while chitosan-hydroxyapatite composite materials show promise in mimicking both the organic and inorganic portions of natural bone [71].

Calcium phosphate ceramics have been used as bone substitutes for a long time, due to its similarity to the mineral part of the natural bone so that they can be recognized, dissolved and remodeled as new bone tissue. More recently, injectable bone substitutes composed of calcium phosphate granules suspended in a polymeric matrix based on chitosan have been developed [72]. These bone substitutes are intended for use in filling bony voids or gaps of skeletal system, such as hands, feet, pelvis, spine, among others (figure 2.6).

Figure 2.6. Ready-to-use injectable bone substitute composed of calcium phosphate granules and chitosan [72].

2.3.5. Potential applications of chitosan-based implants

Chitosan has generated enormous interest for biomedical applications, as seen before, due to its various advantages such as: easy availability, positive charge, biocompatibility, biodegradability and antimicrobial activity. Moreover, it is soluble in weak acids (pK_a=6.3) and can be easily processed into films and porous scaffolds. Due to its osteoconductive properties, it has been also suggested that chitosan can be used as a bioactive coating to improve osseointegration of orthopedic and craniofacial implant devices [73]. In addition, it has been reported that chitosan can be a promising material for temporary mechanical support for bone

fractures since chitin's role in the exoskeleton of crustaceans is analogous to that of collagen in bone [10–12]. Three-dimensional hydroxyapatite/chitosan nanocomposite rods with layer-by-layer structures have been constructed via an *in situ* precipitated method [74–76]. The reported bending strength and bending modulus of these composite rods are 86 MPa and 3.4 GPa, respectively [76]. In general, these materials induce a minimal foreign body reaction, with little or no fibrous encapsulation. Formation of normal granulation tissue associated with accelerated angiogenesis appears to be the typical course of the healing response. This immunomodulatory effect has been suggested to stimulate the incorporation of the implanted material by the host [44]. Therefore, although chitosan has been proving to be one of the most promising biopolymers for orthopedic applications, there are few results for this kind of applications.

This thesis aims to introduce, characterize and assess a novel process, based on a wet gelation process, also known as a precipitation process, for the production of 3D dense chitosan-based products to be used in the future generations of bioabsorbable implants, taking advantage of the promising properties presented above. A review on the currently used polymers-based absorbable implants is presented in chapter 3.

3. Bioabsorbable Polymer-Based Implants for Medical Applications

Nowadays, the traditional biostable implants currently used for total hip and knee replacement, and spine surgery are perceived by many surgeons and patients as reasonably successful. The most common materials used in these implants are metals (e.g. stainless steel, cobalt-chrome and titanium alloys), polymers (e.g. ultra-high molecular weight polyethylene, poly(ether-ether-ketone)) and ceramics (e.g. hydroxyapatite) [77–80]. However, the last two decades of the twentieth century saw a paradigm shift from biostable biomaterials to bioabsorbable or biodegradable (hydrolytically and enzymatically degradable) biomaterials for medical devices that could help the body to repair and regenerate the damaged tissues [81]. Among the multiple medical applications for which biodegradable polymers have been used, absorbable internal fixation devices for orthopedic applications, that require a transient existence of the implant, have had special attention.

Thanks to their outstanding mechanical properties, metal fixation devices made of stainless steel and titanium have been used for bone fracture internal fixation and they are still the *gold standard* method for the majority of the treatments [13]. However, they have several significant drawbacks. First, after the fracture healing, a second operation is often necessary to remove the implants, especially in pediatric procedures, and this has several risks such as infection, removal problems of jammed implants, implants migration and associated extra health care costs [13]. Moreover, when the metallic devices are in contact with bone, they will take most of the load due to their high modulus of elasticity, producing stress shielding in the adjacent bone which may induce local bone resorption that will lead to the eventual failure and loosening of the implant. Thus, these load bearing metallic implants tend to unload the tissues and may necessitate removal because of their migration over time, irritation of the overlying tissues and hypersensitivity reactions against corrosion and released ions (e.g. iron, nickel, chromium) [82–87]. Metal implants also interfere with radiologic imaging of the underlying skeleton. Other complications include poor cosmesis, interference with therapeutic irradiation and the potential for growth restriction in pediatric patients [88]. Furthermore, some of these devices are often difficult to remove, due the fibrous layer formation around the implant, and interventions bear several implications in terms of health and social costs [89–91]. Therefore, biodegradable implants have emerged to overcome the inherent disadvantages of metal implants.

There are many materials that have been considered as potential candidates for biodegradable implants: magnesium metal and alloys, calcium phosphate ceramics and glasses, and polymers [92,93]. The biodegradable polymers have probably generated the most expectation as degradable materials for osteosynthesis [13]. These polymers are either of natural or synthetic origin. Natural polymers can closely mimic the biological environment (e.g. extracellular matrix) and present some biofunctionalities, however none has yet been processed

and modified successfully into strong fixation devices. Synthetic polymers have the advantage to have a controlled and reproducible molecular structure and to be non-immunogenic [94]. Biodegradable synthetic polymers have to be well tolerated upon implantation and during their degradation if considered for orthopedic applications. It should elicit no or a minimal inflammation from the surrounding tissue and no reaction from remote locations.

There are many factors common to any fixation device that can trigger a foreign body reaction: the implant geometry, the geometry and size of the implant, the implant surface properties, etc [84]. The rate of implant degradation, the associated structural and surface property changes, and the biocompatibility of the released by-products are supplementary factors that have to be taken into account for these devices. The implant biocompatibility reflects the body's tolerance to these factors [13].

Products where biodegradable materials are used for temporary fixation of tissue include: suture anchors/tacks for soft tissue to bone fixation; interference screws for ligament repair; meniscal repair tacks; and fracture fixation screws, pins or plates (figure 3.1). More recently, biodegradable cages for spinal fusion have also been reported [95–100]. Ideally, these implants would provide effective initial fixation and ultimately being replaced by autogenous bone. Moreover, bioabsorbable implants have greater biological interaction with the body and, without the presence of a permanent implant, it can more closely restore the native state [101].

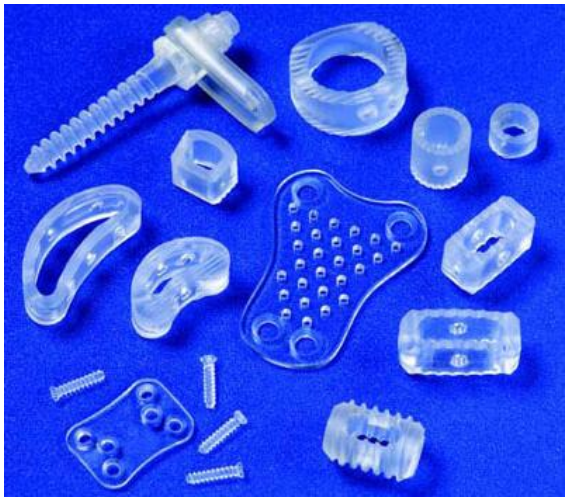


Figure 3.1. Commercially available bioabsorbable implants [102].

Bioabsorbable implants can be defined as materials or devices that, once implanted in the body, break down over time into harmless by-products that can be eliminated from the body via natural pathways and, ideally, leave no sign of the injury or repair, making a removal operation unnecessary [103,104]. The best-known application of bioabsorbable materials is in sutures, where synthetic biodegradable polymers have been used since the late 1960s, whereas for orthopedic devices, these polymers began to be used two decades later [105]. Since then the

number of products and applications has grown steadily and the current market for bioabsorbable products is growing more rapidly than that for their metal counterparts for some applications [106,107]. Every year, more than 3.1 million orthopedic surgeries are performed in the United States and the biodegradable orthopedic and oral/maxillofacial implants already represent a combined $2.8 billion market [108,109].

The purpose of this chapter is to summarize the main materials that have been used in bioabsorbable polymers-based devices, their properties and main applications and present chitosan as a potential material to be used in the future generations of bioabsorbable implants.

3.1. Materials and processing methods of bioabsorbable implants

Currently, there are approximately 40 different biodegradable polymers, being the derivatives of *α*-hydroxy acids, with the structure: HO-CHR-COOH, the most widely researched. The majority of the scientific bioabsorbable polymers are composed of one or more monomers, being glycolic acid and lactic acid, in which the R group is H and CH_3, respectively, the most common [88,95,107,110,111]. It is common to refer to the polymers in terms of their repeating units, e.g., poly (glycolic acid) or poly (L-lactic acid), which may be abbreviated as PGA and PLLA, respectively. ε-caprolactone, p-dioxanone and trimethylene carbonate are also monomers used in the manufacture of highly specialized polymers, forming the polyesters poly(ε-caprolactone) (PCL), poly(p-dioxanone) (PDO or PDS) and poly trimethylene carbonate (TMC), which are also widely studied for medical applications [88,106,107,112,113]. It is noteworthy that copolymers, which are polymers that contain two or more monomer types in their molecular chain, can be produced by ring-opening polymerization of cyclic lactide, glycolide dimers and caproic monomers allowing the synthesis of polyesters with modified molecular structure, mechanical properties and degradation pattern. The vast majority of the commercial biodegradable fixation devices are based on these copolymers [13,114] and have been approved by the Food and Drug Administration (FDA) for several medical applications, such as surgical sutures [115–117]; plates, screws and pins for bone fixation [118–120]; interference screws [97,121]; among others [13,114].

The history of absorbable implants in the repair of bone tissue began in the late 1960s. Schmitt and Polistina (1969) first suggested the use of polyglycolide (PGA) as reinforcing pins, screws, and plates for bone surgery [86]. PGA-based implants were hydrophilic, degraded very quickly and were losing all strength within one month and all mass within 6-12 months. Adverse reactions occurred as the rate of degradation exceeded the limit of tissue tolerance, and the use of PGA alone has gradually been discontinued. A newer generation of materials is now available, created from a blend of polymers, comprising lactides, glycolides and trimethylene carbonate [86,113]. Copolymers, which can permit additional control over properties through careful selection of the identity and ratio of the monomeric constituents, are becoming

increasingly adopted for implant use [88,95,122,123].

α-hydroxy acid chains can be formed by step-growth polymerization whereby monomers are added one at a time to the growing chain, however, better properties are achieved by first preparing an intermediate purified cyclic dimer and then performing a ring-opening reaction in which repeating units are added in pairs [105]. The cyclic dimers that correspond to glycolic acid and (D or L) lactic acid are glycolide and (D or L) lactide, respectively. When a subunit is added, a molecule of water is formed and released as a by-product (condensation reaction). The ester bond that forms makes bioabsorbable polymers polyesters [13,88,110].

Bioabsorbable internal fixation devices, or simply bioabsorbable implants, often perform the same functions as their metallic counterparts. Because form follows function, they may superficially resemble metal implants. These include plates, meshes, screws, pins, staples, interference screws, suture anchors, among others [88]. Although there are numerous methods by which polymers can be processed into finished devices, a limited set of these methods are routinely applied to the fabrication of bioabsorbable implants [105,124]. Many of these are based on their thermoplastic properties, meaning that these polymers can be heated, softened, formed and then cooled to retain their shape. Most fabrication processes include fabrication of heat and pressure [125–127]. In compression molding, a heated cavity is filled with the resin and the top portion of the cavity is applied under pressure. Injection molding is when the heated, liquid polymer is forced, under pressure, through a runner into the enclosed mold cavity. After the molded specimen has cooled, the mold is opened and the specimen is removed and separated from the runner portion. Extrusion is the process by which a heated screw melts and advances the liquid polymer, under pressure, through an orifice to create axially symmetric parts such as cylinders, tubes, or bars. Final geometries can be machined from the extruded part [88,128]. It is also well known that extrusion compounding parameters, such as screw profile, barrel temperature, and residence time, affect the morphology of blends [43]. Similarly, several injection molding processing parameters, such as injection speed, packing pressure, barrel temperature, and mold temperature, may affect the tensile properties [43]. In conclusion, bioabsorbable polymers can be melted and extruded, molded by injection or compression. However, the presence of moisture must be carefully controlled, because their hydrolytic sensitivity leads to a significant decrease in the material's molecular weight. Therefore, the polymer has to be kept completely dry before thermally processing, and its contact with moisture during the processing must be avoided [90]. For example, during the melting processing of PLLA, the heat and the presence of moisture reduce the polymer molecular weight. The consequence is a decrease of mechanical properties and a faster degradation rate of the biodegradable polymer device [13].

As a conclusion, although biodegradability can be a great asset when the implant is intended for temporary use, being the elimination of its removal an advantage because of reduction of infection, pain and cost, the mode and the extent of degradation for a polymer under a set of conditions have to be known to determine the suitability of the material for a given

application. When considering a degradable material for use as a fixation device, it is important that it retains its mechanical properties until the bone has healed. Moreover, the following loss of mechanical properties should be progressive enough to allow the new bone to withstand and remodel under the increasing load [13]. This information can then be used to select a biodegradable polymer composition that may fit the desired degradation pattern for the medical device.

3.2. Properties of bioabsorbable implants

Since the human body consists of a highly corrosive environment, very stringent requirements are imposed on the properties of candidate materials [129]. A desirable material for bioabsorbable implant for orthopedics should provide enough initial mechanical strength, induce or promote new bone formation and possess some bioactivity such as osteoinductive properties. The healing rate will depend on the tissues involved, extent of injury, age, lifestyle and metabolic status of the patient, illustrating the importance of matching the properties of the material to the patient. For example, a healthy pediatric patient may do well with a fast degrading implant, but an elderly, diabetic patient may require a longer strength retention profile to compensate for slower healing [110]. Therefore, when designing biodegradable orthopedic implants, several important factors should be considered. First, the implant should possess the required mechanical characteristics to sustain loads applied to defects during the healing process. Second, it should degrade with the healing process so that load is gradually transferred to the newly generated tissue. Finally, neither the initially implanted biomaterials nor the degraded materials and related products should elicit a serious inflammatory or immunogenic response in the body [109,130].

The existing absorbable implants are generally composed of various combinations of poly (α-hydroxypolyesters), as previously referred. An attempt to obtain strength of absorbable plates and screws but also maintain convenient resorption rates has led various companies to modulate implant properties via changes in morphology, composition, molecular weight, crystalline/amorphous ratio, thermal history, polymer processing methods, distribution of forces, and sterilization technique of the product [131]. Most bioabsorbable implants are made of semicrystalline materials containing both amorphous and crystalline regions, each of which plays a role in strength and absorption rates [95,106]. Semicrystalline polymers are typically stronger and more resistant to degradation in comparison with amorphous polymers. The decreased density of amorphous regions allows faster diffusion of reactants and products (e.g. water, polymer bonds, ions, enzymes) into the polymer, leading to more rapid degradation [113,132].

The biocompatibility of a degradable internal fixation device is strongly influenced by the degradation behavior of the material utilized. The degradation of synthetic biodegradable

polyesters suited for manufacturing of orthopedic implants occurs principally by simple hydrolytic scission and, to a lesser extent, through nonspecific enzymatic action, being respiration the main route of final elimination [84]. Many factors affect the degradation of the polymer and the resulting reaction of the body to the polymer including implant material, porosity, molecular weight of the polymer, implant geometry, site of implantation (an implant is more efficiently depolymerized by richly vascularized cancellous bone than by dense avascular connective tissue), and method of sterilization [13,17,95]. For instance, the length of the polymer chains influences the degradation rate. The longer the polymer chain is, the more hydrolytic chain scissions are necessary to obtain by-products which are able to diffuse out of the device. This reduces the degradation rate of the polymer. The crystallinity of the polymer is another example. Although a polymer cannot be 100 % crystalline, a semicrystalline polymer is well organized at molecular level and present many inter- and intra-molecular bonds (e.g. hydrogen bonds). In contrast, an amorphous polymer does not present a close packed organization. Therefore, small molecules such as water can more readily diffuse in amorphous polymers than in semicrystalline polymeric materials [13]. The consequence is that amorphous polymers are hydrolyzed faster than semicrystalline ones.

In the degradation process, there is first a loss in molecular weight, followed by loss of strength and finally loss of mass [17,88,95,110]. The process of biodegradation of a polymer-based implant begins with the polymer chains being broken into smaller fragments by hydrolysis, through a nonspecific scission of their ester bonds [101]. Therefore, water penetrates the bulk of the implant and attacks preferentially the chemical bonds of the amorphous phase, shortening the polymer chains. Thus, the molecular weight of the implant decreases. Thereafter, the mechanical strength of the implant decreases allowing subsequent mechanical fragmentation and absorption of the implant to begin. Mass loss of the implant occurs then through the release of soluble degradation products, phagosytosis by macrophages and histiocytes, intracellular degradation and finally, metabolic elimination through the citric acid (Krebs) cycle to carbon dioxide and water, which are expelled from the body via respiration and urine [87,88,106,107]. In the case of PLA and PGA, the final products of the polymer degradation are the acidic monomers (lactic acid and glycolic acid, respectively) that are enzymatically converted and used in the metabolic pathway of the tricarboxylic acid cycle that takes place in the mitochondria. The final products are adenosine triphosphate (ATP), water and carbon dioxide (CO_2) that are excreted by the lungs and kidneys [90]

While the polymer degrades, the mechanical performance of the device also deteriorates. The key to develop effective fracture fixation systems based on biodegradable devices is to provide an adequate level of fixation strength for a time frame that exceeds that expected for fracture healing. Once the fracture is healed, the device can be completely absorbed by the body [103]. Therefore, the mechanical properties of bioabsorbable orthopedic implants must be considered both at the time of insertion and throughout degradation. The implants are initially subjected to considerable loads, which gradually decrease with tissue healing.

Table 3.1 summarizes the main properties of the most used polymers, as well as the properties of the most common metallic materials and bone. Poly(glycolic acid) (PGA) was one of the very first degradable polymers ever investigated for biomedical use, as previously mentioned. With a melting point (T_m) greater than 200ºC, a glass transition temperature (T_g) of 35-40ºC, and a considerably high tensile strength, PGA found favor as the degradable suture DEXON, which has been actively used since 1970 [133]. From 1984 to 1996, PGA was marketed as an internal bone pin under the name Biofix, but since 1996 Biofix has been converted to a poly(L-lactide) base for better long-term stability [134]. Although there has been research conducted into a wide range of applications, there exists significant issues regarding the use of PGA. Rapid degradation leads to the loss of mechanical strength and significant local production of glycolic acid. Although glycolic acid is bioresorbable by cells via the citric acid cycle, high level of glycolic acid have been linked to a strong, undesired inflammatory response [2].

Table 3.1. Properties of some biomaterials [13,88,110,127,131,135,136].

Material	T_g (ºC)	T_m (ºC)	Initial Modulus (GPa)	Initial Strength (MPa)	Elongation (%)	Total Strength loss (months)	Total Mass loss (months)
PGA	35 - 40	224 - 230	4 - 18	75 - 142	15 - 20	1 - 2	6 - 12
PLLA	57 - 65	173 - 184	1.7 - 10	40 - 140	5 - 10	3 - 10	24 - 120
PDLLA	55 - 60	-	1.9	42 - 51	3 - 10	3 - 4	12 - 36
PCL	-65 - -60	58 - 63	0.4	20 - 40	300 - 500	> 6	> 24
PDO	-16 - 0	110	1.5 - 2.1	-	-	1 - 2	6 - 12
PGA-TMC	-	-	2.4	-	-	-	< 12
PLLA-PGA (82:18)	57	-	2 - 7	40 - 55	3 - 10	> 8	12 - 18
Bone	-	-	3.3 - 20	51 - 193	1	-	-
Steel	-	1375 - 1400	200	550 - 965	20 - 50	-	-
Titanium alloy	-	1650 - 1700	100 - 110	620	18	-	-

Regarding polylactide (PLA), as far as use in biomedical research, only PLLA and PDLLA have shown promise and have been extensively studied [2]. The additional methyl group in PLA causes the polymer to be much more hydrophobic and stable against hydrolysis than PGA. High molecular weight PLLA has been shown to take greater than 5 years to be completely resorbed *in vivo*. PDLLA is an amorphous polymer due to the random positions of its two isomeric monomers within the polymer chain yielding a slightly lower T_g and lower mechanical strength of 1.9 GPa. Although possessing more desirable degradation properties than PLLA,

PDLLA still takes over a year to properly erode. Like PLLA, PDLLA has been often combined with other degradable polymers such as PLGA, PEG, and chitosan to create composites with desirable material properties [2,137].

Random copolymerization of PLA (both L- and D,L-lactide forms) and PGA, known as PLGA, is the most investigated degradable polymer for biomedical applications. One particular advantage is that because PLA and PGA have significantly different properties, careful choice of copolymer composition allows for the optimization of PLGA for intended applications [2].

PDO is a colorless, semicrystalline polymer synthesized by ring-opening polymerization of p-dioxanone that has a T_g about -10 to 0ºC and a T_m of 110-115ºC. Although quicker degrading than longer aliphatic poly-esters of similar backbone length, PDO can still be considered a slow degrading polymer (6–12 months for complete mass loss). With a low modulus (~1.5 GPa), but good flexibility and strength maintenance (1–2 months), PDO has been commercialized as the monofilament suture PDS [2].

Stainless steel and titanium alloys have modulus of elasticity an order of magnitude greater than bone, hence their ability to induce stress shielding [110]. On the other hand, the modulus of bioabsorbable polymers is smaller than bone, thus stress shielding is not a concern, but the challenge is to design the implant to be sufficiently strong to withstand biomechanical loading. Following polymerization there is no preferred direction to the polymer chains which makes the polymer exhibits an isotropic behavior, meaning that the mechanical properties are equivalent in every direction. There are processing techniques though, that allow the polymer chains to acquire a preferred orientation or direction, in which the mechanical properties become enhanced in certain directions (anisotropic behavior). This is often referred to as self-reinforcement (SR) or orientation. For example, orientation increases the modulus of an 82:18 PLLA:PGA copolymer from 4 GPa to 7 GPa [110]. As far as degradation rate is concerned, some polymers lose strength in less than one month while others require six months or longer. Also, the time required for complete mass loss (from 6 months up to 6 years) is frequently multiples of that required for complete strength loss. Within the family of glycolide- and lactide-based polymers, considerable variation exists in strength and mass loss profiles [17,95,110]. *In vivo* testing of PLLA showed no loss of strength after 5 months, and it is unknown whether or not it ever completely degrades [101].

Fracture healing proceeds in three phases: (i) an inflammatory phase lasting from 3 to 7 days; (ii) a reparative phase lasting 1 month, approximately; (iii) and a remodeling phase that can require a much longer period for completion [138]. The bony union that forms at the end of the reparative phase, through the development of the bony callus, can result in strength equal to or greater than that of the intact bone. Consequently, 6 to 8 weeks after adequate stabilization of a fracture is often thought to be sufficient to result in a strong healing union [122]. Thus, during the first 6 to 8 weeks after fixation, the strength of the biological union increases as the absorbable fixation strength diminishes. If the implant loses strength too rapidly with respect to the rate of healing, then fixation may be compromised. Conversely, an absorbable implant that

retains properties significantly longer than required might result in prolonged “protection” of the fixation site and may possibly lessen the mechanical stimulus for remodeling. Also, a slowly absorbing implant might present long-term palpability problems that may necessitate surgical intervention, as has been reported for PLLA-based screws. Thus, absorbable fixation should ideally lose strength in concert with healing as well as lose all mass as quickly as possible thereafter [122].

In addition, the properties of the biodegradable polymers (as well as any polymers and also metals generally), even if made out of the same raw material components, will clearly depend on their manufacturing processes (e.g. processing temperature, possible self-reinforcing, sterilization method etc.). Conversely, this provides the possibility to develop materials and implants with distinctive desirable properties. However, this also means that differently manufactured products made out of the same raw material can have different product-specific properties such as different mechanical strengths and degradation behaviors. Therefore unambiguous data or conclusions about the properties of biodegradable products (or raw materials) cannot simply be based on test results obtained with other products made out of the same raw material if the detailed processing methods and parameters are not known [135]

The use of bioabsorbable materials has become commonplace in orthopedic surgery. These devices have expanded the implants available for surgeons, especially in the field of sports medicine [101]. Interference screws, suture anchors, meniscal repair devices, and simple fracture fixation devices are the most commonly used biabsorbable implants for anterior cruciate ligament reconstruction, shoulder surgery, meniscal repair and fracture care, as demonstrated in the following section.

3.3. Applications of bioabsorbable implants

The best-known application of bioabsorbable materials is in sutures, where synthetic bioabsorbable polymers have been used since the late 1960s. As previously stated, it was only in the 1980s that bioabsorbable polymers began to be used in orthopedic devices. The first biodegradable bone fixation implant was a biodegradable rod made out of PGA and the world’s first orthopedic patient treated with the biodegradable rods was an ankle fracture patient treated in Helsinki, Finland in 1984 [135]. Since then, the number of products and applications (table 3.2) has grown steadily and the current market for bioresorbable products is growing more rapidly compared to their metal counterparts in some applications [106]. For instance, in the orthopedic trauma market, that consists of legs, arms, hands and feet bone fractures, in 2000, the US sales across trauma implant manufacturers exceeded $540 million, with steady 5% annual growth [91]. These devices included: (1) plates, which were attached with screws to the surface of the broken bones; (2) rods, which were inserted into the center or medullary canal of broken bones; and (3) screws (used without plates), which helped to realign bone fragments.

The market prices for these devices varied greatly according to type, size, and application, with plates priced from $100 to $600, rods from $500 to $1,500, and screws from $5 to $100 [91]

Today, nearly every orthopedic manufacturer has an extensive line of bioabsorbable devices to offer. These devices are manufactured in the form of pins, screws, plates, rods, tacks, and suture anchors and are most often manufactured from PLLA, PGA, PDO, or a copolymer of PLA or PGA [87,122].

Table 3.2. Biodegradable polymers and some of their biomedical applications

Material	Trademark	Description
PGA	DEXON™ S	Surgical sutures [139]
	Medisorb®	Injectable technology for CO_2 absorption [140]
	Biofix®	Rods for bone fixation [141]
PLA	Phusiline®	Membrane for tissue regeneration [142]
	BioSorb™ FX	Plates and screws for bone fixation [120]
	BIORCI™	Screw for arthroscopic surgery [121,143]
	BioScrew®	Interference Screw [97]
PLGA	LactoSorb®	Plating system for craniomaxillofacial fixation [120,144]
	Vicryl®	Surgical sutures [115]
	RESOMER®	Microspheres for controlled release of drugs [145]
	Lactomer®	Surgical sutures [116]
PDO/PDS	PDS™ II	Surgical sutures [117]
	Ethipin®	Pins for bone fixation [118]
	OrthoSorb®	Pins for bone fixation [119]
PGA-co-TMC	Maxon®	Surgical sutures [117]
PLA-co-TMC	Inion OTPS™	Plates, screws and pins for bone fixation [146,147]

3.3.1. Bioabsorbable implants in extremities

Ankle fractures are one of the most common injuries in orthopedics. Biodegradable plates and screws made of biodegradable copolymers composed from PLA and TMC monomers have been successfully used for ankle fractures [148]. Bioabsorbable fixation is also widely used in forefoot procedures such as correction of hallux valgus (bunion) deformity [88,110]. Scaphoid fractures, metacarpal fractures and distal radius fractures have also been successfully treated [88,110].

3.3.2. Bioabsorbable implants in joints

Bioabsorbable polymer screws used in anterior cruciate ligament (ACL) reconstructions have shown acceptable clinical results [113]. Interference screws and suture anchors are commonly used in arthroscopic surgery and sports medicine for fixation of soft tissue to bone. Interference screws provide a press fit between bone, graft/tendon, and screw, whereas suture anchors tie soft tissue to an implant embedded in bone [97,110,113,136,149]. The anterior cruciate ligament (ACL) is the most frequently injured ligament in the knee and consequently, the majority of research into knee ligament injuries has been directed towards the ACL. The patients who experience ACL injuries are significantly younger and more active than those who experience many other orthopedic injuries [150]. Several different types of screws, which vary in polymeric composition, are currently available [151,152]. Graft fixation strength in anterior cruciate ligament reconstruction is critical in the period from initial fixation to osseous integration of the graft. This period ranges from 6 weeks for bone-patellar tendon-bone fixation to approximately 12 to 16 weeks for hamstring fixation. The initial pullout strengths of these implants should exceed the estimated 500 N load for activities of daily living [95,153].

The use of bioabsorbable fixation in the treatment of soft-tissue lesions in the shoulder has been increasing as well. These implants have eased Bankart repairs, as well as the treatment of labral and rotator cuff lesions. The development of bioabsorbable tacks, suture anchors, and screw-and-washer implants with different polymeric compositions has given surgeons more treatment alternatives [95,96,154–157].

3.3.3. Bioabsorbable implants in spine

Bioabsorbable implants for spinal surgery are rather recent in comparison with the applications mentioned previously. Some applications such as interbody cages for specific spinal fusion applications, bioabsorbable ramp type interbody spacers for posterior lumbar interbody fusion procedures and the use of absorbable screws for anterior cervical decompression and fusion processes have been successfully reported [158]. The stabilization of posterolateral lumbar fusion using facet joint fixation with bioabsorbable rods has also been studied [90]. The spine is one of the most difficult anatomic regions to achieve fusion and, hence, is a stringent test of fixation. Anterior cervical discectomy and fusion is performed to treat anterior degenerative or traumatic instability of the cervical spine. Bioabsorbable anterior cervical plates that provide stability for 6 to 12 months can reduce or eliminate the complications associated with metal devices [159]. Bioabsorbable sheets and screws have been successfully used for graft containment in one- and two-level fusions, with intraoperative heating of the mesh enabling it to be shaped to fit the construct [88,110]. The clinical use of bioabsorbable PLLA interbody cages for posterior lumbar interbody fusion has also been reported [160,161].

3.3.4. Bioabsorbable implants in craniofacial skeleton

Biodegradable implants are also frequently used in craniomaxillofacial (CMF) surgeries to overcome the metal implants' drawbacks, such as thermal conductivity, allergic hypersensitivity, chemical carcinogenesis, infection, among others [13,110,148]. Despite the initial skepticism, bioabsorbable implants use was expanded from the cranial vault and midfacial fractures to orthognathic surgery, mandibular fractures, and restorative surgery after tumor ablation. One of their undeniable indications is pediatric craniofacial surgery, because of their absorbable character, which allows for bone development [162–168].

In short, bioabsorbable fixation can be used in the treatments of a variety of fractures, within different medical fields, such as orthopedics, sports medicine, craniomaxillofacial, among others. Moreover, the use of bioabsorbable implants has shown satisfactory results for pediatric fracture fixation. However, although bioabsorbable implants have been employed in a variety of applications, they have several disadvantages over metallic implants, being their low mechanical properties, high cost and undesired biological responses the most frequently mentioned. Furthermore, the uncertainty regarding the predictability of the degradation time interval, especially the time interval in which the mechanical properties are at the level demanded by the application is another concern [16].

3.4. Limitations of bioabsorbable implants

A desirable material for bioabsorbable implants should provide enough initial mechanical strength, induce or promote new bone formation by osteogenic cells and possess some bioactivity by being osteoinductive. Thus, absorbable fixation should ideally lose strength in concert with healing as well as lose all mass as quickly as possible thereafter [10,17]. However, there is the need to improve the degradation profile of the degradable polymers currently used for osteosynthesis in order to match more closely the bone healing process [13]. One of the main disadvantages of biodegradable materials is the premature loss of mechanical properties before the healing process is complete. Because of the viscoelasticity of these materials, they lose a significant amount of their force immediately after application. For example, PLA-based screws lose approximately 20% of their force within 20 minutes. In distilled water, this effect was even more pronounced, with a loss of up to 45% of the initial force of these screws [101]. In addition PLGA undergoes an autocatalytic degradation process which results in an accelerated degradation that leads to hollowing of the implant and its catastrophic failure [99,104]. Another weakness is the sterilization of a polymeric implant. Common dry heat and autoclaving sterilizations cannot be carried out as they significantly modify the biodegradable device's specifications. Typically, ethylene oxide and radiations are employed to minimize degradation during polymer device sterilization. In early studies, these difficulties with the necessity of a careful storage to avoid early degradation may have been the cause of infection related to the

release of by-products [13].

Besides this, reported complications with the use of these materials include sterile sinus tract formation, osteolysis, synovitis, and hypertrophic fibrous encapsulation [107,113]. Another drawback of such polymer implants is that their acidic degradation (e.g., lactic acid) have an effect on the microenvironment pH and may cause local inflammation [11]. The degradation of PLLA has been associated with a foreign-body reaction as late as 3 years after implantation. A foreign-body response reaction to PGA has been seen as early as 3 to 6 weeks. These inflammatory responses occur in fewer than 10% of patients, but may be severe enough to require surgery for resolution of the reaction and affect the normal bone healing [17,95,110,169]. Since most of the existing polymer-based implants are composed of various ratios of PLLA and PGA, their absorption period vary significantly. During the period of biodegradation, complications such as infection or wound dehiscence may occur at a rate comparable to that seen in metallic plates, with some differences noted in the stability of osteosynthesis with bioabsorbable plates showing decreased stability over metallic ones [162].

Therefore, three key challenges that need to be addressed by new bioabsorbable polymers can be identified: optimization of degradation rates to better match the healing processes of each tissue; development of materials with higher strength or stiffness for greater load bearing applications; and improved biocompatibility of breakdown products and long-term *in vivo* performance [18]. Problems associated with the stimulation of chronic inflammatory reaction and toxicity by synthetic polymers can be largely suppressed or eliminated by using natural polymers. Another attractive characteristic of natural polymers is their ability to be degraded by naturally occurring enzymes, implying that the implant will be degraded and eventually metabolized by physiological mechanisms. On the other hand, natural polymers are frequently moderately immunogenic [43].

Enzymatically degradable polymers are materials that possess bonds that despite hydrolytically sensitive, in reality require catalysis to undergo meaningful degradation under physiological conditions [2]. As previously mentioned, polysaccharides are natural polymers composed of monosaccharide units joined together by glycosidic linkages. Their use as biomaterials has become much more common as new biological functions are identified for these materials. Also, the array of materials that can be investigated has increased due to new synthetic routes that have been developed for modifying polysaccharides. Their biodegradability, processability, and bioactivity make polysaccharides very promising natural biomaterials [2]. Chitin, but mainly chitosan, is a good example among polysaccharides exhibiting unique physicochemical properties, as it is a biocompatible, antibacterial, biodegradable and environmentally friendly polyelectrolyte. Therefore, a big variety of applications including water treatment, chromatography, additives for cosmetics, textile treatment for antimicrobial activity, novel fibers for textiles, photographic papers, biodegradable films, biomedical devices, and microcapsule implants for controlled release in drug delivery have been explored [4–9,12,21,44,46,67,70,170].

The following chapters aim to introduce, characterize and assess a novel 3D dense chitosan-based products production process.

4. Chitosan-Based Products Production Process Development

Biomaterials have been extensively developed and applied in an enormously number of medical devices. Among these materials, bioabsorbable polymers have attracted special attention for orthopedic applications where a transient existence of an implant can provide better results, when compared with permanent implants. Although the properties of bioabsorbable implants have considerably improved along the time, most of these implants are fabricated from synthetic polymers and therefore, problems associated with the stimulation of chronic inflammatory reactions and toxicity are common [171,172]. However, these problems can be largely suppressed or eliminated by using natural polymers. Chitosan has generated enormous interest for biomedical applications due to its various advantages such as biocompatibility, biodegradability and osteoconductive properties, as described in chapter 2. Moreover, it has been reported that chitosan can be a promising material for temporary mechanical supporter for bone fractures since chitin's role in the exoskeleton of crustaceans is analogous to that of collagen in bone [10–12]. However, due to a non-accessible glass transition temperature before thermal decomposition, the conventional processing techniques used for thermoplastic polymers are not suitable [14]. Therefore, the *in situ* precipitation method, where the polymer is first dissolved in a solvent (e.g. acetic acid aqueous solution), followed by a precipitation, coagulation, or gelation process in the presence of basic salts (e.g. sodium hydroxide), is used. Three-dimensional hydroxyapatite/chitosan nanocomposite rods with layer-by-layer structures have been constructed via this method [74–76]. In general, these materials induce a minimal foreign body reaction, with little or no fibrous encapsulation [44].

Although chitosan has been proving to be one of the most promising biopolymers for orthopedic applications, there are few results for this kind of applications. Thus, this chapter aims to introduce a novel process, based on a wet gelation process, for the production of 3D dense chitosan-based specimens as a potential material to be used in the future generations of bioabsorbable implants, taking advantage of the promising properties presented throughout chapter 2. The production process of these specimens and its main steps are described, the specimens characterized according to their physicochemical properties and the results are presented and discussed.

4.1. Materials and methods

Free base chitosan powder (deacetylation degree = 90%; average molecular weight = 300 kDa) was provided by Altakitin, S.A. Glacial acetic acid and sodium hydroxide solution (50% w/v) were purchased from Panreac Química S.L.U.

4.1.1. Production of 3D dense chitosan specimens

The production of three dimensional (3D) chitosan specimens involved the dissolution of chitosan (3%, w/v) in an aqueous solution of acetic acid (2%, v/v). After total dissolution, the homogeneous solution was poured into molds with predefined geometry and left at 5 ºC overnight to remove air bubbles, prior to be frozen at - 20 ºC for 24 hours. The frozen solutions were removed from the molds and introduced in a sodium hydroxide aqueous solution (10%, w/v) for 48 hours. After gelation, the 3D specimens were abundantly washed with deionized water until pH~7 and air-dried in oven at 40 ºC for 96 hours. The three-dimensional dried and dense specimens were then ready to be machined into the intended final shape.

A minimum of three specimens for each experiment were prepared and the average value and the standard deviation (SD) are presented.

4.1.2. Morphological analysis of chitosan specimens

The specimens were cut in different sections and observed in a scanning electron microscope (SEM), using a FEG-SEM, model JSM-7001F, from JEOL with energy of 10 kV. The specimens were previously coated with gold due to its nonconductivity. Different cross sections areas were observed and analyzed in terms of porosity (size and distribution), topography and overall density.

4.1.3. Density and porosity of chitosan specimens

The density of the raw material (ρ_r) – chitosan powder – was analyzed by a AccuPyc 1330 Pycnometer, connected to a helium system under pressure (200 bar). Density of 3D specimens was determined by applying the Archimedes' principle. The density of each specimen was determined with the aid of purified water. Thus, the specimen was weighted in air (A) and then in the purified water (B). The density of the specimen (ρ_s) can be calculated from the two weightings as follows:

$$\rho_s = \frac{A}{A-B} \, x \, \rho_{water} \tag{4.1}$$

The porosity (P) of each specimen was calculated from the density of chitosan (raw material) and the density of the specimens that were produced:

$$P(\%) = \left(1 - \frac{\rho s}{\rho r}\right) x \, 100 \tag{4.2}$$

4.1.4. Swelling ratio of chitosan specimens

The water sorption capacity of the specimens was determined by immersing the specimens in phosphate buffered saline (PBS, Sigma-Aldrich) at pH 7.4 for 24 days at 37°C. The swollen specimens were removed at predetermined time intervals (30min, 1h, 2h, 4h, 8h, 24h, 2 days, 3 days, 6 days, 12 days and 24 days) and immediately weighted with an analytical balance after the removal of excess of water by lying the specimens on a filter paper. The swelling ratio (SR) was calculated using the following equation:

$$SR(\%) = (W_t - W_0) / W_0 \times 100 \quad (4.3)$$

Where W_t and W_0 are the weights of the specimens at time t (swelling state) and at time 0 (dry state), respectively.

4.1.5. Mechanical properties of chitosan specimens

Preliminary mechanical tests were also conducted. Prior machining step is needed to obtain test samples with appropriate shape and dimensions – flat plates with a thickness of approximately 3 mm – for running the tests, namely 3 point bending tests, according to the ASTM standard D 790 [173]. An universal testing machine from Instron (model 5566) was used with a load cell of 500 N with extension control. Tests were performed with a crosshead speed of 0.2 mm/min at room temperature. Finally, the tests results were processed using the computer aided software for static systems (Bluehill® 2 Materials Testing Software).

The flexural stress (σ_f) of each point was calculated by means of the following equation:

$$\sigma_f = 3PL / 2bd^2 \quad (4.4)$$

Where P is the load at a given point on the load-deflection curve; L is the support span; b and d are the width and thickness of the tested specimen, respectively. The flexural strength (σ_{fM}) is the maximum flexural stress sustained by the tested specimen.

The flexural strain (ϵ_f) can be calculated for any deflection using equation 4.5:

$$\epsilon_f = 6Dd / L^2 \quad (4.5)$$

Where D is the maximum deflection of the center of the specimen; d is the thickness of the tested specimen; and L is the support span.

The modulus of elasticity (E_B) is the ratio, within the elastic limit, of stress to corresponding strain. It is calculated by drawing a tangent to the steepest initial straight line portion of the load-deflection curve and using the following equation:

$$E_B = L^3 m / 4bd^3 \quad (4.6)$$

Where L is the support span; m is the slope of the tangent; b and d are the width and thickness of the tested specimen, respectively.

4.1.6. NMR spectroscopy of chitosan specimens

The composition and the degree of deacetylation of the chitosan specimens were determined by ^{1}H NMR spectra, according to the method described by Hirai et al [36]. Measurements were performed on a Bruker Avance-III 400 MHz NMR spectrometer, under a static magnetic field of 9,4 T at 70ºC. The software used to analyze the spectrum was the Bruker Topsin 3.1. The concentration of chitosan solution was 10 mg/ml in a DCl/D_2O solution (2%, w/v).

The degree of deacetylation (DD) was evaluated by using the integral intensity of CH_3 residue (I_{CH3}) and the sum of integral intensities of H_2-H_6 (I_{H2-H6}):

$$DD(\%) = \{1 - (\frac{1}{3} I_{CH3} / \frac{1}{6} I_{H2-H6})\} \times 100 \quad (4.7)$$

4.1.7. Gel permeation chromatography of chitosan specimens

The molecular weight of chitosan specimens were determined by gel permeation chromatography (GPC) at room temperature. A PL aquagel 8 µm column was used, with a sodium acetate/acetic acid buffer solution (pH 5) as eluent. The flow rate used for the measurements was 1 mL/min. Calibration curve was previously obtained by using Varian pullulan polysaccharides certified standards in the same chromatographic conditions.

4.2. Results and discussion

In this section, the results and their interpretation are presented. The previously described process (section 4.1.1) developed to produce the chitosan specimens is explained below, highlighting its main steps. The results of produced and tested specimens are also discussed.

4.2.1. Production of 3D dense chitosan specimens

The 3D dense chitosan-based specimens production process is summarized in figure 4.1, being its main steps detailed, one by one, as follows:

1 – Dissolve chitosan powder. The first step consists in dissolving chitosan in an acidic acid aqueous solution. In the standard procedure, a chitosan solution with a concentration of 3% (w/v) is prepared, however, chitosan solutions with higher concentrations are possible to be obtained. As expected, it was observed that the lower the molecular weight of chitosan and the higher its DD, the higher is the mass of chitosan that can be dissolved in the acidic solution. With the chitosan used in the process, homogeneous solutions with concentrations of up to 5% (w/v) were possible to obtain, however, due to direct proportionality between viscosity and

amount of chitosan dissolved in the acidic solution, a concentration of 3% (w/v) of free-base chitosan powder dissolved in a 2% (v/v) acetic acid aqueous solution was the most adequate for the purpose of this work. Solutions with higher concentration of chitosan were hard to handle and exhibited more air bubbles due to a higher air retention. On the other hand, solutions with lower concentrations of chitosan were more fragile and could easily break during the gelation process.

It is suggested that chitosan dimer formed in acetic acid solution has a relatively strong intermolecular interaction to give a tighter film structure [174]. Therefore, since we aim to have tight and dense 3D chitosan structures, the acidic solution needed to dissolve chitosan was always prepared with acetic acid, although other acid solutions could be also used (e.g. citric, lactic). Moreover, it was also reported that chitosan fibers tensile strength showed a 70% improvement in fiber strength when acetic acid was increased from 1 to 2 (v/v), but above this concentration fiber strength decreased steadily [175]. Thus, a 2% (v/v) acetic acid solution was always used and set as standard.

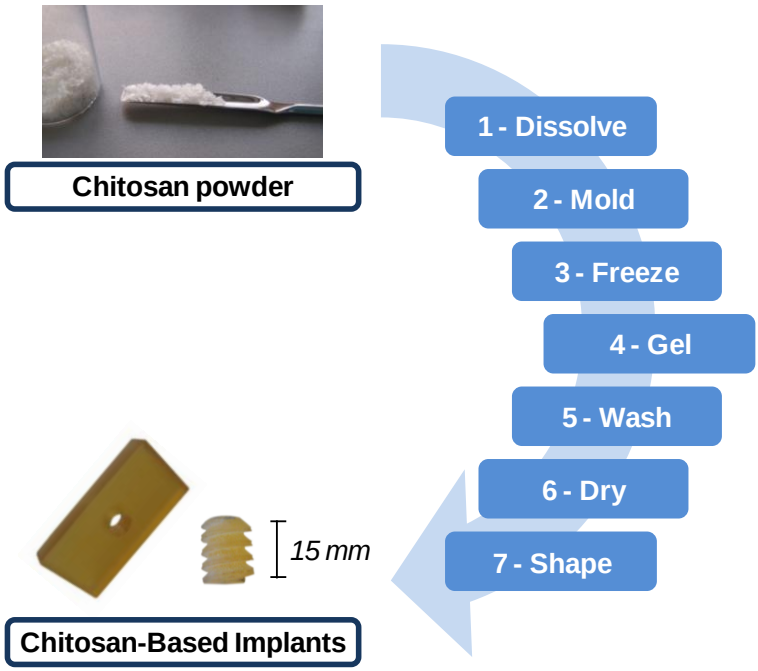


Figure 4.1. Scheme with the main steps of the production process of 3D dense chitosan-based specimens.

2 – Transfer the solution into a mold. After total dissolution of the polymer, the resulting homogeneous solution is poured inside a mold. Before freezing, the molds are left at rest overnight to allow air bubbles to collapse. It has been found that this is a critical step in order to obtain a dense product, since air retention is adverse for the present purpose. The size and shape of the mold may vary, being 150 x 60 x 40 mm rectangular mold or a cylindrical mold with a diameter of 50 mm and 150 mm height the typical standard molds.

3 – Freeze the solution. The air bubble free chitosan solution is frozen at -20 ºC for 24h in order to get a 3D structure with the same shape as the mold. To accelerate the freezing process and to assess the effect of temperature, several solutions previously poured inside the molds, were frozen in liquid nitrogen (approximately -200 ºC), instead of freezing them at -20 ºC. The resulting 3D solid solutions frozen faster when using liquid nitrogen, however, they presented cracks in the structure due to the sudden decrease in temperature. Thus, freezing the solutions at -20 ºC proved to be a better process yielding smooth surfaces without visible cracks.

4 – Precipitate the frozen solution. After removing the frozen solution (*a*) from the mold, it is introduced into an alkaline solution (*b*) to precipitate. During this wet gelation process, also known as *in situ* precipitation, the diffusion velocity of *b* (within *a*) is faster than the defrost velocity of *a*, which allows the gelled solution (*c*) to keep the same geometry of *a*. Taking into account that this wet gelation process is performed in a frozen solution and NaOH aqueous solution is used as a chemical gelation agent, it was crucial to optimize its concentration. On one hand, the lower the NaOH concentration the easier would be to neutralize the specimens. Moreover, a low NaOH concentration would possibly be harmless as far as initial chitosan parameters are concerned (e.g. DD, M_w, viscosity). On the other hand, the greater the gelation agent concentration, the faster the gelation process was expected to be, according to previous studies [176]. By analysis of the finished specimens (transversal cut of gelled solutions), it is possible to assure that 10% (w/v) NaOH aqueous solution can precipitate the entire specimens in less than 48 hours, with the formation of a layer-by-layer structure (figure 4.2), as described elsewhere [10,12]. Several NaOH solutions with lower concentrations were tested with poor results since most of the specimens were breaking during this process. On the other hand, the results obtained with higher concentrations of NaOH solutions presented similar results to NaOH 10% (w/v) after 48 hours, making the use of 10% NaOH the best solution for specimens' gelation. Since neutralization of the specimens is an important step, after the gelation process, an optimized concentration of base is desired towards a less time-consuming neutralization.

In the present study, finding an optimal NaOH concentration was crucial since the gelation agent diffusion rate has to be faster than the defrost rate of chitosan's solutions, as mentioned above. Thus, based on Venault *et al.* experimental approach [176] and in order to validate the visual observation of the formation of a layer-by-layer structure along the whole specimen, a 0.1% (v/v) of a phenolphthalein solution previously prepared (50 mg of phenolphthalein (Sigma-Aldrich) + 50 mL of deionized water + 50 mL of ethanol) was added to the acetic acid aqueous solution of several preliminary specimens that were prepared according to the production process previously described. From figure 4.3 it is possible to observe the gelation process along the time. The fuchsia layer visible in the photos indicates the transition of pH (from acid to basic). When pH is above 11, the outer layers of the specimens turn to semitransparent, because of the formation of a colorless trianion structure [177]. After 12 hours, the gelation agent had diffused through more than half of the specimen. After 48 hours, all the specimens tested with the pH indicator presented a completely semitransparent-white structure, validating the visual observation of a complete layer-by-layer 3D solid structure.

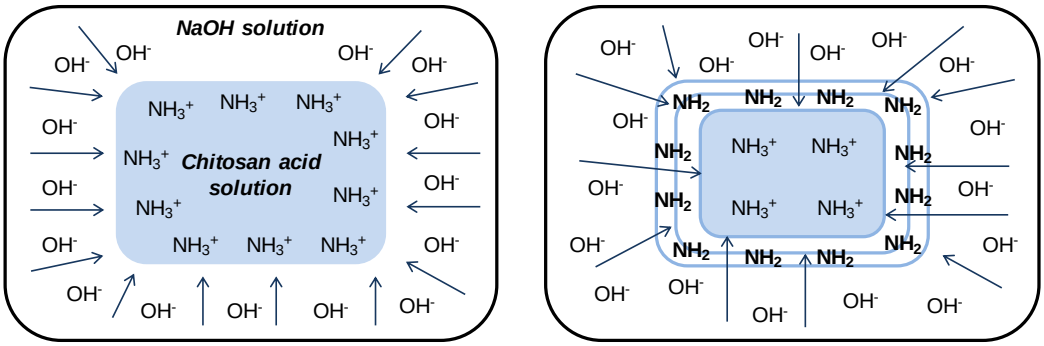


Figure 4.2. Schematic representation of the layer-by-layer structure formed during gelation process.

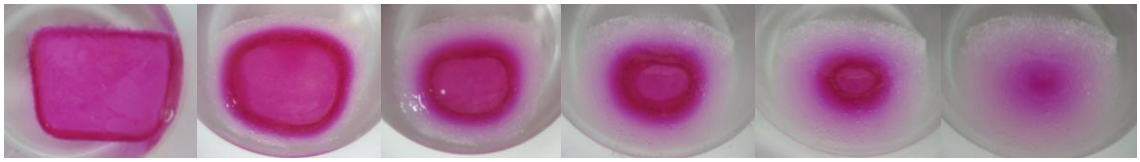


Figure 4.3. Wet gelation process of a 3D frozen chitosan solution.

5 – Neutralize the precipitated solution. Neutralization of specimens is an essential procedure aiming biomedical applications, in order to avoid undesirable tissue reactions. For this reason and in order to get a pH close to 7, the specimens are abundantly washed with deionized water. Due to the relatively high concentration of NaOH, this is usually a time-consuming step. Therefore, depending on the specimens volume, this procedure usually takes 3-5 days.

6 – Dry the precipitated solution. Another key step for achieving the desired 3D dense products is the drying process. In order to obtain a smooth yet controlled, efficient and homogeneous dehydration, the specimens were dried at approximately 40ºC, in which up to 97% hydrated specimens lose their water content. During the process, the specimens shrink rather uniformly and become darker, while losing more than 94% of their initial weight (Table 4.1), yielding a dense chitosan bar with the same shape as before while hydrated (Figure 4.4).

Both lower and higher drying temperatures were also tested. When using a lower temperature (25ºC) the specimens were not completely dried after one week, proving to be a much less efficient drying temperature. On the other hand, when using a higher temperature (60ºC) the specimens were not shrinking as uniformly as they were shrinking when using 40ºC. Thus, a drying temperature of 40ºC proved to be the most adequate for these specimens.

Table 4.1. Weight loss of 3 chitosan specimens.

Specimen code	Weight (g)					Weight loss (%)
	t = 0h	*t = 24h*	*t = 48h*	*t = 72h*	*t = 96h*	
1	168.53	19.97	10.35	8.82	8.68	**94.8**
2	202.12	30.28	13.56	11.23	11.18	**94.5**
3	264.05	32.79	17.37	15.03	14.87	**94.4**

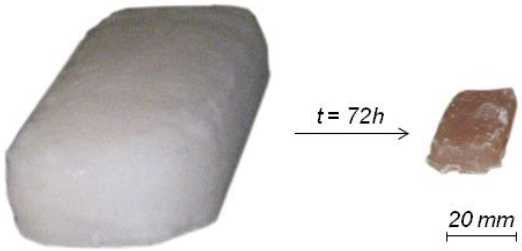


Figure 4.4. Example of a 3D chitosan specimen before dried (left) and after dried (right).

7 – Shape the dried and dense specimen. After drying, the specimens showed high performance towards machining techniques. The products obtained showed good machinability characteristics and it was observed that they can be easily machined into flat surfaces by using a milling machine (Figure 4.5a), drilled and polished (Figure 4.5b), or machined into a screw-shape by using a lathe (Figure 4.5c). If more complex geometries are desired (e.g. suture anchors, cages) a computer numerical control (CNC) machine tool can be used.

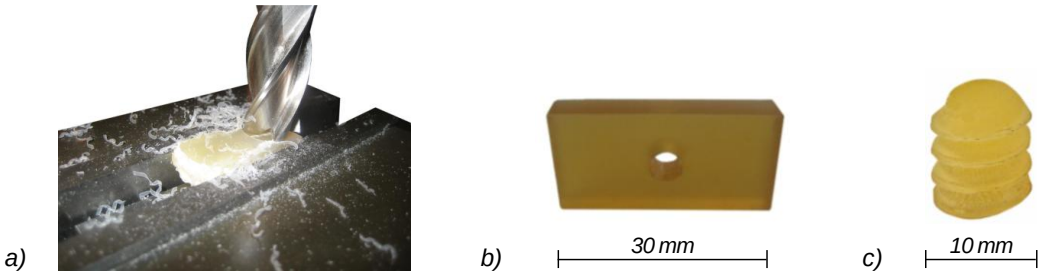


Figure 4.5. Example of 3D chitosan specimens: a) specimen being shaped in a milling machine; b) flat plate with a hole; c) screw-shaped specimen**.**

4.2.2. Morphological analysis of chitosan specimens

In order to study the morphology, SEM technique was used to observe external surface and cross sections of the specimens. As previously mentioned, the resulting specimens shrink and

become dense, with little porosity in their inner part. Unlike other methods like freeze-drying, freeze-extraction, or freeze-gelation used to prepare highly porous scaffolds [63], figure 4.6a) and 4.6b) illustrate the importance of drying the specimens under a constant mild temperature, so that they lose the water and slowly shrink, leaving almost no porous.

In order to better evaluate the influence of the drying step, several specimens were produced while varying the drying method. Some specimens were freeze-dried, after washing, in a freeze dry system (Labconco 7400030 FreeZone Triad Cascade Benchtop). It is clear from figure 4.6c) that when the specimens are freeze-dried instead of dried using a mild and constant temperature, highly porous specimens are obtained.

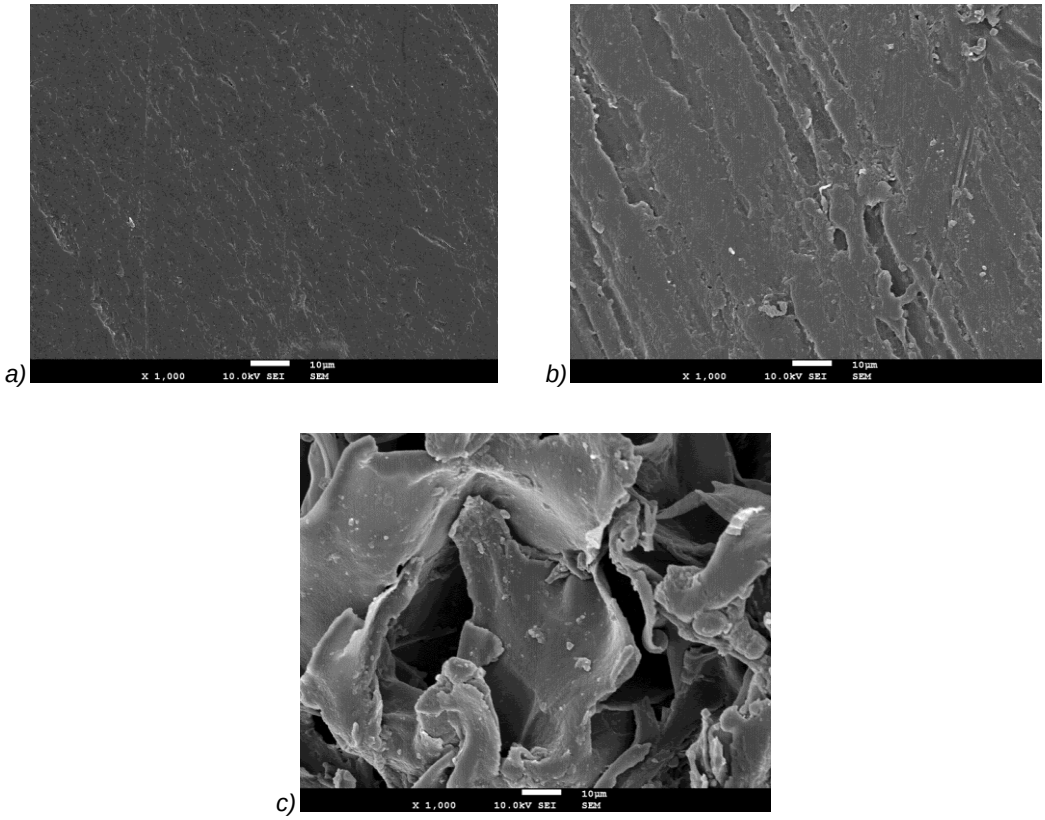


Figure 4.6. SEM images of a 3D dense chitosan specimen: a) external surface and b) cross section; and c) cross section of a freeze-dried 3D chitosan specimen.

4.2.3. Density and porosity of chitosan specimens

The density of the raw material (ρ_r) – chitosan powder – was measured by a gas pycnometer. The average result obtained was 1.453 g/cm^3 (SD = 0.002 g/cm^3). Since helium may demonstrate some measurable permeability through polymers, density of the tested specimens (ρ_s) was determined according to the Archimedes' principle. The results obtained are presented in table 4.2. According to equation 4.2, the porosity of the tested specimens was also

calculated, with an average value of 6.5%. Although the porosity of specimen 1 was noticeably higher than the porosity of specimen 3, the average result corroborates the SEM images and shows that the specimens produced through the developed process are considerably dense and have few micro- and nanoscale pores.

Table 4.2. Density and porosity of 3 chitosan specimens.

Specimen code	Weight (g)		**Density (g/cm^3)**	**Porosity (%)**
	Air (A)	*Water (B)*		
1	5.657	1.281	**1.293**	**11.0**
2	5.705	1.589	**1.386**	**4.6**
3	5.283	1.503	**1.398**	**3.8**

4.2.4. Swelling ratio of chitosan specimens

The swelling ability was evaluated by soaking the specimens in PBS at 37°C for 24 days (figure 4.7).

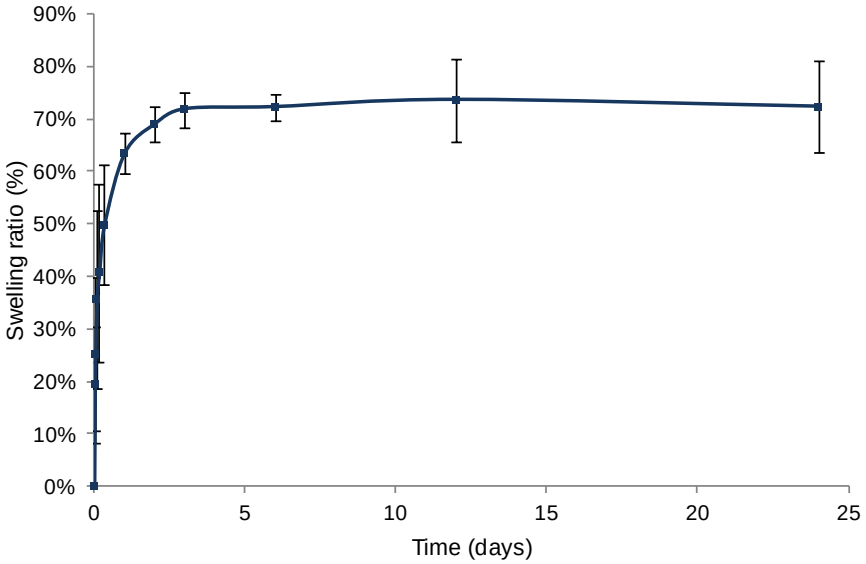


Figure 4.7. Swelling ratio (%) of 3D chitosan specimens.

The water absorption of the specimens reached its highest point after 12 days with an average value of 73.7% (table 4.3). However, the swelling ratio (SR) of the specimens did not increase steadily. In the first 2 hours, all specimens rapidly increased their weight (SR = 36%) and continued until 48 hours (SR = 69%). Then, the swelling ratio of the specimens seemed to

stabilize, and a slight increase could be observed with time. This water absorption behavior was expected due to the amino and hydroxyl groups in chitosan molecules [10,178].

Table 4.3. Swelling ratio of 3D chitosan specimens.

	Time (h = hours; d = days)										
	0.5h	*1h*	*2h*	*4h*	*8h*	*24h*	*2d*	*3d*	*6d*	*12d*	*24d*
SR (%)	**19.4**	**25.3**	**35.7**	**40.8**	**49.9**	**63.4**	**69.0**	**71.9**	**72.3**	**73.7**	**72.4**
SD (%)	11.0	14.5	17.0	16.9	11.4	3.9	3.2	3.3	2.5	7.8	8.8

4.2.5. Mechanical properties of chitosan specimens

Preliminary 3 point bending tests were also conducted to evaluate the mechanical performance of the produced specimens (figure 4.8). The average flexural strength and bending modulus obtained were 18.0 MPa (SD = 7.4 MPa) and 808.7 MPa (SD = 191.6 MPa), respectively. Although higher values were previously reported [10], these results are in the same order of magnitude, making them optimistic results for future experiments.

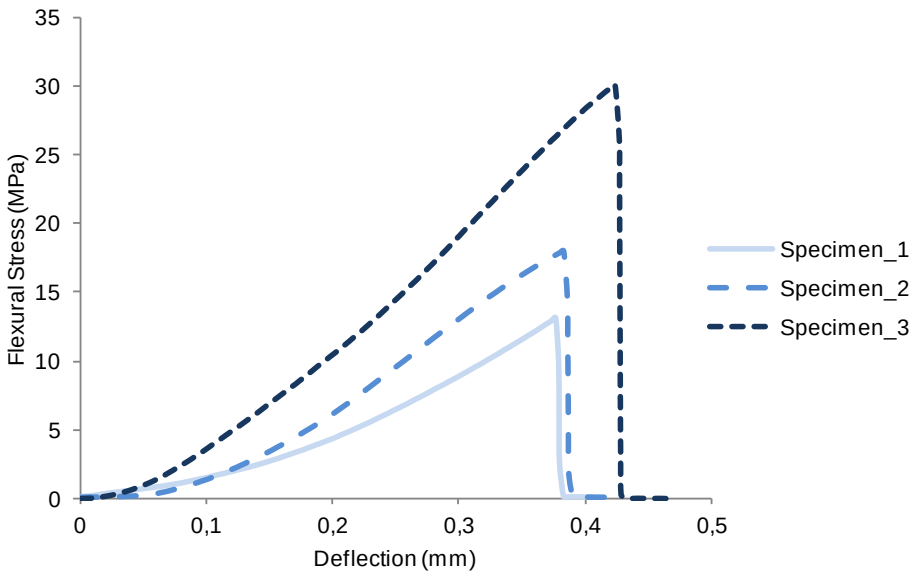


Figure 4.8. Mechanical behavior curves of 3 chitosan specimens tested in 3 point bending.

Although the shape of the curves is similar, both flexural strength and bending modulus of each specimen is considerably different. After testing and careful analysis, it was observed that specimens with higher porosity (refer to table 4.2) yield lower mechanical properties. Therefore,

although all of them presented a brittle behavior, specimens with less micro- and nanoscale pores (like specimen 3) achieved flexural strengths and bending modulus higher than 30 MPa and 1 GPa, respectively, confirming the importance of porosity control during the production process, in order to obtain a product with properties suitable for absorbable implants for orthopedic applications.

4.2.6. NMR spectroscopy of chitosan specimens

The degree of deacetylation (DD) of chitosan is one of its most important features which plays a major role in the biological interaction with living tissues [32]. In order to evaluate the hypothetical influence of the 3D specimens production process in the DD of raw material, an NMR study was conducted. Figure 4.9 refers to the NMR spectrum of a standard specimen. The DD was calculated according to equation 4.7 resulting in 89,4%.

From the acquired data it is possible to conclude that (1) the gelation step does not induce the crosslinking between polymer main chains besides hydrogen bonding and (2) the degree of deacetylation of chitosan did not experience any alteration because the values are similar before and after the production process. This result proves the mild conditions in which the production takes place, meaning that the concentrations of the reagents that are used are not aggressive enough to change this parameter.

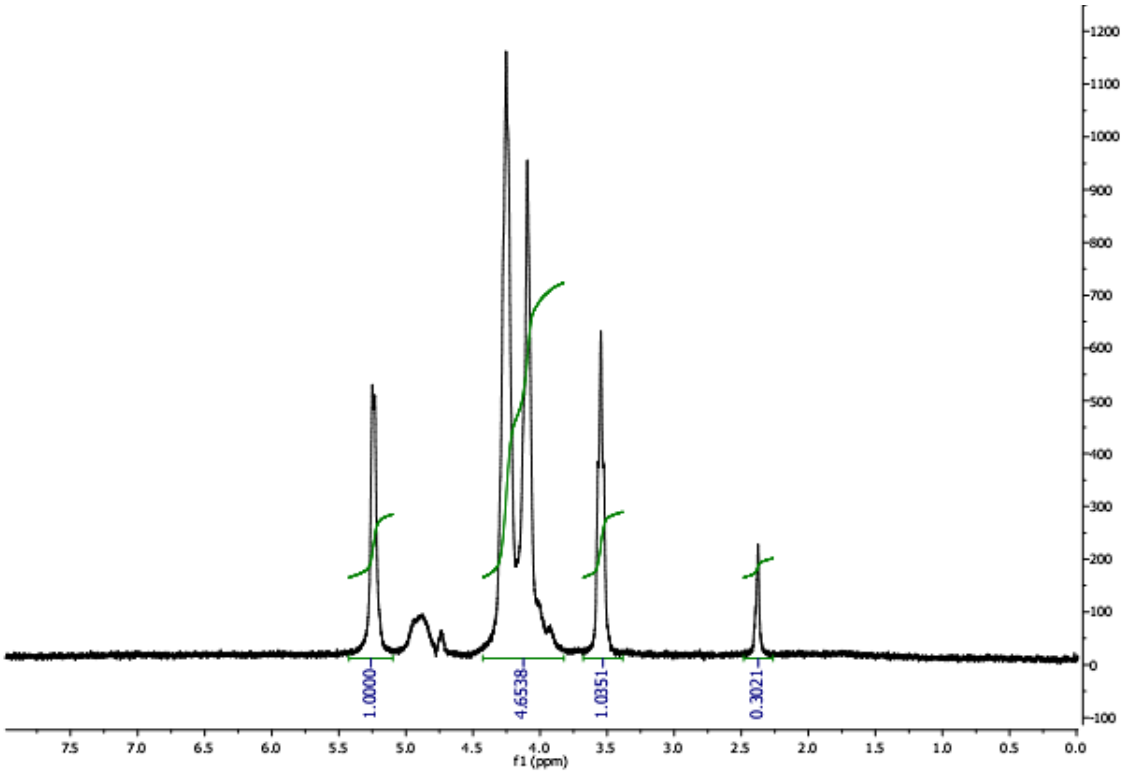


Figure 4.9. ^{1}H NMR spectrum of one 3D chitosan specimen.

Taking into account that the main goal is to use these implants as absorbable medical devices, one important feature is the degradation rate of the material. From previous studies [179,180], it is well known that the degradation rate of chitosan-based devices is highly dependent on the deacetylation degree of the material. Therefore, since the production process presented in this work does not affect the deacetylation degree of the raw material (chitosan powder), the degradation rate of future chitosan-based specimens might be easier to predict. Thus, the production method do not influence the deacetylation degree which helps the the production of chitosan-based implants more reliable and reproducible.

4.2.7. Gel permeation chromatography of chitosan specimens

Another main feature of the polymer is the average molecular weight (M_w) distribution which may be altered during the raw material processing. Using gel permeation chromatography (GPC) it was possible to conclude that the 3D specimen production process does not significantly affect the M_w of chitosan. Despite a slight discrepancy in both retention time and M_w of chitosan before and after the process, the chromatogram (Figure 4.10) shows similar results - 300 kDa. This analysis demonstrates that the mild conditions of the acidic prompt dissolution of the biopolymer followed by a non hydrolytic base gelation step leave the labile glycosidic fairly intact during the whole process.

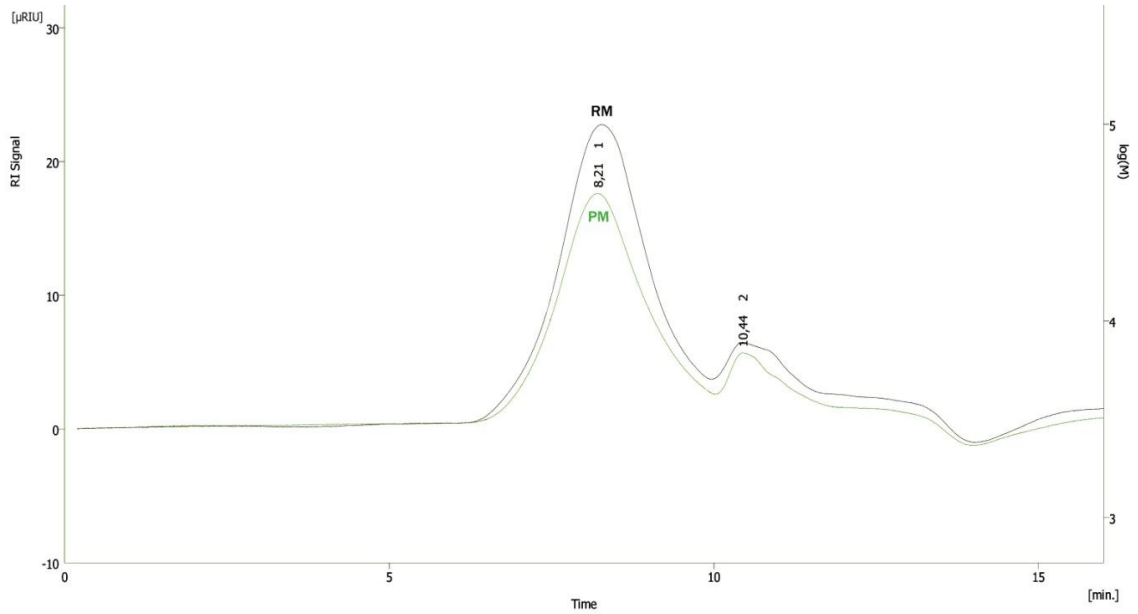


Figure 4.10. GPC chromatogram of chitosan before (RM curve) and after (PM curve) the production process.

As suggested for the degree of deacetylation, the fact that the molecular weight of chitosan remains unchanged after the process, can be considered a good result for future experiments,

since the molecular weight is expected to have a remarked influence on mechanical and biological properties of chitosan-based devices [42,181]. Thus, if the molecular weight of the raw material is the same as the final product, the desired molecular weight for a specific application can be chosen from the raw material, decreasing the variable parameters of the 3D dense chitosan-based products production process.

4.3. Conclusions

An innovative process to produce three-dimensional dense chitosan products was developed and presented in this section. The structures that result from the process are considerably dense and easy to machine. Both physical and morphological results suggest that the production process can yield 3D products with few porous – 6.5 ± 3.9% of porosity – and promising mechanical properties – σ_{fM} = 18 ± 7.4 MPa and E_B = 808.7 ± 191.6 MPa – that with proper design, can be good candidates to be used in absorbable implants for different types of applications, within different medical fields such as orthopedics, sports medicine and maxillofacial surgery. Moreover, both GPC and NMR spectroscopy results proved that the production process presented and described in detail in this work does not change two of the main important parameters of chitosan – deacetylation degree and molecular weight.

The mechanical properties of the produced specimens vary among them and seem to be highly correlated with the size and amount of porous. Thus, it is important to perform more experiments in order to improve the production process so that the results become increasingly predictable and reproducible. In addition, these mechanical properties might be improved by making some changes to the production process (e.g. change key parameters, use cross-linkers; add more materials). Therefore, the following chapter presents the main results of the tens of experiments that were conducted in order to improve the reproducibility of the process and the chitosan-based specimens' properties.

5. Mechanical Behavior of Chitosan-Based Specimens

As demonstrated in chapter 3, the elastic modulus of metals ranges from roughly five to twenty times the elastic constant of cortical bone and so, while this can be advantageous during the reparatory phase of a bone fracture, where stability is required, it becomes problematic during the bone regeneration phase when bone begins to remodel in response to loading. During this phase, the majority of the load is carried by the high stiffness metal device rigidly fixed to the bone. This leads to disuse atrophy in the underlying cortical bone, a phenomenon known as stress shielding. As an alternative to metallic devices, research has been conducted on the use of biodegradable polymeric fixation devices. Such polymeric devices have elastic constants below that of cortical bone but sometimes are also not stiff enough or strong enough to be used in major load bearing applications [85]. Thus, although they offer the advantage of a single surgical procedure avoiding the need for a second surgery for device removal, other disadvantages such as insufficient mechanical properties, premature material breakdown, sterile sinus developed over the implantation site, among others, have been frequently reported [104] and were addressed in chapter 3. Various attempts have been made in order to achieve mechanical stability of biodegradable orthopedic implants, as it has proven to be of utmost importance during their service period [130]. Polymers with high crystallinity have been explored and reinforced with elements such as fibers of crystalline polymers, fibers of carbon in polymeric resins, and particulate fillers, such as hydroxyapatite (HA) [99,182]. However, the lack of reinforcing ability of hydroxyapatite, for instance, with synthetic polymers has been attributed to the lack of chemical interaction of hydroxyapatite with the polymer [183].

While naturally occurring polymers exhibit a range of properties that make them suitable for use as alternatives to currently used biomaterials, they also have less than ideal mechanical properties which prevent their use as materials in load bearing applications [183]. Therefore, as for other biopolymers, many strategies have been explored to improve the mechanical properties of chitosan-based biodegradable structures. Biopolymers, or their derivatives, can be blended with other polymers resulting in a number of new composite materials with enhanced properties and applications in several fields. Blends made of synthetic and natural polymers, as well as some proteins (e.g. soy), can imbibe the wide range of physicochemical properties and processing techniques of synthetic polymers as well as the biocompatibility and biological interactions of natural polymers [15,54,184].

Blending chitosan with hydrophilic compounds such as polyvinylpyrrolidone (PVP), polyvinyl alcohol, phosphomolybdic acid (PMA), cellulose, among others, allows manipulation of the chain flexibility of chitosan [181,185]. The addition of other biodegradable polymers, such as poly-caprolactone (PCL), poly(butylene succinate) (PBS), poly(lactic acid) (PLA), poly(butylene terephthalate adipate) (PBTA), poly(butylenes succinate adipate) (PBSA), and poly(ɣ-glutamic acid) (ɣPGA) has also been investigated to produce materials with intermediate properties between the two components [186,187]. The development of hybrid materials that combine

naturally occurring polymers with biocompatible synthetic polymers is expected to minimize the mismatch of mechanical properties and preserve biocompatibility [183].

Reinforcing chitosan-based structures with chitin whiskers is also a possible approach to improve their mechanical properties. The increase in the tensile strength of these structures with increasing chitin whisker content could be due to the interaction between chitosan molecules and chitin whiskers via hydrogen bonding. Such interaction, however, caused these structures to be more rigid as the whisker content increased and, as a result, the percentage of fracture strain decreased [188]. Similar results were also obtained with cellulose whiskers. The tensile strength of the composite films significantly increased with an increase of cellulose whisker content. Moreover, the composite films displayed excellent thermal stability and water resistance with the incorporation of fillers [189].

Another possibility that has been reported is the use of chitosan in combination with bioactive inorganic ceramics, especially hydroxyapatite (HA), to further enhance tissue regenerative efficacy and osteoconductivity. Composites of biodegradable polymers with hydroxyapatite are especially attractive for bone tissue applications because calcium phosphate–polymer composites create a highly biocompatible and osteoconductive product [190,191]. Furthermore, the sustained release of calcium and phosphate from those composites is an added benefit, where the two ions serve as substrates in remodeling reactions of mineralized tissues [127]. Thus, combining chitosan and HA has the potential to overcome their mechanical limitations, maximize the beneficial properties of each and create a biodegradable scaffold with an organization that mimics the organic/inorganic nature of bone [192]. Composite chitosan/HA-based scaffolds were already produced using a co-precipitation method. Composite scaffolds swelled significantly less than chitosan scaffolds and are therefore expected to maintain their shape better *in vivo*. The compressive modulus of composite scaffolds was higher than the modulus of chitosan scaffolds, and composite scaffolds were also tougher and more flexible than what has been reported for pure calcium phosphate scaffolds [192,193]. Similar results have been obtained for chitosan scaffolds reinforced by β-tricalcium phosphate (β-TCP) [194].

Besides blending chitosan with other materials, or the incorporation of solid-state reinforcement materials, such as the ceramics, in order to improve the mechanical properties of chitosan-based structures, cross-linking is also one effective way used for reinforcement and it can also improve the mechanical properties of chitosan-based implants. Crosslinkers are molecules with at least two reactive functional groups that allow the formation of bridges between polymeric chains. To date, the most common crosslinkers used with chitosan are dialdehydes such as glyoxal and in particular glutaraldehyde. The aldehyde groups form covalent imine bonds with the amino groups of chitosan [195]. Thus, chitosan can be easily crosslinked and this process is able to change drastically some properties, namely water absorption, ion permeability, chemical and mechanical properties [196]. Due to toxicity concernrs, genipin has great potential as a natural crosslinking agent. In comparison with

glutaraldehyde, genipin exhibited comparable crosslinking capability but significantly lower toxicity [197,198].

In order to improve elasticity and overcome problems associated with high deacetylated chitosan specimens' brittleness [199], the addition of a plasticizer can be applied. The main non-volatile plasticizers are glycerol, sorbitol, propylene glycol or polyethylene glycol (PEG). The effects of different plasticizer molecules and the stability of the plasticized films have been investigated, and it has emerged that PEG and glycerol are the best candidates as plasticizers of chitosan films [200]. Other small molecular organic materials have been found to be effective plasticizers, but their efficiency decreases within several weeks. PEG and glycerol form relatively soft and stable films with chitosan [200–202]. Among these two, glycerol is the most used plasticizer due to its good plasticization efficiency, large availability and low exudation [203]. Moreover, the strongly hydrogen-bonded chitosan/glycerol mixtures are as strong as or even stronger than the supposedly unique chitosan. Thus, glycerol is suggested to improve the processability of chitosan specimens and their mechanical properties [204]. Hydrophilic plasticizers can interfere with chain-to-chain hydrogen bonding, thereby improving the mechanical properties of films. Polyols, such as glycerol and sorbitol, have been extensively used for this purpose [205].

The aim of this chapter is to present the main results of several experiments that were conducted in order to improve the mechanical properties of the chitosan specimens that result from the process presented in chapter 4. On one hand, some process parameters were changed in order to study their influence on the mechanical properties of the specimens and, on the other hand, different material blends were tested with the aim of strengthen specimens properties.

5.1. Materials and methods

Chitosan high molecular weight (800 kDa; 90% deacetylation degree; viscosity 1200 cps), chitosan medium molecular weight (300 kDa; 90% deacetylation degree; viscosity 200 cps) and hydroxyapatite (100% pure and crystalline; d0.50 size distribution aprox 3 microns) were provided by Altakitin, S.A. Glacial acetic acid and the sodium hydroxide solution (50% w/v) were purchased from Panreac Química S.L.U. Pharmaceutical grade glycerol (purity degree ≥ 99.5%) was purchased from AMSC. PEG 2000, PVP and glutaraldehyde were purchased to Sigma Aldrich. None of the products were purified prior to use.

5.1.1. Preparation of chitosan-based specimens

As presented in chapter 4, the fabrication process for the production of chitosan-based specimens involved seven main steps: (1) the dissolution of free base chitosan powder in an

aqueous solution of acetic acid (2%, v/v); (2) the molding step, by pouring the chitosan solution in 150 x 60 x 40 mm rectangular molds and leaving them at rest for 24 hours, at 5ºC; (3) the freezing of the solution at -20ºC; (4) the precipitation of the frozen solution in a 10% (w/v) sodium hydroxide (NaOH) aqueous solution; (5) the neutralization of the precipitated solution; (6) the drying step performed in the oven; and (7) the three-dimensional dried and dense specimens shaping process, performed in a milling machine, into flat plates with a thickness of approximately 3 mm.

Table 5.1 and 5.2 summarize the different specimens that were prepared to conduct the experiments, in order to study the influence of some process parameters and the influence of different material blends on the 3D chitosan-based products morphology and mechanical properties. A minimum of three specimens for each experiment were prepared and the average value and the standard deviation (SD) are presented.

Table 5.1. Experiments conducted to assess the influence of some of the production process parameters on the 3D chitosan-based products microstructure and mechanical properties

Experiment	Process step	Description
Concentration of medium M_w chitosan (M)	1	Specimens with different chitosan concentrations - **2%, 3%, 4% and 5% (w/v)** - were prepared
Concentration of high M_w chitosan (H)	1	Specimens with different chitosan concentrations - **2%, 3%, 4% and 5% (w/v)** - were prepared
Mixture of chitosans (H+M)	1	Specimens with both chitosans – **1.5% (w/v) of M + 1.5% (w/v) of H** – were prepared
Air retention in the solution	2	Chitosan solution was poured in molds and, without leaving them at rest, immediately frozen **with visible air bubbles**
Freezing temperature	3	Chitosan solution was poured in molds and frozen in **liquid nitrogen (~ -200ºC)**.
Precipitation time in NaOH	4	The frozen solutions removed from the molds were introduced in a NaOH aqueous solution for **24h, 48h, 72h and 96h**
Crosslinker effect - glutaraldehyde (GA)	4	The frozen solutions removed from the molds were introduced in a NaOH aqueous solution supplemented with **1% (w/v) GA**
Neutralization	5	Chitosan specimens that resulted from the gelation (or precipitation) process were washed until **9 < pH < 10**
Heat treatment	6	Chitosan specimens were **heat treated at 60ºC** for 24 hours, after being dried at 40ºC for 72 hours

Table 5.2. Experiments conducted to assess the influence of blends on the 3D chitosan-based products microstructure and mechanical properties

Experiment	Description
Hydroxyapatite (HA)	After dissolving 3% (w/v) chitosan powder in a 2% (v/v) acetic acid solution, **10% and 50% (w/w) HA** was added. The solution was stirred for more 30 min
Polyethylene Glycol (PEG)	After dissolving 3% (w/v) chitosan powder in a 2% (v/v) acetic acid solution, **10% and 50% (w/w) PEG** was added. The solution was stirred for more 30 min
Poly(vinylpyrrolidinone) (PVP)	After dissolving 3% (w/v) chitosan powder in a 2% (v/v) acetic acid solution, **10% and 50% (w/w) PVP** was added. The solution was stirred for more 30 min
Glycerol (Gl)	After dissolving 3% (w/v) chitosan powder in a 2% (v/v) acetic acid solution, **5%, 7.5%, 10% and 15% (v/v) Gl** was added. The solution was stirred for more 30 min

5.1.2. Mechanical and morphological characterization of specimens

The tested specimens were produced and machined with appropriate shape and dimensions for running the tests, namely 3 point bending tests, similar to what was described in 4.1.5, due to its simplicity and direct clinical relevance [162]. These tests were performed according to the ASTM standard D 790 [173]. An universal testing machine from Instron (model 5566) was used with a load cell of 500 N with extension control. Tests were performed with a crosshead speed of 0.2 mm/min at room temperature. Finally, the tests' results were processed with the use of the computer aided software for static systems (Bluehill® 2 Materials Testing Software). Figure 5.1 shows how each specimen was tested.

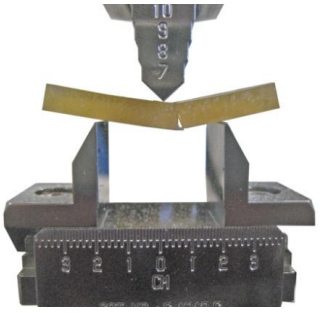


Figure 5.1. Example of a 3D chitosan specimen being tested.

The flexural stress (σ_f) of each point was calculated by means of the following equation:

$$\sigma_f = 3PL / 2bd^2 \quad (5.1)$$

Where P is the load at a given point on the load-deflection curve; L is the support span; b and d are the width and thickness of the tested specimen, respectively. The flexural strength (σ_{fM}) is the maximum flexural stress sustained by the tested specimen.

The flexural strain (ϵ_f) was calculated using equation 5.2:

$$\epsilon_f = 6Dd / L^2 \quad (5.2)$$

Where D is the maximum deflection of the center of the specimen; d is the thickness of the tested specimen; and L is the support span.

The modulus of elasticity (E_B) is the ratio, within the elastic limit, of stress to corresponding strain. It is calculated by drawing a tangent to the steepest initial straight line portion of the load-deflection curve and using the following equation:

$$E_B = L^3m / 4bd^3 \quad (5.3)$$

Where L is the support span; m is the slope of the tangent; b and d are the width and thickness of the tested specimen, respectively.

The cross-sectional morphology of these chitosan-based specimens was analyzed using either a Hitachi Scanning Electron Microscope (SEM) / S 2400 (Hitachi Instruments, Inc.), at an accelerating voltage of 20 kV, or a FEG-SEM, model JSM-7001F, from JEOL with energy of 10 kV. A fine stream of carbon was previously deposited onto specimens due to their nonconductivity. Different cross sections areas were observed and analyzed in terms of porosity (size and distribution), topography and overall density.

5.2. Results and discussion

The main results regarding the mechanical properties of chitosan-based specimens are summarized in table 5.3. All the performed experiments were conducted with either a medium molecular weight chitosan (M) or with a high molecular weight chitosan (H), both with a deacetylation degree of approximately 90%. Due to the available amount of each chitosan, most of the experiments were conducted with M, being H used either in some predetermined studies (e.g. when studying the influence of chitosan concentration, or the influence of freezing temperature), or when the preliminary results were suggesting its use (e.g. the promising preliminary results obtained when blending H with glycerol led to further experiments).

The interpretation and discussion of the main results are specified throughout the following subsections.

Table 5.3. Mechanical Properties of chitosan-based specimens

Experiment	Chitosan (# - %)	Blend (# - %)	Modulus (*MPa*)	Strength (*MPa*)	Strain (%)
Concentration of medium M_w chitosan (M)	M – 2%	-	-	-	-
	M – 3%		808.7 ± 191.6	18.0 ± 7.4	3.2 ± 0.3
	M – 4%		642.2 ± 63.3	20.8 ± 4.8	4.7 ± 2.0
	M – 5%		380.5 ± 23.0	14.2 ± 1.0	6.1 ± 0.6
Concentration of high M_w chitosan (H)	H – 2%	-	1429.8 ± 232.6	23.9 ± 3.0	1.5 ± 0.3
	H – 3%		2867.8 ± 1105.3	26.2 ± 7.4	1.0 ± 0.5
	H – 4%		467.5 ± 168.1	15.2 ± 3.8	6.4 ± 2.5
	H – 5%		-	-	-
Mixture of chitosans (H+M)	M – 1.5% H – 1.5%	-	3588.1 ± 437.5	35.3 ± 8.6	1.2 ± 0.3
Air retention in the solution	M – 3%	-	607.3 ± 370.9	17.0 ± 9.2	4.4 ± 2.6
Freezing temperature	M – 3% H – 3%	-	-	-	-
Precipitation time in NaOH	M – 3%	*72 h*	728.2 ± 67.6	19.1 ± 1.6	3.0 ± 0.3
		96 h	737.0 ± 101.6	14.3 ± 5.2	2.2 ± 0.7
Glutaraldehyde (GA)	M – 3%	1 %	-	-	-
Neutralization	M – 3%	*NaOH*	198.7 ± 62.3	10.4 ± 1.7	8.9 ± 3.1
Heat treatment	M – 3%	-	1283.2 ± 106.9	19.9 ± 0.4	1.6 ± 0.1
Hydroxyapatite (HA)	M – 3%	10 %	1069.1 ± 62.3	20.8 ± 8.2	2.2 ± 1.0
		50 %	1025.5 ± 269.2	14.0 ± 2.3	1.7 ± 0.7
Polyethylene Glycol (PEG)	M – 3%	10 % 50 %	-	-	-
Poly(vinylpyrrolidinone) (PVP)	M – 3%	10 % 50 %	-	-	-
Glycerol (Gl)	M – 3%	5 %	1005.3 ± 8.3	22.8 ± 0.8	3.1 ± 0.3
		7.5 %	997.8 ± 64.0	30.5 ± 2.4	4.1 ± 1.3
		10 %	512.7 ± 46.9	21.0 ± 1.6	7.3 ± 1.9
		15 %	170.0 ± 27.2	8.6 ± 1.6	> 10
	H – 3%	5 %	1967.0 ± 430.8	57.8 ± 23.1	3.0 ± 0.5
		7.5 %	2308.7 ± 408.4	59.3 ± 4.1	2.5 ± 0.8
		10 %	3093.3 ± 339.7	63.7 ± 0.5	2.5 ± 0.1
		15 %	992.8 ± 491.7	32.0 ± 11.8	4.3 ± 1.9

5.2.1. Influence of chitosan molecular weight and concentration used

One of the first experiments conducted was to evaluate how the concentration of chitosan could influence the mechanical properties of specimens. For this purpose, both M and H chitosans were used. Before analyzing the mechanical tests results for each chitosan, it is important to remind that the main difference between them is their M_w and consequently their viscosity. Therefore, while it was possible to dissolve 5% (w/v) of M in a 2% (v/v) acetic acid solution, it was not possible to get an homogeneous solution when using the same concentration of H, due to its greater viscosity. On the other hand, as can be seen from figure 5.2, the specimens that were prepared with 2% (w/v) of M were very fragile and broke while washing. This finding is probably due to the lower extension of chain entanglement and less inter-hydrogen bonding interactions.

Figure 5.2. Example of a broken 2% (w/v) M-based chitosan specimen.

Figure 5.3 shows the mechanical properties of M-based specimens. Specimens with 5% (w/v) of chitosan had the poorest mechanical results, especially when looking for their bending modulus.

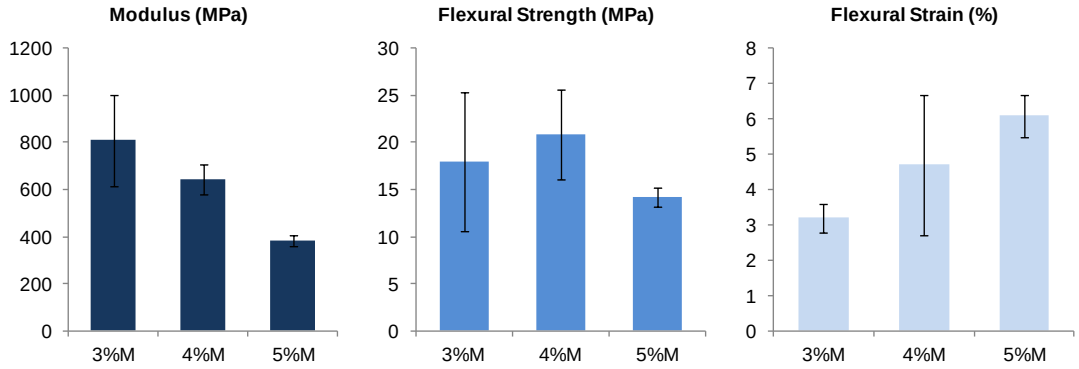


Figure 5.3. Modulus (MPa), flexural strength (MPa) and flexural strain (%) of 3%, 4% and 5% (w/v) M-based chitosan specimens.

The mechanical properties of H-based specimens are presented in figure 5.4. As previously referred, it was not possible to get an homogeneous chitosan solution when using 5% (w/v) of H, and therefore these specimens were not tested. H-based specimens with 4% (w/v) of chitosan had the poorest mechanical results, following the pattern observed for M-based specimens with 5% (w/v).

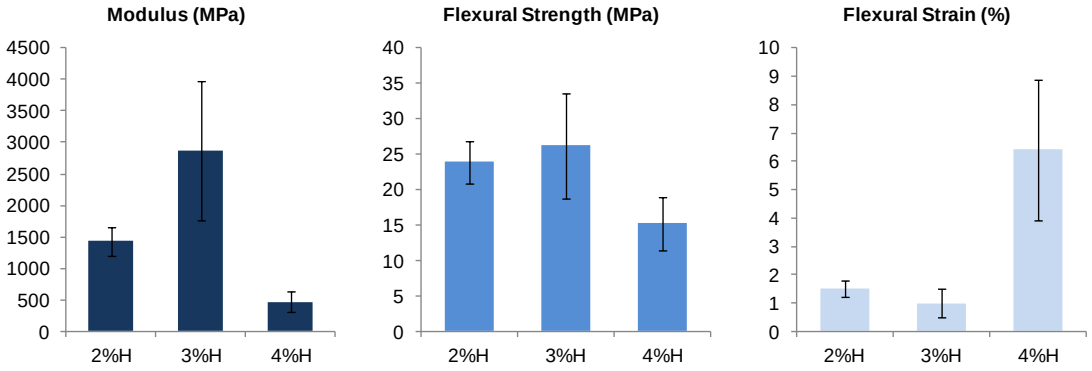


Figure 5.4. Modulus (MPa), flexural strength (MPa) and flexural strain (%) of 2%, 3% and 4% (w/v) H-based chitosan specimens.

During the preparation of these specimens, several details that can help explaining the results were taken into account. For both chitosans, when increasing chitosan content, the viscosity of solutions was clearly increasing as well and consequently the needed chitosan homogeneous solution was getting harder to obtain. Besides this, due to the increased viscosity, these solutions were retaining more air. It was observed that the H-based solution with 4% (w/v) chitosan was so viscous that after 24 hours most of the air bubbles were retained. Thus, the resulting specimens were visibly more porous and their microstructure is clearly different (figure 5.5).

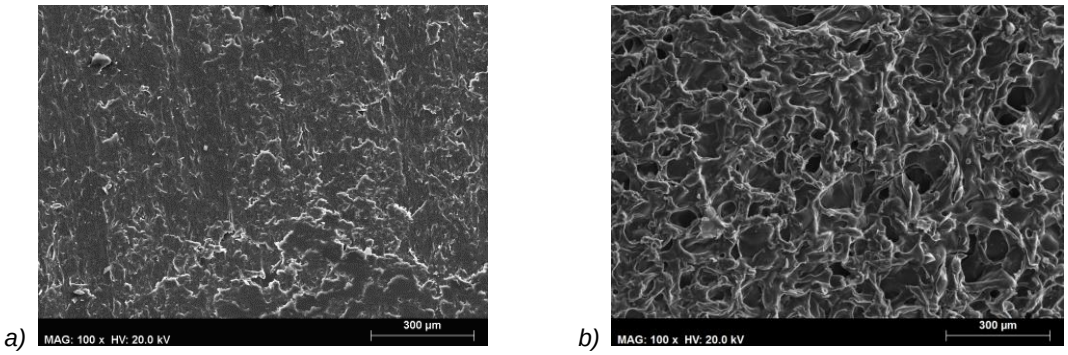


Figure 5.5. SEM images of 3D H-based specimens with different chitosan concentrations: (*a*) 3% (w/v) and (*b*) 4% (w/v).

This structural results can explain on one hand, the significantly decrease in the bending modulus and, on the other hand, the increase of flexural strain, when comparing H-based specimens with 3% (w/v) and 4% (w/v) of chitosan, respectively. A similar behavior was also observed and reported by other authors [62].

The mechanical properties of M+H-based specimens with 1.5% (w/v) of each type of chitosan were also tested and compared with 3% (w/v) M- and H-based specimens (figure 5.6). The results suggest that with similar degree of deacetylation, higher molecular weight chitosan led to higher modulus and flexural strength, but lower flexural strain. Similar behavior was already reported for chitosan membranes, as far as tensile strength properties are concerned [181,206]. It is suggested that the use of lower molecular weight chitosan produced less entanglement. The high M_w chitosan (H-based) specimens showed a higher modulus than the medium M_w chitosan (M-based) specimens, confirming that the decrease of the chitosan degree of polymerization negatively affected the mechanical performance of the specimens, as seen in other reported studies [184].

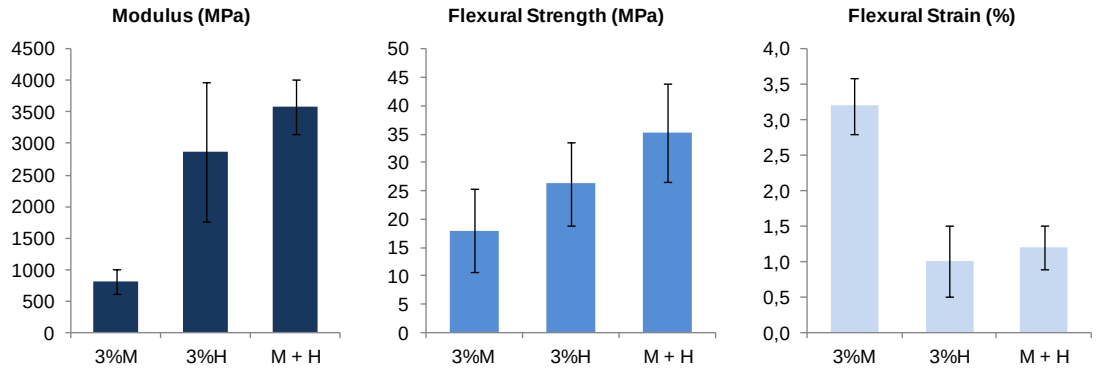
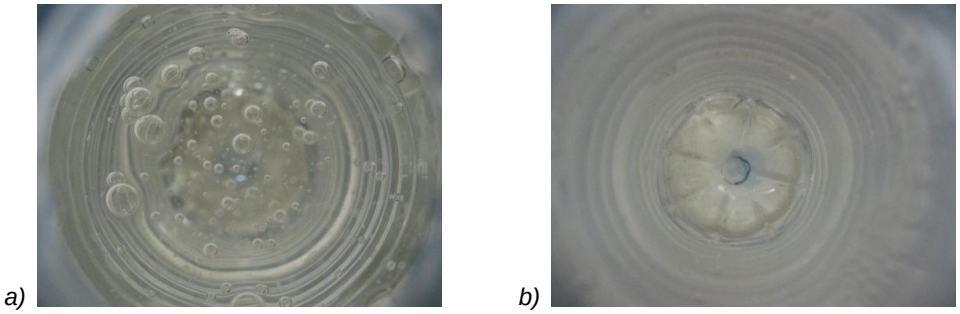


Figure 5.6. Modulus (MPa), flexural strength (MPa) and flexural strain (%) of 3% (w/v) M-based specimens, 3% (w/v) H-based specimens and 1.5% (w/v) M + 1.5% (w/v) H-based specimens.

Interesting results were obtained when mixing M and H chitosans. Both average values of modulus of elasticity and flexural strength increased, while flexural strain average value is similar to the value obtained for H-based specimens. If on one hand the decrease of the chitosan degree of polymerization negatively affected the mechanical performance of the specimens, on the other hand, it was also reported that polymer chains with lower molecular weights may allow stronger intra- and interchain interactions, forming a more homogeneous film [205]. Thus, by mixing chitosans with different M_w, the mechanical performance of specimens seem to be optimized, due to the formation of more intermolecular hydrogen bonding, forming a more compact and homogeneous matrix than H-based specimens and at the same time, with enough long chains to keep and improve the mechanical properties that were obtained with H-based specimens.

5.2.2. Influence of air retention and heat treatment

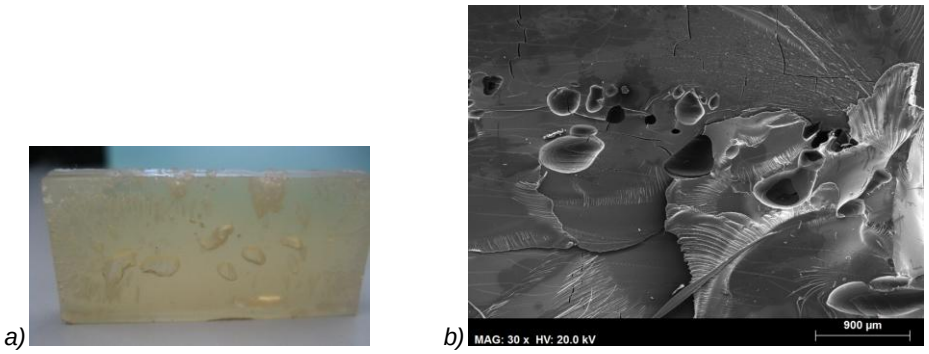
As mentioned in chapter 4, leaving the chitosan solutions resting prior to the freezing step can be a critical procedure in which the air bubbles entrapped in the solution will collapse. One of the purposes of this experiment was to assess the influence of air retention in the microstructure and mechanical properties of specimens, so 3% (w/v) M-based specimens were produced by following the standard process but skipping the resting step, as described in 5.1.1, meaning that the chitosan solution was frozen with air bubbles, as shown in figure 5.7.



a) *b)*

Figure 5.7. 3% (w/v) M-based solution: (*a*) immediately after dissolution, with air bubbles and (*b*) after 24 hours resting at 5ºC, without air bubbles.

The specimens resulting from this process had visible pores, as can be seen from figure 5.8 and consequently their mechanical performance was expected to be poor, as demonstrated by the mechanical tests results obtained and presented below.



a) *b)*

Figure 5.8. Macrostructure (*a*) and microstructure (*b*) of an M-based specimen with air bubbles.

The drying step, which is also crucial, was assessed as well. As previously mentioned, 40ºC was defined as the standard temperature, however, both lower and higher drying temperatures were also tested in order to assess their impact in the mechanical properties of the resulting specimens. When using a lower temperature (25ºC) the specimens were not completely dried after one week, proving to be a much less efficient drying temperature. On the

other hand, when using a higher temperature (60ºC) the specimens were not shrinking as uniformly as they were shrinking when using 40ºC. Moreover, these specimens were difficult to machine, due to their brittleness, and could not be tested.

In order to assess the influence of temperature on the mechanical properties, three specimens that were dried at 40ºC were also submitted to a heat treatment at 60ºC, for 24 hours. As figure 5.9 shows, after being 24 hours under 60ºC, specimens became darker and slightly contracted.

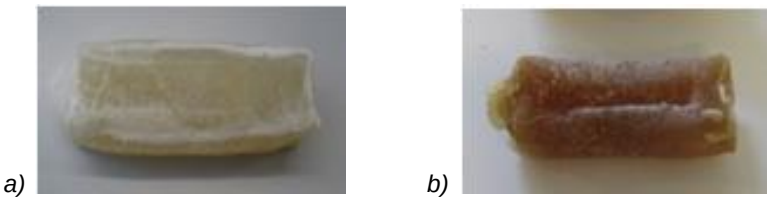


Figure 5.9. Dried M-based specimen: (*a*) before heat treatment and (*b*) after heat treatment.

The mechanical properties of these specimens are presented in figure 5.10. As expected, the mechanical performance of specimens with retained air bubbles is poorer than the standard specimens, mainly due to their inner porosity. As can be seen from the graphics, the average value of flexural strength hardly changed. However, the average modulus of elasticity decreased more than 20%, while the average flexural strain increased approximately 50%. Besides this, the standard deviation of the results also increased, making the mechanical performance of these specimens harder to predict.

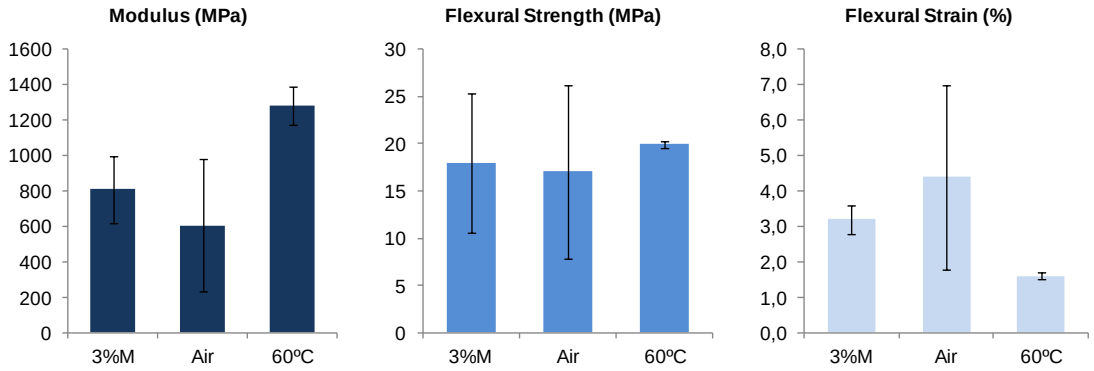


Figure 5.10. Modulus (MPa), flexural strength (MPa) and flexural strain (%) of standard M-based specimens (3%M), M-based specimens with air bubbles (Air) and heat treated M-based specimens (60ºC).

Since the goal is to obtain solid, dense and easy-to-shape chitosan specimens, air retention should be avoided while preparing them, in order to avoid the presence of porous in the resulting 3D shaped chitosan devices. These pores can not only have a negative impact in the

mechanical results of chitosan devices, but can also make the mechanical performance of these devices more unpredictable due to the irregular number and size of porous.

Regarding the influence of the heat treatment in the mechanical properties of specimens, a different behavior can be observed. According to previous studies, an increase in mechanical properties was expected due to the network structure formed by thermal treatment [207]. Although the average value of flexural strength seems not to be affected, the average bending modulus increased more than 50%, while flexural strain declined in the same proportion. Moreover, the standard deviation of these results significantly decreased, making the mechanical performance of these specimens more predictable. Therefore, although the thermal treatment made these specimens more brittle, as suggested by flexural strain, it also made them stiffer and with less disparate results.

5.2.3. Influence of the chitosan solution freezing temperature

It is known that the faster the freezing procedure, the smaller the ice crystals are [208]. Moreover, it was already reported that the tensile strength of chitosan-based structures increased as the freezing temperature decreased [209].

In order to test the influence on the mechanical performance of our 3D dense chitosan specimens by reducing the size of the ice crystals formed during the freezing process, both M and H-based solutions were immersed in liquid nitrogen (~ -196ºC) until they were completely frozen. This process took less than 10 minutes. The remaining production process procedures were unchanged.

After removing the frozen solutions from the molds and introduce them in a 10% (w/v) NaOH solution, the frozen solutions started to crack (figure 5.11). Thus, although the remaining steps of the process were followed until they were dried, the specimens broke while machining and therefore became unable to be tested.

a)  *b)* 

Figure 5.11. Specimens in a 10% (w/v) NaOH solution: *a)* after being frozen in liquid nitrogen *b)* after being frozen in a freezer, at -20ºC.

5.2.4. Influence of frozen chitosan solutions precipitation time in NaOH

The gelation, precipitation, or coagulation process is performed in the chitosan frozen solutions and a 10% (w/v) NaOH aqueous solution is used as the chemical gelation agent, as previously pointed out. Both concentration and immersing time in NaOH solution was a crucial step to optimize.

After performing some preliminary experiments, it was possible to assure that 10% (w/v) NaOH aqueous solution can precipitate the entire specimens in less than 48 hours, with the formation of a layer-by-layer structure, as described in literature [10,12]. Although similar results were obtained with 15% (w/v) NaOH solution, when increasing NaOH concentration, a decrease in the mobility of chitosan molecules is expected and consequently little or even no attachment between the layers [210]. Having these conditions in mind, a concentration of 10% (w/v) NaOH was kept and further experiments were conducted in order to study the influence of immersing time on the mechanical properties of 3D dense chitosan specimens. The importance of the washing step, which follows this gelation process, in the mechanical performance of these specimens was also assessed.

As can be seen from figure 5.12, by immersing chitosan specimens in a 10% (w/v) NaOH solution for different periods of time does not seem to significantly affect the mechanical properties of the specimens, as far as they are properly washed and neutralize (pH ~7). On the other hand, specimens that were intentionally washed fewer times (until 9 < pH < 10) showed poorer mechanical results when compared with the results of neutralized specimens. The impact of sodium ions left inside the specimens in the modulus of elasticity and flexural strain, due to the intentional non-neutralization, was great. These specimens were much less rigid and more flexible than the standard neutralized specimens, with the average modulus of elasticity decreasing more than 70%.

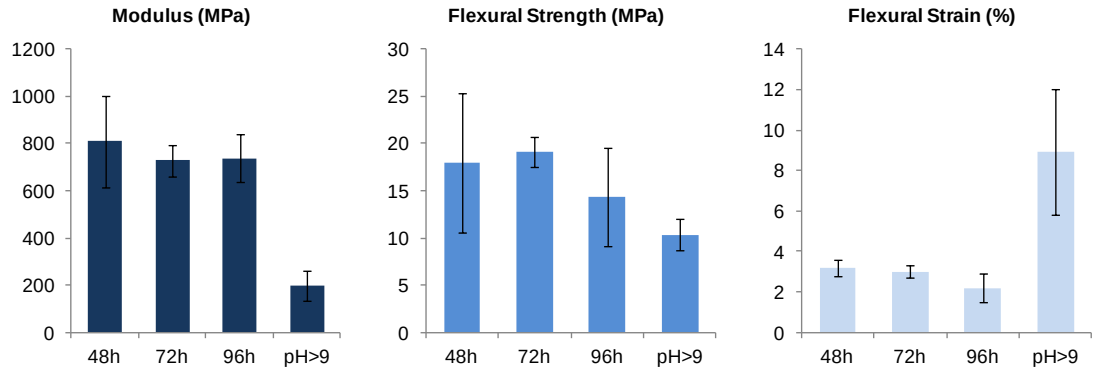


Figure 5.12. Modulus (MPa), flexural strength (MPa) and flexural strain (%) of standard M-based specimens (48h), specimens in NaOH solution during 72 hours (72h) and 96 hours (96h), and specimens that were just washed until 9<pH<10 (pH>9).

Although an inadequate neutralization process can have a negative impact in terms of specimens' cytotoxicity, the purpose of this experiment was just to understand the impact of a basic pH (due to the presence of sodium ions left in the specimens) in the mechanical properties and morphology of these specimens that become whitish after dried (figure 5.13).

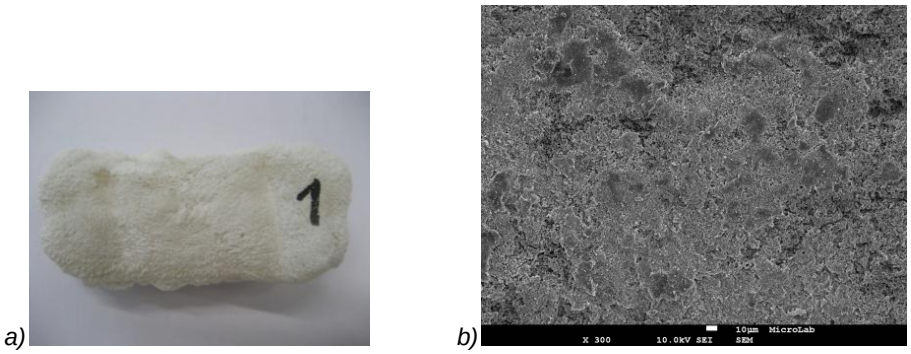


Figure 5.13. Macrostructure (*a*) and microstructure (*b*) of a dried M-based specimen that was washed until 9 < pH < 10 was reached.

In conclusion, immersing the frozen chitosan solutions in 10% (w/v) NaOH solution during 48 hours proved to be adequate to precipitate the solutions; however, an incomplete neutralization of the specimens significantly change their mechanical properties.

5.2.5. Influence of using glutaraldehyde as a crosslinker

Specimens were introduced in a 10% (w/v) NaOH and 1% (w/v) glutaraldehyde (GA) solution, in order to simultaneously precipitate and crosslink them. Moreover, since the best condition of reactivity between chitosan (NH_2) and GA is connected to neutral or basic media [211], this was performed by adding 30 ml of GA (50% in water) in 1500 ml of a previously prepared 10% (w/v) NaOH aqueous solution. The remaining production process was unchanged.

Although the concentration of GA used was in accordance with other reported experiments [196,211], excessive time in contact with GA and an inefficient gelation process, due to a faster reactivity between chitosan and GA, can be the main reasons for the unpredictable bad results that were obtained. The specimens that resulted from this experiment had a burnt appearance and they have scrapped while they were being machined and consequently could not be tested.

In order to overcome these unsatisfactory results, future experiments with glutaraldehyde should be conducted after the gelation process. Furthermore, other crosslinkers, such as genipin, can also be considered. Although it has been reported that chitosan structures crosslinked with GA improved their mechanical properties, the main drawback of dialdehydes as glutaraldehyde is that they are generally considered to be toxic [198]. Other covalent

crosslinkers for chitosan have been investigated as alternatives. Besides dialdehydes, crosslinkers such as diethyl squarate, oxalic acid or genipin can exhibit direct crosslinking mechanisms [195]. The use of genipin is an interesting alternative to dialdehydes, since it is not cytotoxic *in vitro* and has been shown to be biocompatible after injection in rats [195].

5.2.6. Influence of blending chitosan with hydroxyapatite

The goal of this experiment was to evaluate the mechanical performance of 3D dense chitosan-based specimens with different contents of hydroxyapatite (HA). After completely dissolving 3% (w/v) of M in a 2% (v/v) acetic acid solution, different concentrations of HA – 0%, 10% and 50% (w/w) – were added and mixed during 30 minutes. Figure 5.14 presents the results obtained from the bending tests.

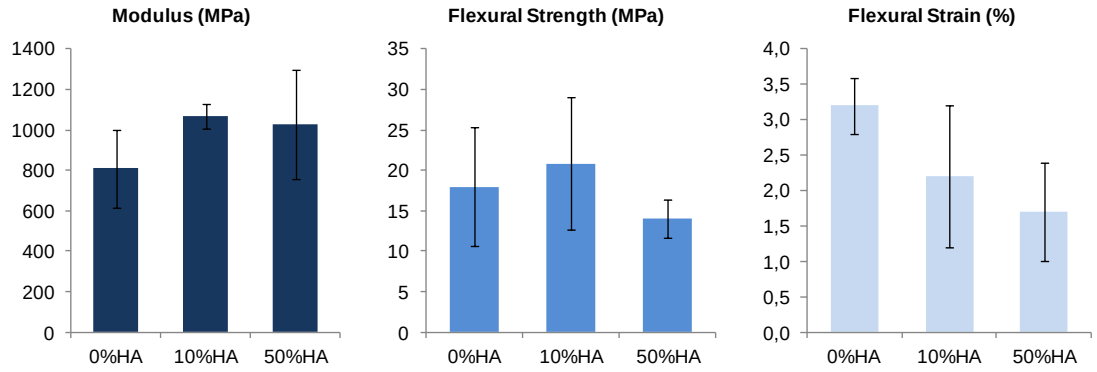
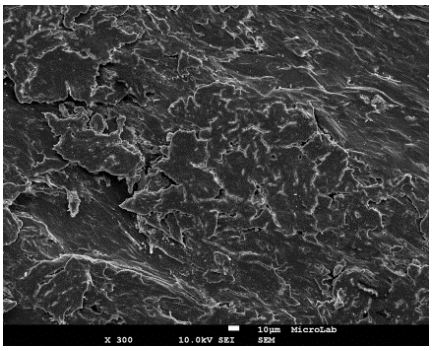


Figure 5.14. Modulus (MPa), flexural strength (MPa) and flexural strain (%) of standard M-based specimens (0%HA), specimens with 10% (w/w) of HA (10%HA) and specimens with 50% (w/w) of HA (50%HA).

From the graphics is clear that the highest average modulus and flexural strength were obtained for specimens with 10% (w/w) of HA. This could be due to the interaction between chitosan molecules and some of the HA particles via hydrogen bonding. Such interaction, however, caused these structures to be more rigid as the HA content increased and, as a result, the flexural strain decreased. On the other hand, when the concentration of HA was 50% (w/w), the specimens decreased their average flexural strength, possibly due to the weaker interfacial bonding between all HA particles and chitosan molecules [10]. Moreover, these specimens were microscopically inhomogeneous, as can be seen from figure 5.15.

A co-precipitation method may allow higher mechanical results due to the coordination bond of chitosan-calcium complex formed in the $Ca(OH)_2$ suspension [190]. Therefore, it is suggested that small HA crystallites are able to align along the chitosan molecule upon aggregation through the interaction between the Ca ions on the HA surface and the amino groups of the chitosan molecule.

a) 

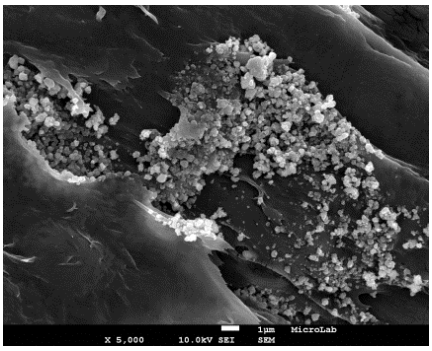
b) 

Figure 5.15. SEM images of a dried M-based specimen with 50% (w/w) HA with different magnifications: 300x (*a*) and 5000x (*b*).

5.2.7. Influence of blending chitosan with PEG or PVP

Similar to what was carried out with hydroxyapatite, the goal of these experiments was to assess the mechanical performance of 3D dense chitosan-based specimens with different contents of PEG or PVP. After completely dissolving 3% (w/v) of M in a 2% (v/v) acetic acid solution, different concentrations of PEG or PVP – 0%, 10% and 50% (w/w) – were added and mixed during 30 minutes.

After dried, the visual appearance of both chitosan/PEG and chitosan/PVP specimens was similar to the standard M-based specimens. Unfortunately, none of these specimens could be tested since they showed a very brittle behavior and broke while machining. These behavior was not expected since polymeric blends of chitosan and PVP or PEG were previously prepared and tested, using similar composition of blends [201,202,212,213]. As can be seen in the next section, the use of glycerol as a plasticizer can enhance the mechanical properties of 3D chitosan specimens. Thus, similar results were initially expected when using PEG. However, since PEG is considered an external plasticizer, only weak second-order bonds are developed between the plasticizer and the polymer, while internal plasticizers, as glycerol, are covalently bound to plasticized material [174]. Therefore, the external plasticizers can migrate in the polymer, which can lead to recrystallization of the material and a loss of elasticity [200], justifying the brittle behavior of these specimens.

5.2.8. Influence of blending chitosan with glycerol

Due to the brittle behavior of most 3D dense chitosan specimens, the addition of glycerol as a plasticizer was tested. After completely dissolving 3% (w/v) of chitosan in a 2% (v/v) acetic acid solution, different concentrations of glycerol (Gl) – 0%, 5%, 7.5%, 10% and 15% (v/v) – were added and mixed during 30 minutes. Due to the initial promising results, both M and H chitosans were tested.

Figure 5.16 shows the results of the bending tests performed in M-based specimens with different contents of glycerol. From these data, one can say that the highest results were obtained for specimens with 7.5% (v/v) of glycerol. When comparing the results with M-based specimens without glycerol, the modulus average value increased around 20%, while flexural average value nearly doubled. For higher concentrations of glycerol, both modulus and flexural strength decreased, while flexural strain started increasing significantly. Specimens with a concentration of glycerol of 15% (v/v) were noticeably flexible and malleable, and did not break during the bending tests. Thus, from a certain concentration, it was expected that glycerol could interfere with chitosan chains, decreasing intermolecular attraction and increasing polymer mobility, facilitating specimens deformation [214–216].

It is also important to highlight that the standard deviation of modulus and flexural strength decreased considerably, suggesting that the addition of glycerol, to some extent, not only help improving the mechanical performance of 3D dense chitosan-based specimens, but also make their mechanical behavior more predictable.

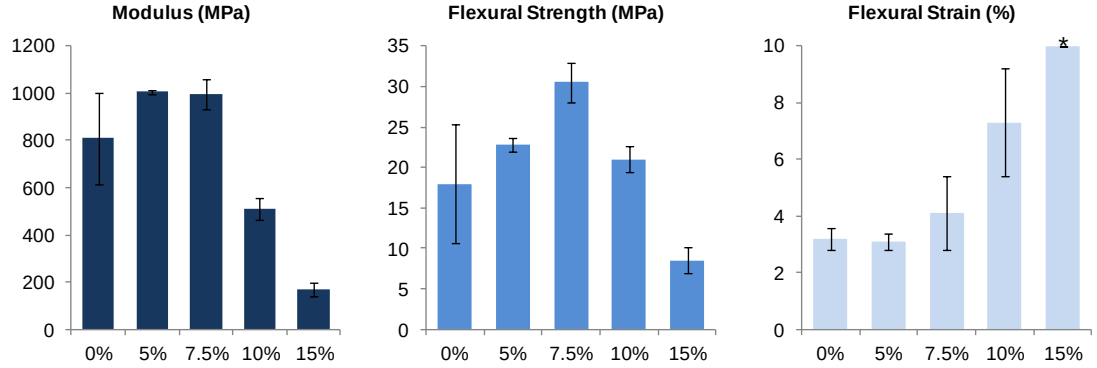


Figure 5.16. Modulus (MPa), flexural strength (MPa) and flexural strain (%) of standard M-based specimens (0%), and specimens with 5%, 7.5%, 10% and 15% (v/v) of glycerol. (*) Specimens with 15% (v/v) of glycerol did not break.

Interesting results were also obtained when doing the same experiments with H-based specimens (figure 5.17). When using a more viscous chitosan, with a higher molecular weight, the highest results were obtained for specimens with 10% (v/v) of glycerol, particularly when looking for the modulus of elasticity, suggesting that the higher the viscosity of chitosan, the higher the concentration of glycerol needed to achieve the highest results. However, when looking at the flexural strength, surprising results were already obtained for specimens with 5% (v/v) of glycerol. When comparing the results of H-based specimens without glycerol with specimens with 5% and 7.5% (v/v) of glycerol, the modulus average value decreased, while both flexural strength and strain average values more than doubled. For specimens with 10% (v/v) of glycerol, modulus and flexural strength reached the highest average values. Specimens with a concentration of glycerol of 15% (v/v) started having a similar behavior to the M-based specimens with 10% (v/v) of glycerol, meaning that they were starting having a more flexible

and malleable behavior, with lower values of modulus and strength, while rapidly increasing their strain.

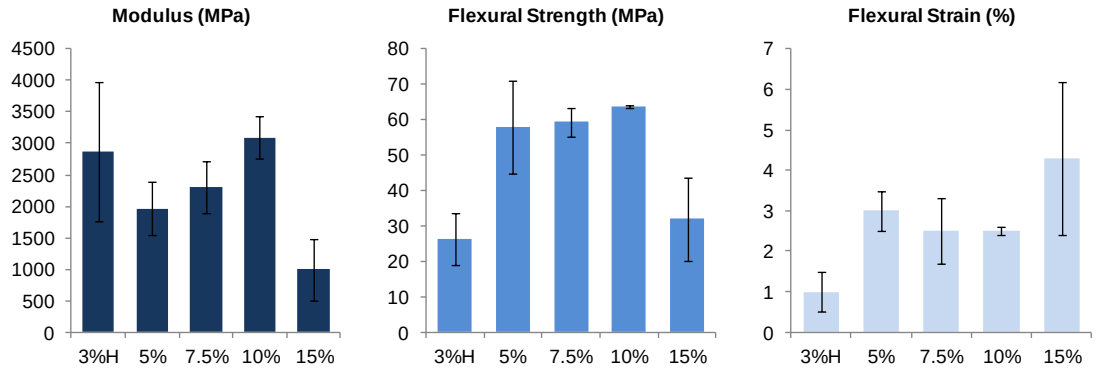


Figure 5.17. Modulus (MPa), flexural strength (MPa) and flexural strain (%) of standard H-based specimens (0%), and specimens with 5%, 7.5%, 10% and 15% (v/v) of glycerol.

Similar to what was observed for M-based specimens, the standard deviation of modulus and flexural strength decreased considerably, especially for specimens with 10% (v/v) of glycerol, suggesting that the addition of this plasticizer not only help improving the mechanical performance of these specimens, but also make their mechanical behavior more predictable.

On one hand, it is known that glycerol, acting as plasticizer, reduces the intermolecular forces, increasing the mobility of the biopolymer chains [186,214]. On the other hand, it was also reported that the strongly hydrogen-bonded chitosan/glycerol mixtures are as strong as or even stronger than the supposedly unique chitosan [205]. Thus, glycerol modifies the hydrogen-bonding network within the material and allows better interaction between filler and matrix, facilitating the stress transfer to the reinforcement phase and improving its mechanical properties [186,204]. Therefore, depending on chitosan molecular weight and its intrinsic viscosity and until a certain concentration of glycerol, chitosan-based specimens blended with this plasticizer presented higher mechanical properties. The smooth and dense specimens' microstructure help explaining the plasticizing effect of glycerol (figure 5.18). The plasticizer may have contributed to an ordering of the polymer chains according to their degree of polymerization, favoring the interactions between it and increasing its crystalline character, explaining the mechanical behavior of the specimens [217].

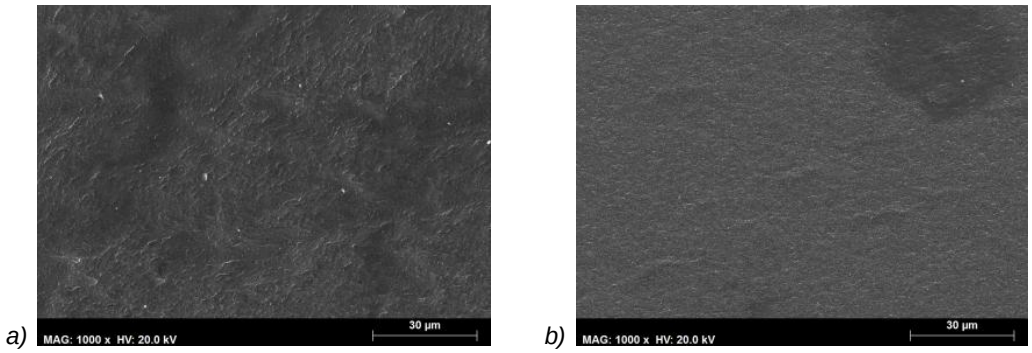


Figure 5.18. SEM images of two M-based specimens with (*a*) 5% (v/v) and (*b*) 7.5% (v/v) of glycerol.

5.3. Conclusions

The aim of this chapter was to present the main results of several experiments that were conducted in order to assess the mechanical behavior of the chitosan specimens that result from the process presented in chapter 4. On one hand, some process parameters were changed in order to study their influence on the mechanical properties of the specimens and, on the other hand, different material blends were tested with the aim of improving the mechanical properties of chitosan-based specimens.

As far as chitosan-based specimens production process parameters are concerned, the results obtained suggest that when using chitosans with the same DD, higher molecular weight chitosan-based specimens led to higher modulus and flexural strength, but lower flexural strain. The most interesting results, however, were obtained when mixing M and H chitosans. For a concentration of 3% (w/v), both average values of modulus of elasticity and flexural strength increased, while flexural strain average value was similar to the value obtained for H-based specimens. Thus, the mechanical performance of specimens seem to be optimized when mixing chitosans with different M_w, due to the formation of more intermolecular hydrogen bonding, forming a more compact and homogeneous matrix than the 3% (w/v) H-based specimens. As expected, the mechanical performance of specimens with retained air bubbles is poorer than the standard specimens, mainly due to their inner porosity. Thus, air retention should be avoided while preparing chitosan-based specimens, in order to avoid the presence of porous in the final 3D shaped chitosan devices. Regarding the influence of the heat treatment in the mechanical properties of specimens, although it made these specimens more brittle, as suggested by flexural strain results, it also made them stiffer, making heat treatment an easy and fast way to increase the modulus of elasticity of chitosan-based specimens, if needed. Finally, the influence of freezing temperature of the chitosan solution as well as the utilization of glutaraldehyde as a crosslinker were also tested, resulting in brittle and inappropriate specimens for mechanical tests.

Blending chitosan with other biomaterials, such as ceramics (hydroxyapatite) and polymers (PEG, PVP and glycerol), in order to improve the mechanical performance of 3D chitosan-based products, was also conducted. While both PEG/chitosan-based specimens and PVP/chitosan-based specimens broke while they were being machined, due to their extremely brittle behavior, preventing mechanical testing, chitosan-based specimens with either hydroxyapatite or glycerol in their content were successfully prepared and tested. Specimens with 10% (w/w) of HA resulted in specimens with higher average modulus and flexural strength, when compared with the tested chitosan specimens without HA. However, when the concentration of HA was 50% (w/w), the specimens decreased their average flexural strength. The most interesting results were obtained when using glycerol. Depending on chitosan molecular weight and its intrinsic viscosity and until a certain concentration of glycerol, chitosan-based specimens blended with this plasticizer presented a mechanical performance improvement. Moreover, these specimens had a translucent appearance without visible porous and were much easier to machine, due to their less brittle behavior.

In conclusion, the mechanical performance of 3D chitosan-based products can be easily changed and potentially customized, within a range of values, in order to match different implant applications requirements.

6. *In Vitro* Assessment of Chitosan-Based Specimens

Bioabsorbable implants have been used successfully in craniomaxillofacial, neurological, general surgical and orthopedic procedures. Their use continues to increase in orthopedic subspecialties such as sports medicine, foot and ankle surgery, shoulder surgery, and in the specialty of spine surgery [88,161]. These implants were developed to eliminate the need of a second surgical intervention for removal of the devices. In addition, the possible risks of metallic implants, such as corrosion and stress-shielding, due to mechanical incompatibilities between host bone and metallic implants, as well as the trouble with radiographic follow-up, have been recognized and can be avoided [84,218,219]. On the other hand, polymer-based implants allow optimal postoperative radiographic evaluation because of their radiolucency and the absence of artifacts associated with similar metallic devices exposed to advanced imaging equipments. Moreover, bioabsorbable implants may offer advantages with respect to bone healing because of their unique biomechanical properties. With a modulus of elasticity closer to that of bone and with its gradual degradable properties, bioabsorbable implants gradually decrease the stress shielding seen with rigid metallic implant systems [100,102]. Thus, the primary advantage of these new bioabsorbable materials is that they confer initial and intermediate-term stability that is adequate for bony healing in various applications. This is followed by gradual implant degradation and resorption, ideally after biologic fixation has occurred. Thus, the load is gradually transferred to the healing bone as they degrade [158]. However, the mode and the extent of degradation for a polymer under a set of conditions have to be known to determine the suitability of the material for a given application [130]. Therefore, the control of the rate and extent of degradability of a polymeric biomaterial is critical for its intended function. For instance, for an orthopedic fracture fixation application, where the implanted polymeric biomaterial is needed for a limited duration, the ideal rate of resorption or degradation should not exceed the rate of bone formation and the reduction of strength of the implant should match, as good as possible, the increase in tissue strength [130].

Although there are many benefits when using bioresorbable implants, there has been a concern about the potential inflammatory response due to bulk erosion, acidic byproducts, and poor clearance of some of these degradation products. Other reported complications with the use of these bioabsorbable materials include sterile sinus tract formation, osteolysis, synovitis and hypertrophic fibrous encapsulation [158]. Thus, one of the major drawbacks associated with absorbable implants is foreign-body reaction. Although the incidence of this reaction varies among the existing implants, it has been reported with most of the currently available materials [87]. Due to the improved biocompatibility and the non-toxic nature of their degradation products, natural polymers are likely to replace synthetic polymers for some applications. Among natural polymers, chitosan is currently receiving substantial attention due to its biomedical applications that have been widely studied owing to chitosan's excellent biocompatibility, biodegradability and osteoconductive properties [6,37,50,179,220].

Degradation of chitosan is controlled by the residual amount of acetyl content and it can degrade rapidly *in vivo* according to its deacetylation degree. In addition, porosity of chitosan specimens can be controlled which can affect their strength and elasticity [47,192]. Lysozyme is the primary enzyme responsible for *in vivo* degradation of chitosan through hydrolysis of acetylated residues [44]. This enzyme breaks down the chitosan polymer chain, diminishing its molecular weight until it becomes small enough to be processed by cells. Lysozyme is abundant throughout the human body, being present in lymphocytes and also secreted by monocytes, macrophages, and granulocytes, which account for the largest source [221]. Monocytes and macrophages are the dominating contributors to the lysozyme content in serum [222]. Lysozyme commonly exists in various human body fluids and tissues with concentrations from 4 to 13 mg/L in serum and from 450 to 1230 mg/L in tears [45]. The degradation rate of chitosan is inversely related to the degree of crystallinity and therefore, highly deacetylated forms may last several months *in vivo*, being chitosan oligosaccharides of variable length the degradation products [21,44,223].

In the previous chapters it was described a novel method for the production of 3D dense chitosan-based specimens, followed by their characterization. The results obtained and presented throughout chapter 5 showed that chitosan molecular weight and the addition of glycerol can have great influence in future chitosan-based devices. The aim of this chapter is to present the results related to the cytotoxicity and *in vitro* enzymatic degradation studies that were performed in order to further assess the influence of chitosan molecular weight and the addition of glycerol in 3D dense chitosan-based products.

6.1. Materials and methods

Chitosan of high (800 kDa; 90% deacetylation degree; viscosity 1200 cps) and medium (300 kDa; 90% deacetylation degree; viscosity 200 cps) molecular weight were provided by Altakitin, S.A. The glacial acetic acid and the sodium hydroxide solution (50% w/v) were purchased from Panreac Química S.L.U. Pharmaceutical grade glycerol (purity degree ≥ 99.5%) was purchased from AMSC. Lysobac, a recombinant human lysozyme produced in an animal-free production system, was purchased from Sigma-Aldrich.

6.1.1. Preparation and characterization of chitosan-based specimens

Similar to what was previously described in chapter 4, the fabrication process for the production of chitosan specimens involved the dissolution of free base chitosan powder (3%, w/v) in an aqueous solution of acetic acid (2%, v/v). After total dissolution, the homogeneous solution was poured in 60 x 40 mm cylindrical molds and left at 5 ºC overnight to remove air bubbles, prior to be frozen at - 20 ºC for 24 hours. The frozen solutions were removed from the

molds and introduced in a sodium hydroxide aqueous solution (10%, w/v) for 48 hours. The specimens that resulted from the gelation process were abundantly washed with deionized water until pH~7 and air-dried in oven at 40 ºC for 96 hours. The three-dimensional dried and dense specimens were then machined into cylindrical-shaped objects with a diameter of approximately 14 mm and 6.5 mm height.

The fabrication process for the production of chitosan specimens with glycerol (Gl) involved the dissolution of free base chitosan powder (3%, w/v) in an aqueous solution of acetic acid (2%, v/v) and glycerol (7.5%, v/v), as described in chapter 5.

Table 6.1 resumes the four different types of specimens that were prepared for this study.

Table 6.1. Name and composition of chitosan-based specimens

Specimens Name	Molecular Weight (M_w)	Glycerol (Gl)
H	800 kDa	No
H+Gl	800 kDa	Yes
M	300 kDa	No
M+Gl	300 kDa	Yes

The cross-sectional morphology of these chitosan-based specimens was analyzed using a Hitachi Scanning Electron Microscope (SEM) / S 2400 (Hitachi Instruments, Inc.), at an accelerating voltage of 20 kV. A fine stream of carbon was previously deposited onto specimens due to their nonconductivity. Different cross sections areas were observed and analyzed in terms of porosity (size and distribution), topography and overall density.

The composition and the degree of deacetylation of the chitosan-based specimens were determined by ^{1}H NMR spectra, according to the method described by Hirai et al [36]. Measurements were performed on a Bruker Avance-III 400 MHz NMR spectrometer, under a static magnetic field of 9,4 T at 70ºC. The software used to analyze the spectrum was the Bruker Topsin 3.1. The concentration of chitosan solution was 10 mg/ml in a DCl/D_2O solution (2%, w/v). The degree of deacetylation (DD) was evaluated by using the integral intensity of CH_3 residue (I_{CH3}) and the sum of integral intensities of H_2-H_6 (I_{H2-H6}):

$$DD(\%) = \{1 - (\frac{1}{3} I_{CH3} / \frac{1}{6} I_{H2-H6})\} \times 100 \quad (6.1)$$

The water sorption capacity of the produced specimens was determined by immersing the specimens in phosphate buffered saline (PBS, Sigma-Aldrich) at pH 7.4 for 24 days at 37°C. The swollen specimens were removed at predetermined time intervals (30min, 1h, 2h, 4h, 8h, 24h, 2 days, 3 days, 6 days, 12 days and 24 days) and immediately weighted with an analytical

balance after the removal of excess of water by lying the specimens on a filter paper. The swelling ratio (SR) was calculated using equation (6.2):

$$SR(\%) = (W_t - W_0) / W_0 \times 100 \quad (6.2)$$

Where W_t and W_0 are the weights of the specimens at time *t* (swelling state) and at time *0* (dry state), respectively.

6.1.2. Enzymatic degradation of chitosan-based specimens

Prior to start this experiment, all the specimens were sterilized by immersing them in ethanol (70%, v/v), for 72h, followed by UV exposure overnight. Ethanol was chosen as a sterilizer due to its negligible effect on the physical properties of materials, as reported in the literature [175]. Chitosan-based specimens were then placed in PBS (control) or in an enzymatic solution containing 500 mg/L of lysozyme and incubated (37ºC, 5% CO_2, fully humidified) for different time periods and the medium replaced every week. At predetermined time intervals (15, 30, 45 and 60 days) specimens were taken from the solutions, washed with distilled water to remove salts and dried for 24 hours at 40ºC.

The weight loss (WL) of the specimens was calculated according to equation (6.3):

$$WL(\%) = (W_i - W_f) / W_i \times 100 \quad (6.3)$$

Where, W_i is the initial dry weight of the specimen and W_f is the weight of the dry specimen either after incubation in PBS, or in the enzymatic solution.

Mechanical compression tests of these specimens were performed, according to the ASTM D695 standard, using a universal testing machine from Instron (model 5566) equipped with a load cell of 10 kN and the loading rate was set as 1.5 mm/min. The tests' results were processed with the use of the computer aided software (Bluehill® 2 Materials Testing Software). Compressive stress was defined as the compressive load per unit area of minimum original cross section carried by the test specimen at any given moment, being the compressive strength defined as the maximum compressive stress carried by a test specimen. The compressive modulus of elasticity was calculated by the slope of the initial linear portion of the stress-strain curve, being the compressive strain defined as the change in length per unit of original length along the longitudinal axis.

6.1.3. *In vitro* cytotoxicity of chitosan-based specimens

The specimens produced were tested for cytotoxicity according to the international standard ISO 10993-5:2009(E) for medical devices. Specimens were sterilized by immersing them in ethanol (70%, v/v), for 72h, followed by UV exposure overnight, as previously described. Triplicates of each type of specimen were placed in 6-well plates containing 2 mL of 10% (v/v) Iscove's Modified Dulbecco's Medium (IMDM) + 10% (v/v) Fetal Bovine Serum (FBS)

and incubated (37ºC, 5% CO2, fully humidified). After 72h of incubation, this media was transferred to 24-well plates and used to culture mouse fibroblasts (L929 cell line) for 48h, plated at an initial density of 80×10^3 cells/cm^2. Fresh IMDM 10% medium not exposed to any material was used as negative control and IMDM 10% medium which has been left 72 hours in contact with a piece of latex glove used as positive control. The cell metabolic activity was analyzed by using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) and the results were normalized to the negative control for cytotoxicity and compared to the positive control.

In order to perform direct contact assays, L929 cells were plate in 24-well plate, 20×10^3 cells per well for 72h. Specimens were placed on the top of the cell layers, with IMDM 10% medium, and incubated (37 ºC, 5% CO_2, fully humidified) for 48h. The morphology and confluence of cells in contact with materials were analyzed by using an optical microscope.

6.2. Results and discussion

The aim of this chapter is to further assess the impact of using chitosans with different molecular weights (300 kDa and 800 kDa) and the addition of glycerol in 3D dense chitosan-based products properties. Thus, the main results related to the cytotoxicity and *in vitro* enzymatic degradation studies that were performed are detailed and discussed throughout the following subsections.

6.2.1. Characterization of chitosan-based specimens

During the preparation of specimens, the difference of viscosity between the high molecular weight chitosan (H) acid solution and the medium molecular weight (M) acid solution was notorious. Although it was possible to dissolve 3% (w/v) for both types of chitosan, H took twice the time to dissolve in 2% (v/v) acetic acid solution when compared to M due to the difference in viscosity, being the viscosity of H solution greater than the other. Similar results were obtained when preparing the H+Gl and M+Gl specimens. Prior to start the experiments, cylindrical specimens were machined in a milling machine in order to give them smooth and parallel surfaces. During this process, a highly differentiated behavior between plasticized and unplasticized specimens was observed. All specimens with glycerol were much easier to shape when comparing to the others that presented a much more brittle behavior, due to the high degree of deacetylation of both chitosans used [199], which sometimes resulted in cracking of the specimens.

In order to study the morphology of specimens, SEM technique was applied at this point to observe their cross sections. As previously mentioned, the resulting specimens should shrink and become dense, with little porosity in their inner part. As can be seen from figure 6.1, there is

a significant difference between specimens with and without glycerol in their content. Thus, when comparing H with H+Gl, or M with M+Gl, it is notorious the difference in their microstructure, being the plasticized specimens more dense and without visible porous. This behavior was expected since glycerol molecules form strong H-bonds with chitosan and in parallel destroy the inter- and intramolecular H-bonds between the polymer chains, making them moving closer to each other [224]. On the other hand, both H and M specimens presented a more irregular and rough surface and in both cases small and randomly distributed pores were also found, as shown in figure 6.1.

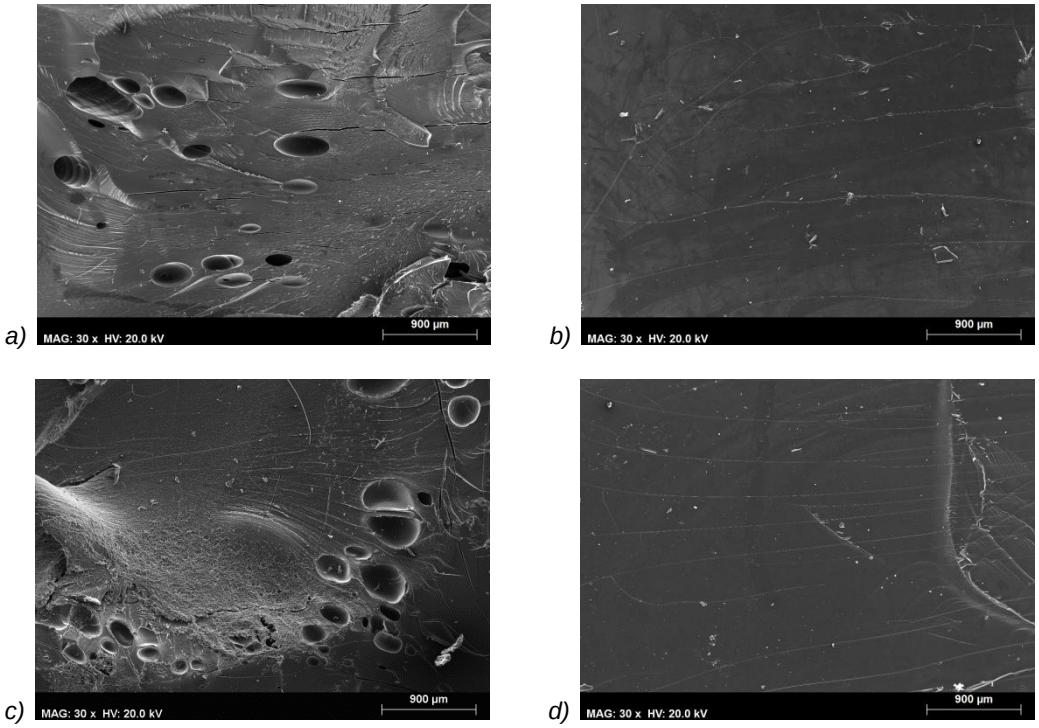


Figure 6.1. SEM images of 4 chitosan-based specimens: (*a*) H; (*b*) H+Gl; (*c*) M; (*d*) M+Gl.

Figure 6.2a refers to the NMR spectrum of an H specimen. The DD was calculated according to equation 6.1 resulting in 90.8%, meaning that the degree of deacetylation of chitosan did not experience any alteration during the specimens production process, as previously confirmed (refer to section 4.2.6). The resonances of the plasticizer can be clearly detected beneath the signals of the chitosan (figure 6.2b).

There are numerous possibilities to form intra- and intermolecular H-bonds between chitosans OH, NH, and carbonyl groups and the plasticizer molecules [200]. The acetamide group plays an important role in the formation of intermolecular bonds between adjacent chains. The proton environment of the carbonyl atoms does not change significantly when plasticizer is added to chitosan specimens, meaning that the carbonyl atom accept polarization predominantly from the chitosan H atoms [200]. Glycerol is a very good H-bond donor and acceptor, and therefore it increases the number of H-bonds by donating protons to carbonyl

groups and accepting chitosan OH and NH protons [200]. The amide NH proton is in close proximity to C3 carbon if the carbonyl group forms an H-bond with the OH group on C6 carbon. Glycerol molecules are probably bound to the acetamide group of chitosan by H-bonds, which prevent the acetamide groups from forming interchain H-bonds with other chitosan molecules, and leads to break down of the intermolecular connectivity between the polysaccharide chains [200].

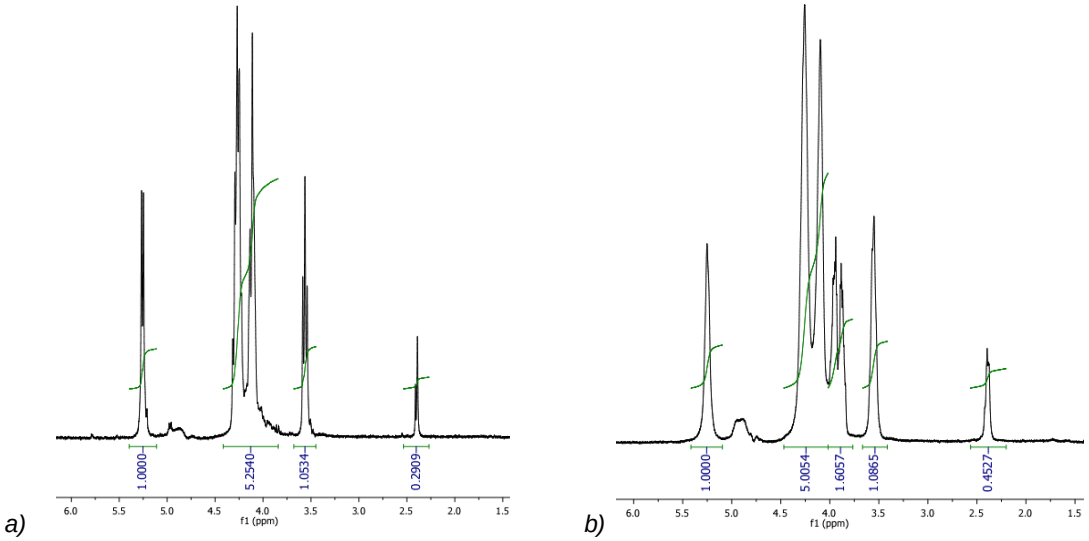


Figure 6.2. ^{1}H NMR spectrum of an H specimen (a) and an H+Gl specimen (b).

As a general behavior, it is known that both plasticized and unplasticized samples have a great water sorption capacity through hydrogen bonds with some chitosan sites. The swelling ratios for the four chitosan-based specimens are shown in figure 6.3, where it is visible that the majority of the water uptake took place in the first 2 days. When comparing the H-based and the M-based specimens, with and without glycerol, the highest water absorption values were obtained with unplasticized samples. As glycerol interacts through hydrogen bonds with chitosan and with water molecules, it was expected that it could lead to higher water contents [203], however, the porous microstructure of unplasticized specimens seems to favor the water uptake. On the other hand, the dense microstructure of plasticized specimens, as can be seen from SEM images, made them more swelling-resistance. Furthermore, the swelling ratio of H-based specimens was higher than the M-based ones. Thus, taking into account that both chitosans have the same deacetylation degree (DD), the results suggest that for the same DD, the lower the molecular weight of chitosan the lower the swelling rate of these chitosan-based specimens.

Increasing swelling-resistance of chitosan-based specimens can be considered a critical step to ensure its use as a functional load bearing material [75]. There are several approaches to decrease the swelling ratio of chitosan-based specimens. Adding hydroxyapatite, or cross-linking chitosan specimens (e.g. with glutaraldehyde or genipin) are some of the reported

approaches to decrease their swelling ratio [54,170,198]. A heat treatment can also be an effective approach to prevent swelling. This fact is attributed to the formation of a more rigid network [225]. From the results, a similar behavior is obtained with plasticized specimens. Due to a more dense and rigid structure, these specimens presented a lower swelling ratio when compared to their unplasticized counterparts.

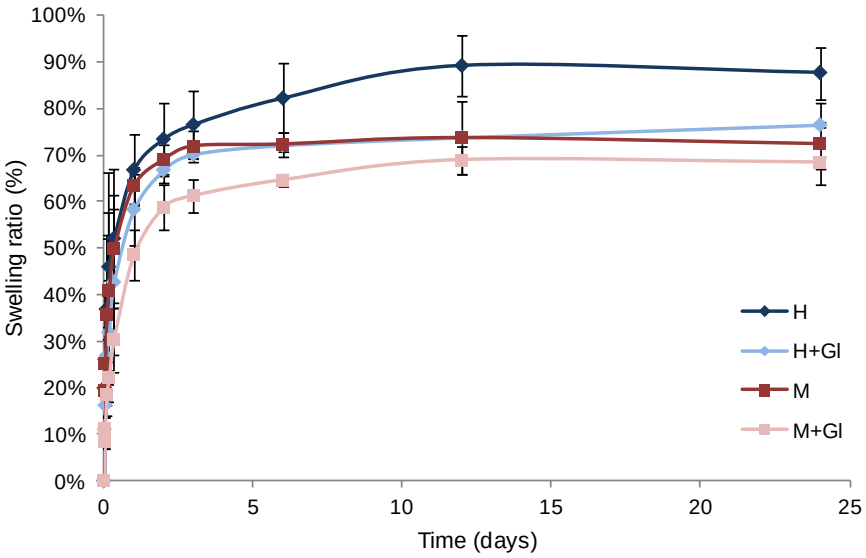


Figure 6.3. Swelling ratio (%) of 4 different chitosan-based specimens over 24 days.

6.2.2. Enzymatic degradation of chitosan-based specimens

The *in vitro* degradation of chitosan-based specimens was conducted with lysozyme to mimic these specimens *in vivo* degradation behavior, however, a concentration of lysozyme much higher than the concentration in human serum was used, as previously reported [45,220,226,227], mainly to accelerate the degradation process since a slow degradation rate was expected due to the high deacetylation degree (DD) of both chitosans. The DD highly influences the degradation behavior and mechanism by enzymes and is well known that the higher the DD of chitosan, the slower its degradation process becomes. Thus, highly deacetylated forms exhibit low degradation rates and may last several months *in vivo* [21,45,81,179,226–228].

Lysobac, the enzyme used in this study, is a recombinant human lysozyme produced in an animal-free production system. Animal-free production eliminates the safety risk and inconsistent lot-to-lot performance of the frequently used hen egg white lysozyme. Moreover, Lysobac has significantly higher bioactivity than hen egg white lysozyme since one gram of Lysobac replaces 4 grams of hen egg white lysozyme [229].

To distinguish between enzymatic degradation and simple dissolution, both the weight loss (figure 6.4) and the mechanical properties of specimens (figures 6.5 and 6.6) that had been placed in PBS supplemented with lysozyme were compared with those that had been placed in PBS used as a control solution.

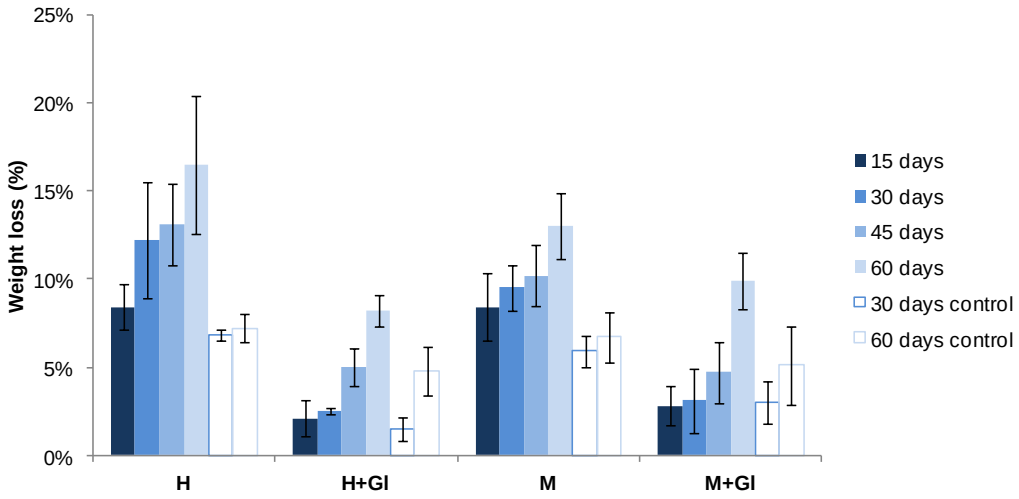


Figure 6.4. Weight loss (%) of chitosan-based specimens immersed in PBS supplemented with lysozyme (*15, 30, 45 and 60 days*) and immersed in a PBS control solution (*30 and 60 days control*) over 60 days.

Weight loss of chitosan-based specimens increased along with time of degradation, as expected [230]. Moreover, the degradation rate of porous structures is expected to be faster than films or other nonporous structures forms owing to the larger contact area with enzymes [228]. Therefore, although the number and size of the randomly distributed pores in H and M specimens were small, the greater weight loss results obtained from these specimens when compared to the plasticized specimens was also expected. For these H and M specimens it is also clear that their weight loss rate was greater in the first days: more than 8% weight loss, on average, after 15 days. On the other hand, the weight loss of all specimens significantly increased after 60 days. Taking into account the overall results, there is a notorious difference regarding the weight loss of chitosan-based specimens and their control counterparts. After 60 days, there is a significant difference between the weight loss of specimens that were in the lysozyme solution than their control specimens counterparts, pointing out the enzyme action in the degradation process, even for high DD. Besides the expected weight loss, the porosity of specimens is expected to increase with increasing immersion time in the prepared enzymatic solutions [231]. *In vivo*, the bone tissue cells could use this increasing porosity to improve the osteointegration of the implant and to accelerate its complete transformation into real bone tissue, since a pore structure of the post-implantation biomaterial can increase the vascularization and nutrient exchange between the surrounding tissues, and accelerate the material degradation [231,232]. Therefore, the chitosan-based porous structure that is expected to result from this degradation process can help the proliferation of cells. However, the initial

mechanical performance of a chitosan-based implant also has to be taken into account, since an increase in the number of pores lead to a decrease in the mechanical properties [231].

To further investigate the effect of degradation on the above mentioned chitosan-based specimens, their modulus of elasticity (figure 6.5) and compressive strength (figure 6.6) were assayed. To start it is important to highlight that after 30 days none of the M specimens could be tested due to its severe physical degradation, since after drying these specimens were so brittle that broke before testing. However, examination of the results for control specimens after 60 days, where degradation is slower due to absence of lysozyme, significant decrease in modulus of elasticity and compressive strength can be quantified. On the other hand, M+Gl specimens just suffered a slight decrease in the modulus of elasticity over 60 days. The 60-day modulus of elasticity declined from an average value of 467 MPa to 413 MPa when immersed in PBS solution containing lysozyme. The same specimens immersed in the control solution (PBS without lysozyme) presented a modulus of elasticity of 450 MPa, on average, after 60 days.

As far as H-based specimens are concerned, the variations in the modulus of elasticity with immersion time revealed that, after immersion for 15 days, both H and H+Gl specimens largely declined the modulus of elasticity. For H specimens, the modulus of elasticity was significantly reduced from the initial average value of 328 MPa, down to 112 MPa, after 60 days immersion in the solution containing lysozyme. A similar trend can be observed for H+Gl specimens, although much higher absolute values of modulus of elasticity were obtained.

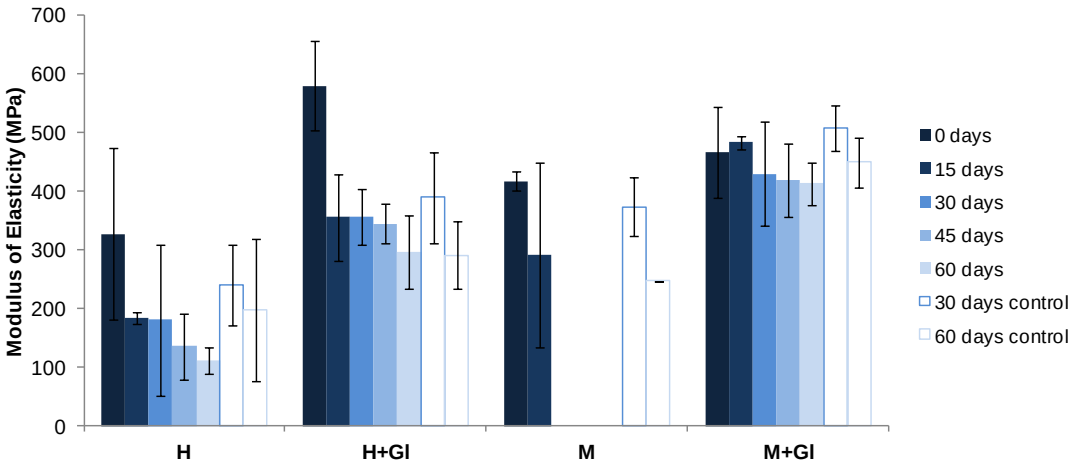


Figure 6.5. Modulus of elasticity (MPa) of chitosan-based specimens immersed in PBS supplemented with lysozyme (*15, 30, 45 and 60 days*) and immersed in a PBS control solution (*30 and 60 days control*) over 60 days.

For the compressive strength, both H+Gl and M+Gl just faced a slight decrease throughout the experiment, confirming the protective effect of glycerol. The highest changes were observed in the H specimens, for which compressive strength reduced to one-third of its initial value after 60 days. SEM images of H and H+Gl specimens after 60 days immersed in a PBS solution

containing lysozyme and after being mechanically tested help explaining these results (figure 6.7). While H specimens become more and more brittle over time and therefore crack and fail at lower compressive stresses, H+Gl specimens compressive strength did not significantly changed since most of these specimens could handle the maximum compressive load – 10 kN – without breaking. As previously mentioned, most of M specimens could not be tested, however, when looking for the results of the control specimens after 60 days, it is expected that these specimens would have a similar behavior as H specimens. Therefore, the plasticized effect of glycerol seems to help maintaining the initial mechanical properties of specimens, over time, when these are immersed in a degradation solution.

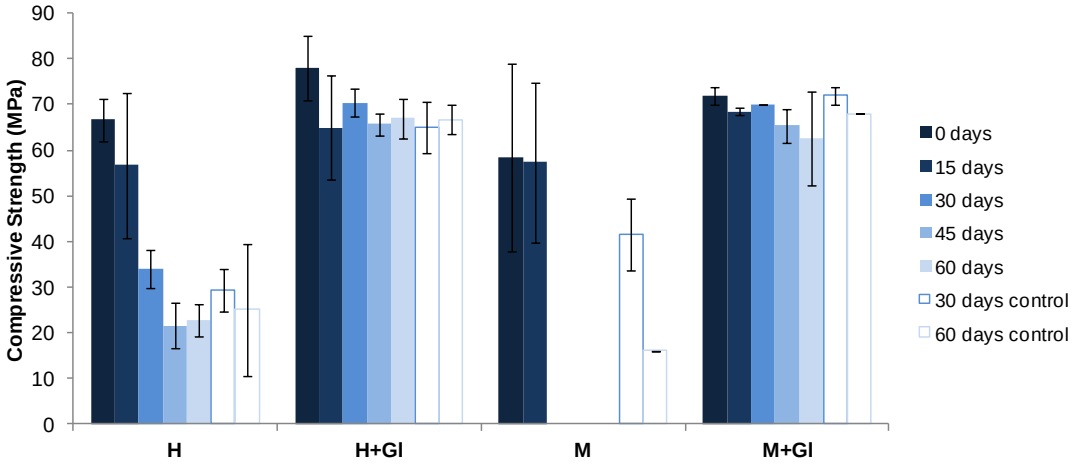


Figure 6.6. Compressive strength (MPa) of chitosan-based specimens immersed in PBS supplemented with lysozyme (*15, 30, 45 and 60 days*) and immersed in a PBS control solution (*30 and 60 days control*) over 60 days.

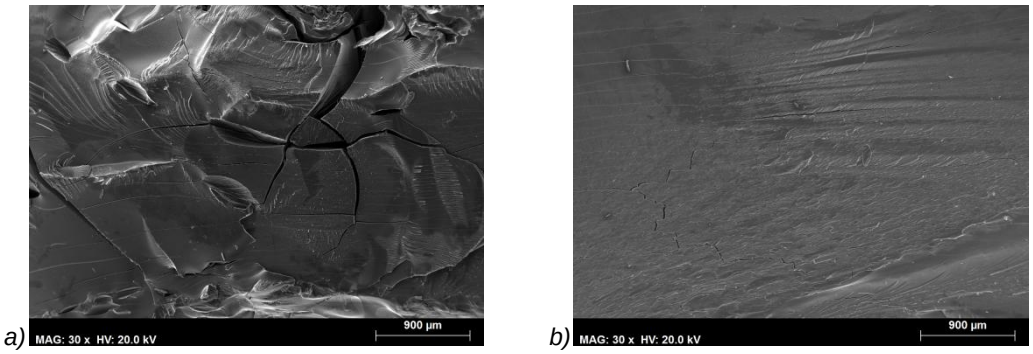


Figure 6.7. SEM images of (*a*) H and (*b*) H+Gl mechanically tested specimens, after 60 days immersed in a PBS solution containing lysozyme.

In general, unplasticized chitosan specimens clearly presented a more brittle behavior than the plasticized ones. Moreover, the penetration of water/ions resulting from the solution could reduce the adhesion between interfaces, explaining the compressive strength decrease of pure chitosan specimens [231]. The addition of glycerol influenced the mechanical properties of the specimens, particularly their strength. Both modulus of elasticity and compressive strength of M+Gl specimens underwent slight changes during the 60 days period. Thus, these specimens' composition might be an optimal material in terms of initial strength and degradation behavior for applications that need to keep the mechanical properties of the absorbable implant, at least, during the first two months. It is known that for a long-term stability *in vivo*, a chitosan-based specimen would have to be composed by a chitosan with a high DD. Moreover, a high DD is also crucial to achieve high mechanical strength [180,181]. Besides that, there are several strategies to maintain the mechanical strength and preventing the premature collapse of specimens, such as the incorporation of polymer coils into the specimens [180], hydroxyapatite [233,234], gelatin [235], the use of various cross-linking reagents (e.g. glutaraldehyde, ethylene glycol diglycidyl ether; diisocyanate; genipin) [198,236], among others. From the results obtained, it is also possible to assume that the incorporation of a low concentration of glycerol – 7.5% (v/v) – can improve the mechanical properties of chitosan-based specimens and slow down their degradation as shown in figure 6.8, where:

$$\Delta\ Weight\ (\%) = (W_0 - W_{60}) / W_0 \times 100 \quad (6.4)$$

$$\Delta\ Compressive\ strength\ (\%) = (\sigma_0 - \sigma_{60}) / \sigma_0 \times 100 \quad (6.5)$$

Where W_0 and σ_0 are the initial average weight and the initial average compressive strength of specimens, respectively, while W_{60} and σ_{60} are the average weight and the average compressive strength of specimens, respectively, after being immersed in PBS supplemented with lysozyme for 60 days.

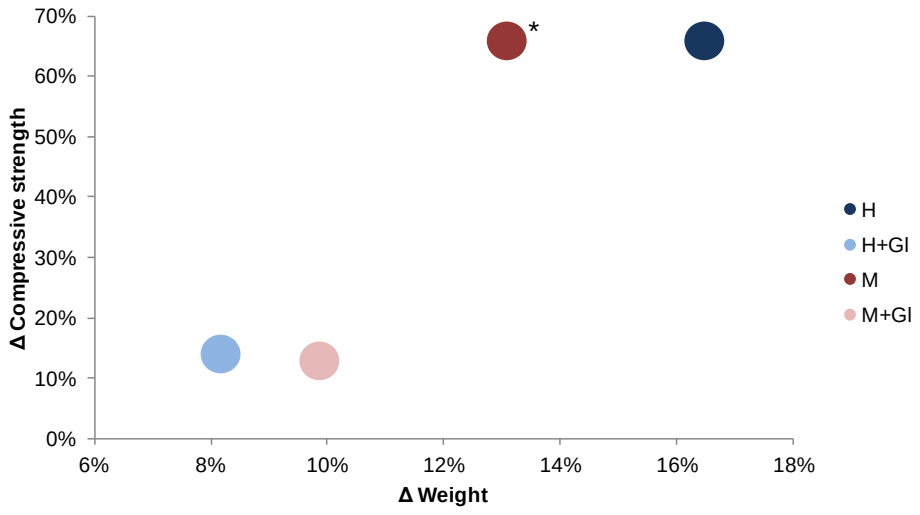


Figure 6.8. Variation of weight (Δ Weight) and variation of compressive strength (Δ Compressive strength) of tested chitosan-based structures after being immersed in PBS supplemented with lysozyme for 60 days (*structures broke before being mechanically tested).

6.2.3. *In vitro* cytotoxicity of chitosan-based specimens

In order to verify the cytotoxicity of the chitosan-based specimens, extract and direct cytotoxic assays were performed according to the frequently used ISO 10993-5 guidelines for medical devices [54,232]. The cell line used for these assays was fibroblast L929. Chitosan-based specimens were tested in order to confirm that their production process and the variation in composition (chitosans with different molecular weight and glycerol) do not turn these specimens toxic. The results in figure 6.9 show that cells' metabolic activity is maintained at high levels, regardless the specimens composition, at values comparable with the negative control (culture in tissue culture plate) and significant higher than the ones observed for the positive control (presence of cytotoxic material). This results indicated that the potential lixiviates from the chitosan-based specimens had no obvious cytotoxic effect, regardless their composition, and therefore further tests where the specimens were put in direct contact with the cells were performed.

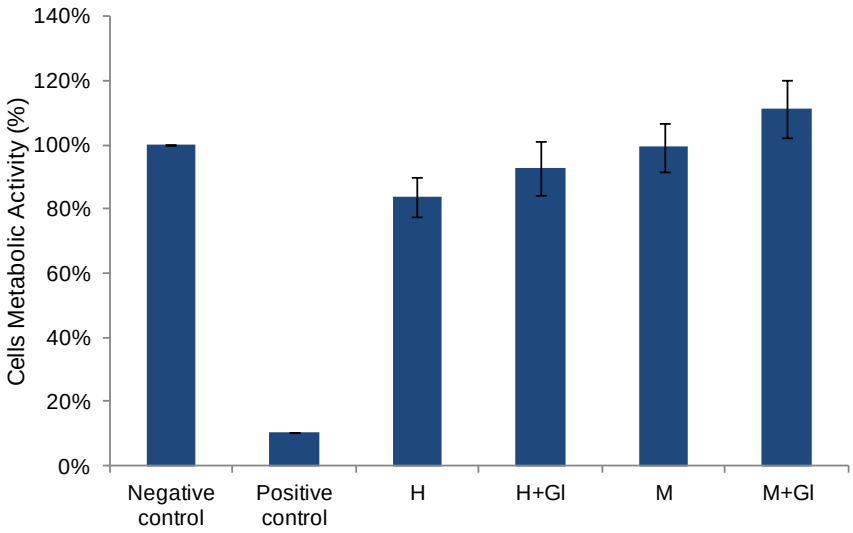


Figure 6.9. Cytotoxicity results for MTT assay of chitosan-based specimens.

The result of the direct contact assay is shown in figure 6.10, where no inhibition halo resulting of cells dead around the chitosan-based specimens was observed. These specimens remained fully transparent by light microscopy, allowing for a direct observation of the cell morphology and growth throughout the material. Since no morphologic alteration or proliferation disorder was observed, it was considered that the tested specimens were neither cytotoxic towards the cells, nor released cytotoxic substances in the culture medium.

Previous studies have demonstrated that DD has no significant influence on the *in vitro* cytocompatibility of chitosan films towards fibroblasts, confirming the biocompatibility of chitosan-based materials whatever the DD. It is also suggested that DD would also have no

effect *in vivo*, or towards other cell types [32,237]. However, at high DD, the toxicity is related to the molecular weight and polymer concentration [237]. In this study, none of the chitosan-based specimens induced a cytotoxic effect. However, when comparing the cells metabolic activity in contact with M and H specimens and also with M+Gl and H+Gl specimens, it is notorious that the M-based specimens induced a higher cellular activity. Thus, for the same DD, chitosan with a molecular weight of 300 kDa induced a higher cellular activity when compared to chitosan with 800 kDa.

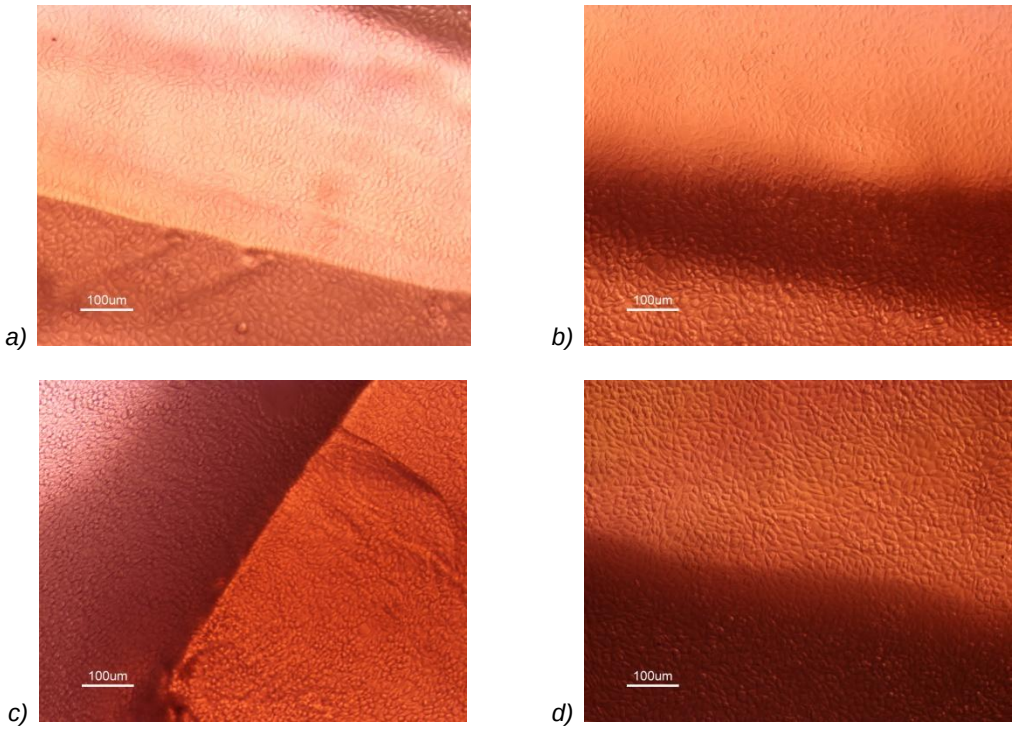


Figure 6.10. Cytotoxicity assays for direct contact assay of chitosan-based specimens: *a)* cells cultured on H; *b)* cells cultured on H+Gl; *c)* cells cultured on M; *d)* cells cultured on M+Gl.

Besides the nontoxic nature and biocompatibility of chitosan, its processability into geometrically different structures is an important aspect for medical applications. Structures made from pure chitosan are rigid and brittle, and it is therefore important to use plasticizers in order to obtain more favorable mechanical properties. Because of the potential medical applications, the plasticizer materials must be biocompatible as well [200]. The results presented in this chapter showed that glycerol is an effective and biocompatible plasticizer for rigid and brittle chitosan specimens, when used in such low concentrations.

Preliminary experiments were also conducted to study the adherence and proliferation of Bone Marrow (BM) Mesenchymal Stem Cells (MSCs) on these chitosan-based specimens. MSCs were cultured with an initial cell density of 5000 cells/specimen during 10 days. As a control, MSCs were plated and cultured on common Polystyrene (PS) tissue culture plates. It

was noticed that cells have a tendency to proliferate more on plasticized specimens that could be explained by the fact that glycerol increases the hydrophilic character of the material [203]. Chitin, chitosan and their derivatives have been reported to stimulate cells proliferation and enhance collagen synthesis [68]. It is suggested that DD plays a key role in the cell adhesion and proliferation. Indeed, the lower the DD of a chitosan film, the lower is the cell adhesion. Cell adhesion also seems to be related to the surface morphology of the films. Although DD plays a major role, the superficial properties, especially the morphology of the surface is an important parameter which must be accounted for. If the general rule is a continuous increase of the cell adhesion on increasing DD, this behavior can be locally contrasted by the superficial physical state which also seems to depend on the nature of the cells. Therefore, it was demonstrated that smooth surfaces favored the cell proliferation much better than rough surfaces [14]. In the present study, specimens with glycerol had smoother surfaces when compared to the equivalent specimens without glycerol and therefore the results obtained suggest that the greater cells activity observed for plasticized specimens are also related to their smooth surfaces. Nevertheless, although several studies have shown that chitosan-based specimens support cell adhesion and proliferation [62,238], further experiments need to be performed for plasticized specimens. No differences were noticed regarding the molecular weight of the chitosan.

From the results obtained for these chitosan-based specimens, several potential biomedical applications could be pointed out. Taking into account the mechanical properties of the tested specimens and the mechanical properties of bone, one can assert that they are in between cancellous bone - compressive strength: 2-12 MPa and Young's modulus: 50-500 MPa - and cortical bone - compressive strength: 100-230 MPa and Young's modulus: 7-30 GPa [239]. With these in mind and knowing that the vertebral cortical bone *in vivo* has a thickness often less than 0.4 mm and the apparent Young's modulus computed by a finite element method (FEM) inverse analysis was equal to, on average, 374 MPa (SD = 208) [240], chitosan-based implants for spine, such as absorbable spinal cages, could be an appealing application. Spinal fusion is considered to be one of the most challenging applications for bone graft substitutes, since even autologous bone, the golden standard, has a relatively high rate of failure [241]. An ideal scenario for interbody fusion is a cage device that has a modulus of elasticity that is the same as or close to that of vertebral bone, that will be absorbed after interbody fusion and that will be replaced by cancellous bone, not leaving foreign body material in the spinal segment, but only a bony fusion between the vertebrae [242]. Thus, the plasticized chitosan-based specimens seem to be an appealing alternative to the existing materials. Moreover, MSC, which are the osteoprogenitor cells responsible for bone fusion and have been identified in vertebra [243], have shown to proliferate well on 3D plasticized chitosan-based structures.

6.3. Conclusions

Four different kinds of chitosan-based specimens – H; H+Gl; M; M+Gl – were successfully prepared and characterized in this study. Both physical and biological results obtained suggest that chitosan-based specimens, particularly the M+Gl specimens with a low concentration of glycerol – 7.5% (v/v) – might be an optimal material in terms of initial strength and degradation behavior for applications that need to keep the mechanical properties of the absorbable implant, at least, during the first two months. Furthermore, besides the non-cytotoxic effect of these specimens, preliminary experiments showed that MSC have a tendency to proliferate more on plasticized structures. In conclusion, although interesting results were obtained, suggesting that chitosan-based spinal cages for interbody fusion could be an appealing application, further *in vitro* and *in vivo* long-term experiments are needed not only to optimize the biological properties, but also the degradation and biomechanical properties of these structures. It is required to guarantee that these degradable devices possess adequate mechanical properties that are gradually lost during the degradation process to progressively transfer mechanical loads to the newly forming bone. They should also provide appropriate surface chemistry to facilitate cell attachment, proliferation, and differentiation throughout the bone healing (or fusion) process.

7. Competitiveness of Chitosan-Based Implants

Over the past decades, there have been significant advances in the development of new biomedical materials. Although current treatments using “passive” materials have proven efficacious, tissue-engineering approaches using materials that actively interact and integrate with their biological environment are being considered as promising future alternatives for both failing tissues and organs [109,244,245]. The multitude of materials studied to perform this task include synthetic polymers like polycaprolactone, poly (lactic-co-glycolic acid), poly(ethylene glycol), poly(vinyl alcohol) and polyurethane; and natural polymers, such as alginate, gelatin, collagen, starch and chitosan, as referred throughout chapters 2 and 3. Among them, natural derived polymers are of special interest due to, as natural components of living structures, their biological and chemical similarities to natural tissues.

In 2012, the global biomaterial market was valued at $44 billion and is expected to grow at a compound annual growth rate (CAGR) of 15.0% from 2012 to 2017 [246]. This indicates a great opportunity in terms of revenue and market growth. Moreover, more than 22% of the global population in 2050 is expected to be over 60 years [246]. Indeed, the innovation opportunities made possible by changes in the numbers of people – and in their age distribution, education, occupations, and geographic location – are among the most rewarding and least risky of entrepreneurial pursuits [247]. The critical need to maximize outcomes from the substantial investments in health and biomedical research that some countries have been making is more reachable now than ever [248].

Motivated by these trends, Altakitin, the industrial partner of this PhD project, was created. Due to the well-known and frequently reported chitosan’s broad spectrum of applications along with unique biological properties including biocompatibility, biodegradability to harmless products, nontoxicity, remarkable affinity to proteins, antibacterial, haemostatic, antitumoral, among other properties [6], Altakitin had decided to focus its research on this polymer. Moreover, according to PubMed, that comprises over 22 million citations for biomedical literature from MEDLINE, life science journals and online books, there are over 12,500 publications referring to chitosan and this number has been increasing over time [249], as shown in the graphic of figure 7.1. Accordingly, taking into account this growing trend of biomedical research related to chitosan and the increasing gap between research and chitosan-based products development and commercialization, Altakitin has been focusing its activity on the production of biomaterials, namely chitosan, and on the development and production of chitosan-based medical devices.

The aim of this chapter is to assess the potential of an innovative production process of 3D solid and dense chitosan-based products for biomedical applications, developed under the PhD project, which resulted in a patent application. The following section starts with a brief explanation of the technology, previously presented throughout the former chapters, highlighting its main features. Then, several potential applications and their markets were identified and

assessed. After choosing a primary application and market, its potential as well as its uncertainties and risks were identified. A business model suggesting how to materialize the value from the application was also sketched, followed by a brief description of the market as well as the identification of the main competitors and their distinctive features. The supply chain analysis and the go-to-market strategy were the following steps. To conclude, a final recommendation based on the assessment of the information gathered during the last 3 years and on a deep analysis was prepared.

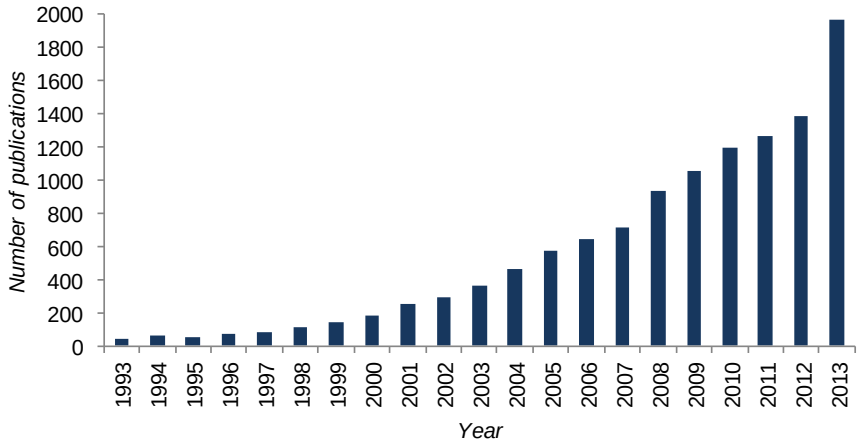


Figure 7.1. Number of publications related to chitosan over time [249].

7.1. Technology

Before start thinking about the potential applications of a certain technology, product, or even an idea, it is crucial to identify its main features. Thus, for the new production process of chitosan-based solid and dense products that was developed, it is very important to state from the very beginning its main properties and, compared to the known state-of-the-art, its key distinguishing characteristics. By doing this, the product development team is able to start listing potential applications.

The present technology relates to a novel process, based on a wet gelation process, which was developed for the production of 3D dense chitosan-based specimens to be used in the future generations of bioabsorbable implants, mainly for orthopedic applications. The specimens that result from the process are considerably dense and easy to machine. The results suggest that the production process can yield 3D structures that, with proper design, can be good candidates to be used as absorbable implants for different types of applications, within different medical fields such as orthopedics, sports medicine and maxillofacial surgery. As a result, this technology allows the:

- production of 3D dense natural polymer-based specimens, avoiding the problems

associated with the stimulation of chronic inflammatory reaction and toxicity by synthetic polymers, on one hand, and taking advantage of the previously mentioned appealing properties of chitosan, on the other hand;

- production of specimens with different sizes, shapes and properties;
- control of specimens‘ degradation rate and mechanical performance through an intrinsic attribute of their raw material (DD), but also by blending chitosan with other materials.

In conclusion, the technology presented here is a simple production process of chitosan-based implants with potential use in many different medical applications where features as biocompatibility, biodegradability, bioactivity and biomechanical integrity are of crucial importance.

7.2. Potential applications

Coming to an understanding of what customers value is usually a more fruitful exercise than merely asking them to submit their own solutions. It is said that the process of innovation begins with identifying the outcomes that customers want to achieve and it ends in the creation of items they will buy [250,251]. Consequently, this stage demands a lot from the product development team and includes a mix of external search, creative problem solving within the team and systematic exploration of the generated solutions. At this stage, a list of potential applications and their assessment is usually made, using the brainstorming tool, by doing a literature review (review papers, research papers, books, patents, among others) and by interviewing some potential customers, users and experts in the field. For each application, it is important to think and assess different application scenarios. This assessment can be made by listing the pros and cons of each scenario.

To understand the potential acceptance of chitosan-based medical devices, as well as the key decision drivers and their decision makers, it is recommended to conduct several interviews. Among these interviews, it is important that some can be conducted to experienced physicians with know-how with bioabsorbable devices in surgery. Moreover, it is also essential to understand from them whether they would use chitosan-based implants if they were readily available in the marketplace. Thus, in addition to understand about surgeons’ willingness to use the implants, the aim of this research is to understand where these surgeons envisioned using them (e.g. low-weight-bearing orthopedic applications) and what are the chitosan-based implants’ factors of success (e.g. the product itself, chitosan’s properties, company’s sales force, etc.). From these interviews, the main concerns regarding chitosan-based implants and how they can be overcome should be also addressed.

Table 7.1 resumes some possible applications, as well as their pros and cons. The list of applications and their assessment were made by doing a literature review, including brochures and websites of the commercially available bioabsorbable implants, and by interviewing some potential customers/users/experts in the field. Please refer to Appendix to see the transcription of some of the conducted interviews.

Table 7.1. Pros and cons of potential applications of chitosan-based implants.

Application	Pros	Cons
Arthroscopy: use of chitosan-based implants (e.g. tacks, anchors, arrows, needles, screws) in shoulder (e.g. Bankart repair, rotator cuff tears repair) and knee (e.g. meniscal repair, anterior cruciate ligament reconstruction)	• Biocompatibility, biodegradability and cell affinity • No cytotoxic effects • Small and easy to shape implants • Complications with existing absorbable implants have been reported [95,154]	• Big bioabsorbable implants competitors in the market: Smith & Nephew (Suretac) [252]; Arthrex (Meniscal DartStick System; Bio-Interference screw) [253,254]; DePuy Mitek (Panalok; Spiralok; RapidLoc) [255–257]; ConMed (Bio-Anchor; Contour Meniscus Arrow; BioScrew) [258–260] • Lack of knowledge (e.g. pull-out strength properties, cyclic loading tests properties)
Bone Fixation: use of chitosan-based implants (e.g. pins, nails, screws) in lower extremities (e.g. osteotomies, foot, ankle and tibial fractures) and in upper extremities (e.g. hand, clavicular, humeral and radial fractures)	• Biocompatibility, biodegradability and cell affinity • No cytotoxic effects • Initial flexural mechanical properties • Complications with existing absorbable implants have been reported [95]	• Big bioabsorbable implants competitors in the market: DePuy Orhopaedics (OrthoSorb Pin) [261]; Bioretec (ActivaPin; ActivaNail; ActivaScrew) [262–264]; Biomet (ReUnite Orthopedic Pin) [265] • Difficult to achieve big implants sizes required for some applications • Lack of knowledge (e.g. long-term mechanical performance)
Craniomaxillofacial Reconstruction: use of chitosan-based implants (e.g. plates, screws, mesh panels) in facial surgeries (e.g. pediatric and adult fractures, plastic surgeries)	• Biocompatibility, biodegradability and cell affinity • No cytotoxic effects • Initial flexural mechanical properties • No bone growth restriction	• Big bioabsorbable implants competitors in the market: Biomet (LactoSorb) [266–268]; Inion (CPS System) [269]; DePuy Synthes (Rapid Resorbable Fixation System) [270] • Difficult to achieve some implants sizes and geometries required for some applications • Lack of knowledge (e.g. how to allow bending of the plates to fit facial curved surfaces during surgeries?)
Spine: use of chitosan-based implants (e.g. plates, screws, cages, meshes) in spine surgeries (e.g. cervical and lumbar fusion, decompression)	• Osteoconductive, absorbable and bioactive • No cytotoxic effects • Compressive mechanical properties • Few bioabsorbable implants competitors in the market: Inion (screws and plates) [271]; SBM (cages) [272]	• Lack of knowledge (e.g. what is the best chitosan-based cage design for spinal fusion surgeries?)

7.3. Primary application

Having in mind that innovation is not looking at need alone, but looking at need and opportunity, among all the potential applications identified and studied, and after careful analysis of all the information gathered during the previous step, the most attractive one should be chosen, in order to focus in one specific market, since the establishment of target specifications for a product is very important from the beginning [273].

7.3.1. Chitosan-based intervertebral fusion cages

Orthopedic implants market is *per se* extremely attractive. The global market is projected to reach $46.5 billion by 2017 from an estimated $21.1 billion in 2007, growing by a CAGR (2007-2017) of 8.2% [274]. The spinal implants market is considered as a very important and lucrative sub segment of orthopedic industry. Spinal devices are observed to be the fastest growing segment with a CAGR of 9.3% [275], being Medtronic (USA), DePuy Spine (USA), Synthes (USA), Stryker (USA), Orthofix International (Netherlands), Biomet Spine (USA), Zimmer (USA), Orthovita (USA), among other companies, the key players in this market [276]. Within this market, lumbar interbody fusions that have been performed in patients with degenerative disk disease and discogenic pain syndromes have been increasing as well [277], making it an attractive market for chitosan-based devices.

The spine, also known as the vertebral column or spinal column, is a column of 26 bones in an adult human body: 24 separate vertebrae interspaced with cartilage, and then additionally the sacrum and coccyx [278]. The vertebrae are named by the first letter of their region (cervical, thoracic, or lumbar) and with a number to indicate their position along the superior-inferior axis. For example, the fifth lumbar vertebra (which is the most inferior one, located beneath the fourth lumbar vertebra) is called the L5 vertebra. Degenerative disc disease occurs when the intervertebral disc between two vertebrae begins to wear out. This intervertebral disc degeneration usually takes place asymptomatically in early life in most human beings and is one of the most common causes of chronic pain [279,280]. The process of degeneration causes the disc to lose its ability to act as a shock absorber between the vertebrae. This can lead to pinching and irritation on the nerves, which causes pain into the legs [281]. Thus, fusion of the degenerative and unstable spinal motion segment can give significant relief from this disabling and often progressive condition [282]. In September of 1996, the FDA approved interbody cages for use in the intervertebral disc space, providing a new technique that allows the spine to be fused with less morbidity (e.g. less post-operative discomfort) than in the past [283]. Nowadays, these devices are commonly used to treat problems such as disc degeneration, disc herniation, spine instability, among other problems [284–287].

The intervertebral fusion implants are designed as “cages” so that bone graft can be placed inside them, allowing the bone to grow from the vertebral body through the cage and into the

next vertebral body [283]. Not only the cage design, but also the choice of cage materials plays crucial roles in the long-term results [288]. Nowadays, hollow horizontal cylinders, vertical rings, and open boxes are standard designs and these cages are made of metal (e.g. titanium), polymer (e.g. PEEK), carbon fiber, or allograft bone [283,289]. This surgical technique in which one or more of the vertebrae of the spine are united together so that motion no longer occurs between them, can be performed in different spine regions - cervical, thoracic and lumbar - by using several surgical approaches [277,290–292]. To achieve a higher stabilization degree and to relieve the load supported by the cage, thus reducing the risk of subsidence and collapse of the intervertebral space, interbody cages are frequently supplemented with posterior instrumentation (posterior rods and pedicle screws most commonly) [277,280], as represented in figure 7.2.

The advantages of interbody fusion include direct removal of the dysfunctional disc and preservation or restoration of the disc height [293]. Thus, these devices allow the restore and maintenance of disk space height and normal sagittal contours by stimulating the vertebrae to grow together into one solid bone. This fusion creates a rigid and immovable column of bone in the problem section of the spine [294].

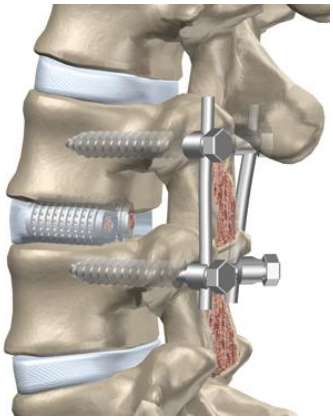


Figure 7.2. Example of an interbody fusion procedure with a fusion cage [294].

The ideal interbody graft combines a strong mechanical construct to withstand compressive loads across the disc space while providing an osteogenic, osteoinductive, and osteoconductive matrix [295]. The *gold standard* for this matrix is autogenous cancellous bone. However, due to the poor compressive strength of this bone along with other disadvantages, including donor site pain and occasional poor bone quality, especially in the elderly, led to the development of interbody fusion cages [293]. A wide variety of spacers or cages for interbody support in spinal fusion are in clinical use today. Metallic devices have the advantage of being biocompatible, but lack of radiolucency obscures evaluation of fusion status. Moreover, these devices are associated with excessive rigidity that may increase postoperative complications such as stress shielding, device-related osteopenia, and subsidence [285]. Non-metallic spacers (e.g., PEEK)

are radiolucent, but again do not load share as they persist in the interbody space. Allograft bone is radiolucent and shares load with the developing fusion mass, but consistency, availability and potential disease transmission are still some of the issues associated with the use of allografts. As a result, biodegradable polymer-based fusion cages have gained increasing attention [296,297]. Among the potential advantages, the most distinctive are related with the material degradation over time, being replaced by newly grown tissue, and the material mechanical properties that are closer to those of vertebrae bone, thereby distributing the load more evenly to the ingrown bone and the device.

Taking into account the mechanical properties of the tested chitosan-based specimens (chapter 5 and 6) and the mechanical properties of bone, one can assert that they are in between cancellous bone - compressive strength: 2-12 MPa and Young's modulus: 50-500 MPa - and cortical bone - compressive strength: 100-230 MPa and Young's modulus: 7-30 GPa [239]. With these in mind, and knowing that the vertebral cortical bone *in vivo* has a thickness often less than 0.4 mm and the apparent Young's modulus computed by a finite element method (FEM) inverse analysis was equal to, on average, 374 MPa (SD = 208) [240], chitosan-based implants for spine, such as absorbable spinal cages, can be an appealing application, as referred in chapter 6.

7.4. Uncertainties and risks

In this process assessment phase, all different kind of uncertainties and risks regarding the product, the production process, the application and the market have to be evaluated. A common problem with new technologies and products is the capability to be scaled up to reach the required yearly volumes that are needed (or expected). Another common important issue is the cost associated with the production. Therefore, since the price is usually an important feature of almost any product, some considerations should be made in order to offer the technology at an attractive and competitive price.

Concerning the medical devices market, many considerations have to be taken into account, such as the reliability of the process regarding the quality of the product. Furthermore, medical devices and products, or applications, before getting out into the market must be approved by health authorities. Consequently, any medical device placed on the market must comply with the legislation and fulfill the more and more demanding requirements defined by FDA, to be marketed in the United States; the European Commission directives to be marketed in the entire European Union (EU), in the European Economic Area (EEA) and in Switzerland [298]; among other markets' requirements. This often requires the validation of the manufacturing process according to best practices (in medical, pharmaceutical and food it must be assured the cGMP – current Good Manufacturing Practices). Many times this takes a long time and is a costly process. Thus, there is no certainty that all of the requirements are fulfilled

and the technology is approved for use and commercialization, since it evolves many different aspects, not only technological about production process, but also about the general evaluation of the facilities where the product is obtained, procedures in place to assure product quality, equipment qualification and qualified people.

Another concern that has to be taken into account is the time to market. Health authorities' requirements are more and more demanding and sometimes, when the product is approved, the time to market can be lost, being other product competitor, or substitute, already in the market. So, the know-how in this health authority's submission is also crucial.

7.4.1. Chitosan-based intervertebral fusion cages uncertainties and risks

Undeniable promising results as far as 3D chitosan-based products biomechanical properties are concerned were already obtained and presented in previous chapters. However, a more long-term characterization both *in vitro* and *in vivo* is still required. It is known that interbody fusion requires a long healing time of more than one year [285]. New bone formation within or adjacent to the fusion device is typically seen by 3 months after the fusion procedure and usually progresses for 18-24 months [277]. Thus, further experiments need to be conducted in order to guarantee that these chitosan-based structures are appropriate for future generations of spine cages, otherwise fast degradation and loss of structural integrity may cause poor fusion performance.

Another concern is the design of chitosan-based cages. Conventional hollowed cylindrical cages or vertical ring types may not be adequate design candidates for biodegradable cages. The thin-wall geometry originally designed for metallic cages may collapse under physiological loading conditions when simply replacing permanent materials, such as titanium or PEEK, with significantly less-rigid biodegradable polymers [285]. During daily activity, the lumbar spine is exposed to significant biomechanical forces. Studies indicate that a motion segment may experience axial compressive loads ranging from 400 N during quiet standing to more than 7000 N during heavy lifting [289] and, therefore, when designing future spinal cages, this information should be taken into account.

Like other technologies, products, or devices, there is always the risk of substitutes. Although spinal fusion has remained the gold standard for the treatment of spinal degenerative disorders, it can cause restriction of motion and degeneration of adjacent spinal segments through stress which can further delay recovery and in some cases, even lead to unwanted additional back surgery [276]. This has led surgeons and patients to adopt spinal non-fusion or motion-preserving technologies, which maintain the patient's spinal mobility while alleviating severe back and leg pain, offering clinical benefits over arthrodesis or spinal joint fusion [299].

In conclusion, although chitosan-based cages (or spacers) seem to be promising absorbable implants for spinal fusion applications, the above mentioned risks and uncertainties, related with the process, the product and the market, have to be taken into account.

7.5. Business model

The business model is an important tool that helps a concept goes from an idea to the market. That means it is an useful tool to explain how to materialize the value from the technology to a specific application to put in the market. The business model stage usually includes the value propositions of a company, meaning the company's offer. Besides that, it usually has three more clusters of information: (i) customer, with the description of target customer segments, customer relationship and distribution channel; (ii) infrastructure, describing the core capabilities, partner network and value configuration; (iii) finance, with the cost structure and revenue streams description [300].

Figure 7.3 represents a template of a business model and it is useful to explain the value proposition of the selected application.

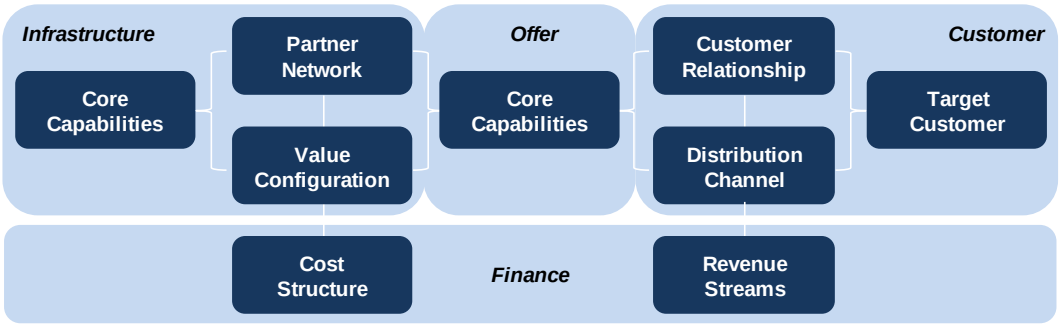


Figure 7.3. Business model template [301].

The value proposition of the presented application could be a totally absorbable chitosan-based intervertebral cage with better biomechanical properties than any other cage, avoiding the need of using autograft bone and promoting a faster fusion, due to the high level of cells adhesion and proliferation.

From the technology – the innovative 3D dense chitosan-based structures production process (core capability) – it is possible to obtain the chitosan-based intervertebral cages with the features mentioned before. Then, through the expertise of partner(s), the product could be offered in individual and customized packages, with the appropriate surgical instruments, intended for different spinal fusion surgeries.

The target customers of these medical devices would be orthopedic surgeons specialized in spine surgery, working in hospitals and clinics. These medical care organizations, specially the hospitals, are usually characterized by periodic purchases of large volumes, being cost awareness and looking for the specificity and the quality of the product (e.g. healthcare authorities' certification; surgeons/patients satisfaction).

In order to build a close and trustful relationship with customers, a certified distributor with a strong sales force could be used. Besides that, the product could be regularly presented and communicated in strategic medical congresses, conferences and seminars (e.g. International Society for the Advancement of Spine Surgery, Congress of Neurological Surgeons, Global Spine Congress, European Spine Congress) to promote and spread its use and to create a strong brand within the medical community. By doing this, it would be easier to introduce new products in the market and/or find new applications for the same chitosan-based implants production process.

7.6. Primary market

Although the choice of the primary application can be supported by the global market size and its attractiveness, in this stage is important to identify and decompose the market by country, for instance, and carefully select the primary target market(s), as well as the primary group of users that should be targeted. This process stage can result in a document, known as mission statement, which helps keeping the product development team focused on the project goals. The formulation of this document is important, since it sets from the beginning the product vision. This document usually includes the product description, the key business goals, the selected primary market(s), as well as the secondary markets, assumptions and constraints, and the identification of the main stakeholders [302].

7.6.1. Chitosan-based intervertebral fusion cages primary market

As previously mentioned, the global spinal implants market is considered as a very important and lucrative sub segment of orthopedic industry. Taking into account that the innovative technology presented throughout this document was developed in partnership with two Portuguese corporations – Ceramed and Altakitin – the primary target market should be Portugal and the remaining countries that require the CE marking, which is a mandatory conforming marking for medical devices sold within Europe, as previously explained. By taking advantage of Ceramed and Altakitin market know-how, customers and other useful contacts, spinal surgeons are the primary group of users that should be targeted. Attending and participating in international meetings and conferences is also highly recommended in order to strengthen these contacts.

Entering the market with chitosan-based bioabsorbable, bioactive and osteoconductive cages for spinal fusion surgeries is a good differentiation strategy to enter in an existing market under an expected dramatic transformation, primarily driven by innovation, globalization and commoditization of products [276]. Thus, within the spinal implants market, there are some niche markets and groups of surgeons specialized in specific surgeries (e.g. intervertebral

fusion surgeries) that can start using these innovative chitosan-based cages and help spreading their use.

Finally, since the Asian countries represent the fastest growing markets, due to large population, growing physicians and patient awareness about the new technologies, improving reimbursement coverage, booming medical tourism and increased purchasing power of hospitals [276], these markets cannot be neglected and therefore should make part of the secondary markets list.

7.7. Competitors

An understanding of competitive products is critical to the successful positioning of a new product and can provide a rich source of ideas for the product and production process design. Knowing the market and its main players, including the existing products, or the potential future products, is crucial [302]. Thus, a comparison of the new product with the main existing solutions should always be carried out. Taking into account the key features and main characteristics of each product, it is possible to identify the strengths and weaknesses of each of them and see if the new product is able to overcome the identified weaknesses.

There are essentially two types of competitors for the technology presented here: (i) those who produce and/or commercialize other spinal fusion devices and (ii) other techniques used as alternative treatment options for low back pain. Although the following subsections will focus on the existing spinal fusion devices, alternative treatment options such as artificial disks have emerged during the last decade and should not be neglected, since it may reduce the need for interbody fusion in the future [303].

7.7.1. Titanium-based cages for spinal fusion

Originally, interbody fusions were all performed with the patients' own bone from their iliac crest. Besides the bone graft site pain, there was a high nonunion rate associated with these procedures. As a result, the threaded cylindrical titanium cages (figure 7.4) became popular in the late 1990's, since they helped the success rate of the procedure by providing more firm fixation of the disc space [283]. Moreover, the amount of bone that needed to be harvested from the iliac crest was greatly reduced because only the soft inner cancellous bone was needed for the fusion. Currently, there are also several bone graft substitutes that may even eliminate the need for bone graft harvests [283].

Originally developed to treat race horses with wobbler syndrome (cervical spinal stenosis), the BAK cage (Zimmer Spine) is a cylindrical, hollow, porous, titanium alloy cage that is screwed into position within the disk space [304]. A second-generation cage developed by

Charles Ray, the Ray Threaded Fusion Cage (Stryker Spine) is a cylindrical, hollow, titanium, threaded device that contains less metal than the BAK cage [277,305]. The LT-CAGE (Medtronic Sofamor Danek), a third-generation device, is one of the most widely used interbody implant in North America [277,306,307]. Its shape allows increased surface area for bone growth. It is a thin-walled, threaded cage with truncated side walls that facilitate radiographic assessment of new bone formation inside and outside the implant, when compared to the other metallic cages [277]. ST MESH (DePuy Spine), a surgical titanium mesh implant, has an open diamond configuration to maximize the area of bone graft and allow for load sharing [277,308].

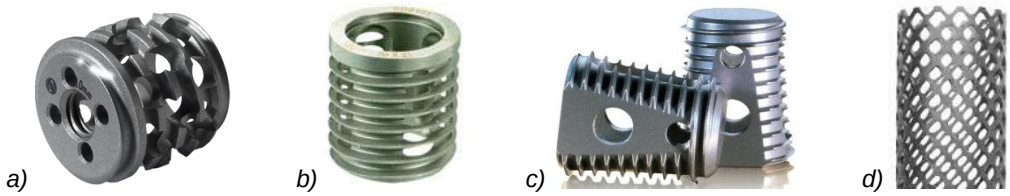


Figure 7.4. Titanium-based cages: (*a*) BAK/C Anterior Cervical Interbody Fusion System [304], (*b*) Ray Threaded Fusion Cage [305], (*c*) LT-Cage Lumbar Tapered Fusion Device [306] and (*d*) ST MESH [308].

One conceptual problem associated with these devices is their geometric shape. The volume available for bone graft in cylinders is less than that in vertical ring devices, such as the femoral ring allograft. A tapered device as opposed to a cylindrical shape better restores lordosis and sagittal balance [289]. Another concern associated with these metallic-based devices is the production of severe artifacts on magnetic resonance (MR) and computed tomography (CT) imaging [277]. Images from CT scans cannot be used because of the scattering effect of the metal. Despite some new techniques, CT scanning is still insufficient for evaluation of bone density and the level of incorporation of the cancellous bone into the cage [309].

As previously mentioned, there are already several bone graft substitutes that may eliminate the need for autologous bone grafts, commonly used in combination with these cages. This has resulted in the use of different materials such as bioceramics, corals, allografts, and constructs made from carbon fibre or metal. However, the results of fusions performed using these techniques are inconsistent and not convincing [309]. Approved in July 2002 by FDA as the first bone graft substitute equivalent to iliac crest autograft for spinal fusion, for use only with the LT-CAGE, INFUSE Bone Graft combines recombinant human bone morphogenic protein (rhBMP-2) with an absorbable collagen sponge carrier [277]. The rhBMP-2 acts as a signaling molecule to attract mesenchymal stem cells, binding to cell receptors and causing these stem cells to differentiate into osteoblasts and initiate bone formation. However, it has been recently associated with many severe problems, including: difficulty breathing, swallowing or speaking; compression of the airway; respiratory depression; nerve damage; among others [310].

7.7.2. PEEK-based cages for spinal fusion

As previously pointed out, PEEK refers to polyetheretherketone, a plastic substance with biomechanical properties similar to those of cortical bone [277,311]. This compound can be machined into any shape and size and is radiolucent on CT and plain radiographs. Depending on the shape, it can be placed through any surgical approach [311]. The main advantages of PEEK-based cages (figure 7.5) when compared with the metallic devices include their lack of artifacts on CT imaging [277]. On the other hand, they do not provide as good fixation. Generally, posterior pedicle screw supplementation is also necessary [283].

a) *b)* *c)*

Figure 7.5. PEEK-based cages: (*a*) PEEK Prevail Cervical Interbody Device [312], (*b*) Adaptive Vertebral PEEK Spacer [313], (*c*) SpineNet ACC – Anterior Cervical Cage [314].

7.7.3. Carbon fiber-based cages for spinal fusion

The JAGUAR I/F CAGE (DePuy Spine) is one of the available carbon fiber-reinforced polymer implant (figure 7.6), which can be machined to meet size and shape requirements. It is predominately radiolucent and produces fewer artifacts on CT and MR images, when compared with metallic implants. This device was designed for the posterior lumbar interbody fusion approach and is always used with supplemental posterior instrumentation. The disadvantage of a rectangular cage placed through a posterior approach is the tendency toward an over-curvature of the thoracic vertebrae [277].

a) *b)* *c)*

Figure 7.6. Carbon fiber-based cages: (*a*) JAGUAR I/F CAGE [315], (*b*) Brantigan ALIF I/F CAGE [316], (*c*) LEOPARD System [317].

7.7.4. Allograft bone-based cages for spinal fusion

The main benefit of allograft bone is that there are no surgical risks for the patient associated with harvesting their own bone. Moreover, the absence of imaging artifacts and the placement of a completely biologic device are two more advantages of these devices. However, there are two main drawbacks: (i) lower chance of fusion - since allograft bone does not contain living bone cells, it is not as effective at stimulating fusion as the patient's own bone - and (ii) risk of disease transmission - despite rules and regulations for tissue banks regarding processing and procedures of human tissue, there is still a small potential risk of disease transmission from using cadaver bone [318]. Furthermore, unlike titanium interbody cages, threaded cortical bone dowels (figure 7.7) are subject to supply shortages and processing problems [289].

a) *b)*

Figure 7.7. Allograft bone-based cages: (*a*) AlphaGRAFT Cervical Ring Spacer [319] and (*b*) AlphaGRAFT Lordotic and Parallel Bone Spacer [320].

7.7.5. Absorbable polymer-based cages for spinal fusion

Novel uses of bioabsorbable technology are constantly evolving. Bioabsorbable implants are already frequently used in sports medicine surgeries, especially in shoulder and knee ligaments reconstructions [87], and their use is now expanding to the realm of spinal surgery with the aim of help reducing many of the complications associated with the use of non-resorbable implants (e.g. stress shielding, pseudarthrosis), since they have a better match of strength and elasticity to bone [88,161]. In addition, these absorbable implants are radiolucent, offering the ability to assess fusion radiographically and eliminating long-term residual hardware [100,158].

Initial results showed that the reduced stiffness of PLA-based cages (figure 7.8) can enhance interbody fusion, as compared with titanium cages [321]. Part of the available clinical and radiographic results have supported the use of interbody devices manufactured from this bioabsorbable polymer for structural interbody support [322,323]. For similar PLA-based implants, however, concerns of early device failure were also raised, with too rapid *in vivo* degradation being the suspected reason [324,325]. Furthermore, other studies showed an increased incidence of nonunion and post-surgical cage migration in patients undergoing

lumbar interbody fusion with PLA-based biodegradable cages versus carbon-fiber implants [326] and PEEK-based implants [327].

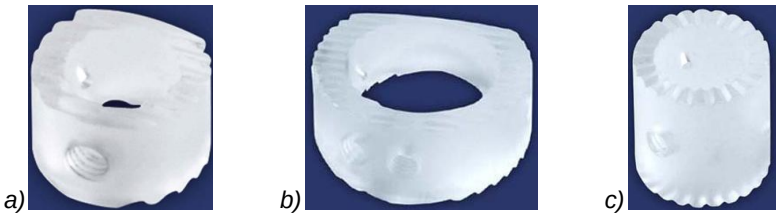


Figure 7.8. Absorbable polymer-based cages: (*a*) anterior cervical interbody spacer, (*b*) anterior lumbar interbody spacer and (*c*) anterior lumbar interbody spacer and vertebral body replacement implant [102].

As spinal interbody implants need to maintain mechanical integrity for a period of at least six months [328], this has serious implications for the clinical application of absorbable polymer-based implants in load bearing situations. Moreover, some authors declare that the disintegration of absorbable polymer-based implants into particles with a very slow hydrolytic degradation rate, as recommended for spinal fusion applications, can induce and maintain a clinically detectable swelling, with the occurrence of foreign body reactions, allowing skepticism regarding the value of these bioabsorbable implants [329,330].

An ideal scenario for interbody fusion is a cage device that has a modulus of elasticity that is the same as or close to that of vertebral bone, that will be absorbed after interbody fusion, maintaining the strength under continuous and alternate loading throughout the period of time required for full spinal bony fusion (a minimum of 6 months) and that will be replaced by cancellous bone, not leaving foreign body material in the spinal segment, but only a bony fusion between the vertebrae [242,331]. Therefore, the plasticized chitosan-based specimens seem to be an appealing alternative to the existing materials that have just been mentioned, as far as biomechanical properties are concerned, although further *in vitro* and *in vivo* long-term experiments are needed.

7.8. Industry dynamics

As the name suggests, study the industry dynamics means identifying the forces driving a specific industry evolution. Taking into account the medical devices example, this industry is very dynamic since it is focused on improving the healthcare of the patients but, at the same time, it is also very competitive because manages a large volume of money. It is an industry characterized by having a complex supply chain – raw-material suppliers, big manufacturers, but also small and medium enterprises (SMEs), distributors, hospitals/clinics, patients [246]. A

short representation of the overall and usual supply chain for medical devices is presented in figure 7.9, being the production process of chitosan-based implants, within the overall manufacturing process, represented in red.

The goal of the manufacturer is to provide a high valued product to their customers and increase market share, either by existing process improvements, or by offering new products for different applications and getting new businesses. Thus, from the manufacturer perspective, the goal is to have a different product to offer to medical devices companies and distributors, or to have an effective production process by lowering costs and increasing margins. Many companies in the medical devices business incorporate several supply chain blocks and usually, the closer it is from the product utilization the higher is the value. Therefore, it is common that large medical devices companies have their own manufacturer facilities and distribution centers. They also have strong sales force to marketing their products in hospitals and clinics. In order to increase their portfolio, these large companies are constantly looking to different technologies that allow better innovative products that can easily enter in the market and be market leaders [332]. Normally, the Intellectual Property (IP) of the product belongs to the medical devices company, but the production process and its technology IP is owned by the manufacturer, when they are not the same company.

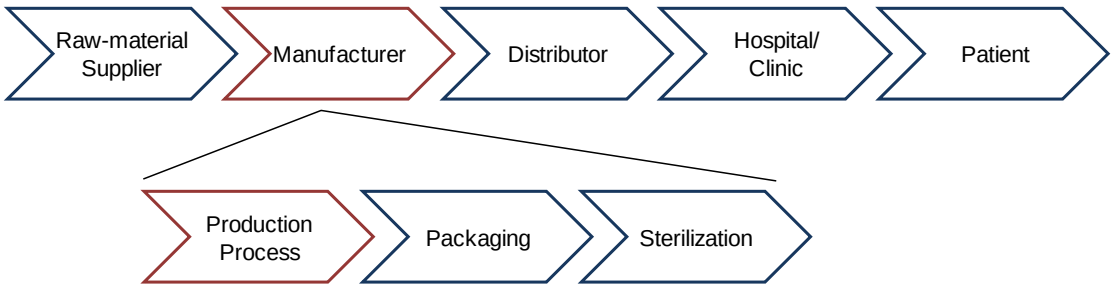


Figure 7.9. Chitosan-based implants supply chain.

Hospitals and clinics want to have products that can improve the healthcare of patients at a reasonable price. The decision-maker of what product to use for each situation, usually the surgeon, has an enormous impact in all of this supply chain. However, particularly in public hospitals, there are constraints regarding costs, so new medical devices must have a better cost-effective ratio when compared to the available ones. Besides that, the purchasing department and the evaluation of budgeting from hospital executives have an important role in the application decisions. In (private) clinics, the medical devices and their applications tend to be state-of-the-art to attract patients that expect a high level of service, although there is already some pressure from insurance companies. In this case, the buying decision is more focused on a surgeon-patient agreement and decision.

7.9. Go-to-market strategy

A Go-to-market strategy is a mechanism, or an action plan, that a company can use to propose how to deliver its value proposition to the target market. Therefore, the three main questions to be answered are: (i) what to sell; (ii) how to sell; (iii) who to sell (figure 7.10). Although these three questions were answered along the previous development stages, it is crucial to define a strategy from the state-of-the-art.

The technology, with a patent submitted, consists of a novel 3D dense chitosan-based structures' production process, developed under this PhD project carried out within the MIT Portugal Program and in partnership with Ceramed and Altakitin, which allows to efficiently producing chitosan-based implants for spinal fusion. In order to bring the intended medical implants to the market, several ways could be adopted, being the one presented here one possible solution.

Figure 7.10. Go-to-market strategy mechanism [333].

While defining the strategy, the product development team should not only take into account the available resources, but also overcome some of the existing limitations and identified needs. Therefore, in order to make chitosan-based implants for spinal fusion applications a real product, several risks and barriers have to be overcome, such as:

- Lack of market know-how
- Large initial investment required
- Need for process scale-up
- Need for plant, product, packaging and sterilization validation and certification
- Find adequate and certified distribution channels
- Entrance of new competitors

With this in mind, and in order to bring the invention to the market, a partnership with a medical devices industrial manufacturer with know-how in the field and interested in increasing its product portfolio may be considered. Although both Ceramed and Altakitin have a great experience within the orthopedic medical devices market, at this stage and to go further with the technology, looking for another partner with know-how in the field that could not only co-develop the technology, but also share the financial investment risks, could be the next step. To do so, crafting a business plan so that it thoroughly and candidly addresses the ingredients of success - people, opportunity, context, and the risk/reward picture - is vitally important [334]. By having the money and the market know-how, the certification process can be done much faster, reducing the time-to-market [335–337].

Surgeons wishing to perform interbody procedures using bioabsorbable cage devices should understand the fundamental differences between the non-absorbable and bioabsorbable cages and should be properly trained in patient selection, surgical technique and correct device handling and placement [161]. Consequently, finding a specialized medical devices distributor with a strong sales force to couch surgeons and sell the products in hospitals and clinics is also very important, not only to gain market share, but also to create a strong brand name and a good reputation in the market. This can be complemented by actively participating in congresses, conferences and by publishing regular papers in the most known spine surgery journals.

In conclusion, after analyzing the market, its stakeholders and its externalities, the presented strategic steps are one possible way to bring new chitosan-based implants for spinal fusion to the market (WHAT) that can be sold through a strong sales force team (HOW) to hospitals and clinics where spine surgeries are regularly performed (WHO).

7.10. Final recommendation

After identifying some of the potential applications for the innovative technology presented in this thesis, as well as a deep analysis of the market and the medical industry for the specific application that was identified and studied, it can be concluded that the potentiality of the technology and the product that can be developed is enormous. However, in order to place it successfully in the market by following the steps presented in the go-to-market strategy section, further information and studies need to be performed.

Firstly, more inquiries to the medical community and particularly to spine surgeons have to be made in order to complete and compile information about market needs, surgical procedures currently being used, advantages and problems associated with the existing devices already in the market.

A detailed cost analysis of the process, from the raw material to the final product, is also highly recommended. Furthermore, the sustainability of the process has to be assessed, as well as its environmental impact. Expanding the IP protection of the technology to other strategic markets must be weighted as well.

Besides strengthening the partnership between the university and the companies involved in the project (Ceramed and Altakitin), a partnership with a bigger medical devices company already established in the market can be advantageous in order to:

- share the required high investments in equipments/ installations to produce the product and know-how in processes scale-up
- reduce the timing for process submission and to increase the possibility to be approved by health authorities
- easily enter in the market, taking advantage of the existing customers and connections
- understand the market dynamics and needs, competition and further explore connections to other companies in the business
- take advantage of the partnerships and of the potentiality of the technology and start thinking and exploring new products and new applications

In conclusion, although the technology seems to have a great potential, the short-term recommendation is to make a deeper research in the field to quantify the main key features of the technology in order to design and perform future studies to validate the premise that chitosan-based implants provide better results than the existing bioabsorbable implants used in spinal fusion surgery.

8. Conclusions

The key objectives of this thesis were to develop a novel production process of 3D chitosan-based products, characterize the physical, chemical and biological properties of the successfully prepared specimens and assess the competitiveness of these chitosan-based products to be used as bioabsorbable implants within the enormous range of potential orthopedic applications. Thus, this work starts providing evidence in that there is the need to develop 3D dense chitosan-based implants mainly for orthopedic applications, by presenting a bibliographic revision on chitosan, focusing on its main properties and applications, on one hand, and providing context information on the existing bioabsorbable implants, identifying their main characteristics and drawbacks, on the other hand.

An innovative process to produce three-dimensional dense chitosan products was developed and presented throughout chapter 4. The specimens that result from the process are considerably dense and easy to machine. Both physical and morphological results suggest that the production process can yield 3D chitosan products with few pores and promising mechanical properties that, with proper design, can be good candidates to be used as absorbable implants for different types of applications. Moreover, both GPC and NMR spectroscopy results proved that the production process does not change two of the main important parameters of chitosan – deacetylation degree and molecular weight.

A fully mechanical and morphological characterization of the produced chitosan-based specimens that resulted from different process parameters changes and from different material blends was exhibited in chapter 5. As far as chitosan-based specimens production process parameters are concerned, the results obtained suggest that when using chitosans with the same DD, higher molecular weight chitosan-based specimens led to higher modulus and flexural strength, but lower flexural strain. The most interesting results, however, were obtained when mixing two chitosans with the same DD but different molecular weights. For a concentration of 3% (w/v), both average values of modulus of elasticity and flexural strength increased. Thus, the mechanical performance of specimens seem to be optimized when mixing chitosans with different M_w, due to the formation of more intermolecular hydrogen bonding, forming a more compact and homogeneous microstructure than the 3% (w/v) high M_w chitosan-based specimens. As expected, the mechanical performance of specimens with retained air bubbles is poorer than the standard specimens, mainly due to their inner porosity. Thus, air retention should be avoided while preparing chitosan-based products. Regarding the influence of the heat treatment in the mechanical properties of specimens, although it made these specimens more brittle, as suggested by flexural strain results, it also made them stiffer, making heat treatment an easy and fast way to increase the modulus of elasticity of chitosan-based products. The influence of freezing temperature of the chitosan solution as well as the utilization of glutaraldehyde as a crosslinker were also tested, resulting in brittle and unsuitable specimens for further mechanical tests. Blending chitosan with other biomaterials, such as ceramics

(hydroxyapatite) and polymers (PEG, PVP and glycerol), in order to improve the mechanical performance of 3D chitosan-based products, was also conducted. While both PEG/chitosan-based specimens and PVP/chitosan-based specimens broke while they were being machined, due to their extremely brittle behavior, preventing mechanical testing, chitosan-based specimens with either hydroxyapatite or glycerol in their content were successfully prepared and tested. 3% (w/v) chitosan-based specimens with 10% (w/w) of hydroxyapatite resulted in specimens with higher average modulus and flexural strength, when compared with the tested chitosan specimens without HA, but when the concentration of HA was 50% (w/w), the specimens decreased their average flexural strength. Results with the most significant difference, however, were obtained when using glycerol. Depending on chitosan molecular weight and its intrinsic viscosity and until a certain concentration of glycerol, chitosan-based specimens blended with this plasticizer presented a mechanical performance improvement. High M_w chitosan-based specimens blended with 10% (v/v) glycerol reached an average modulus of elasticity of approximately 3 GPa and an average flexural strength higher than 60 MPa. Moreover, these plasticized specimens had a translucent appearance without visible porous and were much easier to machine, due to their less brittle behavior. In conclusion, the mechanical performance of 3D chitosan-based products can be easily changed and potentially customized, within a range of values, in order to match different implant applications requirements.

Due to the influence of chitosan molecular weight and the addition of glycerol on the mechanical performance of the tested chitosan-based specimens presented in chapter 5, the aim of chapter 6 was to further assess the influence of chitosan's molecular weight and the addition of a plasticizer (glycerol) on 3D dense chitosan-based products' biomechanical properties. Therefore, several specimens were produced and tested in order to mainly assess their degradation behavior and cytotoxicity. Both physical and biological results obtained suggested that chitosan-based products, particularly the plasticized ones, might have an optimal microstructural composition, mechanical performance and degradation behavior for applications that need to keep the mechanical properties of the absorbable implant, at least, during the first two months. Furthermore, the results of both the extract assay and the direct contact assay proved that chitosan-based specimens, regardless their composition, were neither cytotoxic towards the cells, nor released cytotoxic substances in the culture medium. Preliminary experiments to study the potential for cell integration were also conducted, using human mesenchymal stem cells as a cell model to study cell adhesion and proliferation on these specimens, showing promising results. In conclusion, the results suggested that plasticized chitosan-based products may have an adequate composition, as far as biomechanical properties are concerned, to further develop absorbable chitosan-based spinal cages for interbody fusion, for instance.

Taking into account the promising results obtained and presented throughout chapters 5 and 6, an assessment of the potential of the developed innovative production process of 3D solid and dense chitosan-based products for biomedical applications, was performed and

exhibited in chapter 7. It started with a brief explanation of the technology, highlighting its main features. Several potential applications and their markets were identified and assessed. After choosing a primary application and market, its potential as well as its uncertainties and risks were identified. A business model suggesting how to materialize the value from the application was also sketched, followed by a brief description of the market as well as the identification of the main competitors and their distinctive features. The supply chain analysis and the go-to-market strategy were the following steps. To conclude, a final recommendation based on the assessment of the information was prepared. In conclusion, the technology showed great potential for the development of chitosan-based bioabsorbable implants to be used in spinal fusion applications.

The use of naturally occurring biopolymers for biomedical and pharmaceutical applications is increasing and the potential of chitosan was shown and highlighted throughout the thesis. Chitosan-based devices, either blended or not with other biomaterials, can be an appealing alternative to other advanced polymers-based devices used as bioabsorbable implants. Nevertheless, there are still several medium- and long-term achievements that need to be accomplished, including:

- **Improve the production process of chitosan-based products in order to guarantee the reproducibility of the results.** Although 3D dense chitosan-based specimens were successfully obtained, the standard deviations of the mechanical results of the tested specimens were high, in general, making the mechanical performance of chitosan-based products more difficult to predict. Glycerol, due to its plasticizing properties, made not only the produced chitosan-based specimens less brittle and easier to machine and shape into different geometries, but it also helped improving the mechanical performance of specimens. As a result, the standard deviation of their modulus of elasticity and strength decreased while the average values increased. Nevertheless, further experiments must be conducted in order to optimize the initial properties and the reproducibility of chitosan-based products.
- **Conduct further *in vitro* and *in vivo* studies.** The results obtained and presented in the thesis regarding the degradation behavior of the tested specimens, as well as their non-cytotoxicity and cell affinity, are undoubtedly promising. However, further *in vitro* experiments complemented with future *in vivo* studies are needed in order to assess the long-term degradation behavior of chitosan-based products. Moreover, since some intrinsic characteristics of chitosan (e.g. deacetylation degree) can highly influence the degradation behavior of these products, these future studies should contemplate several specimens made from different chitosans with different deacetylation degrees, for instance. In addition to the recommended enzymatic degradation studies, more experiments with cells are also desirable. The preliminary tests with mesenchymal stem cells showed promising results as far as cells adhesion and proliferation is

concerned, however, due to the potential of using chitosan-based devices as bioactive absorbable implants, an exhaustive biological characterization is required.

- **Establish more partnerships to better define product requirements.** The competitiveness of the developed production process was assessed and presented. Among several potential applications for the chitosan-based products, a primary application – chitosan-based absorbable cages for spinal fusion – was chosen and assessed based on several criteria: experts and potential customers feedback, market analysis and trends, required biomechanical properties, among other factors. Nevertheless, in order to develop, produce, certify and sell the product, several intermediate achievements need to be accomplished. Thus, establishing partnerships with key orthopedic surgeons (including spine surgery experts) is highly recommended to be able to design and develop a device that meets both market needs and requirements. Strengthen the existing industrial partnerships and establish new ones may also help understanding the market dynamics and needs, on one hand, and may also be useful to help improving the process and its scale-up, on the other hand.

To sum up, eventhough medical progress is generally expected to be a continuous process leading to improved medical treatments, chitosan-based implants should be developed under demanding criteria not only to meet current medical needs, but also to outperform the existing bioabsorbable implants, contributing for the quality growth on healthcare treatments.

"Innovation distinguishes between a leader and a follower" *Steve Jobs*

As a future Leader for Technical Industries, particularly within medical devices industries, I truly believe that innovation should always outperform the present to improve the future.

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Appendix

Interview with an orthopedic surgeon

Name: Dr João Gamelas

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Q1: Are you familiar with these internal fixation devices, specially the biodegradable ones? What is their market size in Portugal, or in Europe?

Yes, I am familiar with them and I use bioabsorbable interference screws for anterior cruciate ligament and other ligament repairs. I also know the screws and plates used for internal bone fixation. Absorbable pins could also be used in osteochondral fractures. There are also more specific screws, called Herbert screws (from Zimmer) used in foot surgeries, for instance, where extreme precision in fragment alignment is imperative.

The Portuguese and even European markets are difficult to find, but in some papers from medical journals you can find some numbers, especially related with global market, or American market. Try PubMed to find papers with that information.

Q2: Do you use them? Why? If you use them, in which situations do you use these biodegradable implants instead of other procedures (e.g. metallic implants)?

I use them mainly for ligament repairs. They are very useful especially when a second surgery, or a revision surgery, is needed. Since they are absorbable they disappear, or at least they are easier to break and remove when compared with metallic screws.

Q3: What are the main advantages and disadvantages of these biodegradable implants?

The advantages are related with their degradable properties. They make revision surgeries easier. Besides that, the tendon or ligament incorporation is better when compared with metallic screws.

Some disadvantages are related with degradation time. In some cases the implant is still in the implantation site after some years. The precision of the surgical instruments could also improve.

Q4: What are the essential features of these biodegradable implants and what could be improved?

They have to be reliable, biocompatible, biologically inert, resistant for specific surgeries/applications, among others.

Q5: Is the price a key decision feature when choosing the type of implant to use? If you decide to use a metallic implant over a biodegradable one, do you take into account the cost of a second surgery to remove it?

Price is also very important. We always have to balance very well the price of the implant with its quality, or reliability.

In the private hospitals the decision of buying one implant instead of others is commonly taken by doctors and managers, however, there are usually several health insurance companies constrains that can make the decision harder.

In the public sector I would say that doctors have more flexibility in terms of choosing the best implant for a specific patient. However, if the chosen implant is more expensive than others, we have to justify very well our decision. Usually, if a more expensive technology is chosen is because it usually requires less revision surgeries and is more durable.

Q6: Are you familiar with chitosan? And with chitosan-based medical devices (e.g. wound dressings, bone substitutes, drug delivery systems, etc.)?

I am familiar with chitosan and I have some colleagues working with it, namely Dr Nuno Ribeiro that is using some chitosan-based scaffolds in some animals' surgeries.

(Brief introduction of Altakitin; Ceramed and its medical devices; and 3D dense chitosan-based specimens)

Q7: Among the different surgeries made nowadays for different types of bone fractures and/or ligament repairs, which ones do you think can benefit from these chitosan-based absorbable implants?

It could be very interesting to have chitosan scaffolds with a specific porosity to grow cells. I have a friend called Jiri Adler working in this field. He is using scaffolds to grow chondrocytes.

Regarding absorbable implants for bone fractures, I would say that if you are able to develop kind of long pins, with some flexibility, to be used in long bones, like in humerus, or even in the femur, could be interesting. Nowadays, the existing pins are sometimes hard to implant due to their low flexibility. Stents or tubes for osteochondral fractures are another possibility.

Chitosan-based spine cages used in spinal fusion procedures might be interesting as well.

Another group of patients that could benefit from these implants are the pediatric patients. Bone fractures in children heal much faster and if we have an absorbable implant we can avoid a second surgery and they do not interfere with children's bones growth.

Q8: How many devices (screws, plates, pins, etc.) you usually require for a specific surgery? Do you know their size/measures and the materials they are made from? What are the brands that you are familiar with and their prices?

In ligament repair surgeries I usually use 3 implants with 6-10 mm. The majority of implants I know are made from a copolymer of PLLA and PGA.

There are several companies producing and selling degradable implants, namely Arthrex and Zimmer (Herbert screws).

Q9: Imagine that tomorrow some company launches chitosan-based implants in the market. Would you use them instead of the ones you are using today? Why?

It depends. You would have to show me evidences and advantages of these new products. Publications and clinical results are very important, but if you know the brand and if you know it is a reliable brand it is easier for us (doctors) to make that decision and use them.

Of course I am very happy and willing to participate in the development of new medical devices, especially in collaboration with Portuguese companies and students! I think it is great to have people thinking and developing new medical devices in Portugal. We need it!

Thank you very much for your availability.

Interview with an orthopedic surgeon professor

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Q1: Are you familiar with internal fixation devices, specially the biodegradable ones?

Yes, I know the metallic and I also know the biodegradable implants, mainly the PLA/ PLGA ones.

Q2: Do you use them? Why? If you use them, in which situations do you use these biodegradable implants instead of other procedures (e.g. metallic implants)?

I am just a Professor so I don't use them. However, I know that they are usually used in small bone fractures (e.g. hand, foot, ankle) and for ligament repair, for instance.

Q3: What are the main advantages and disadvantages of these biodegradable implants?

In my opinion the greatest advantage of the biodegradable implants over metallic implants is that there are no constraints regarding MRI (Magnetic Resonance Imaging) procedures. With these implants you don't have image distortion or potential risks related to movement of the devices. After that, I would say that their mechanical properties might be more interesting for some applications (closer to bone properties when compared with metallic implants) and in the end the absorbable feature can be a plus.

The disadvantages are associated with local inflammation due to their acidic hydrolysis (synthetic polymers) and the fact that they might degrade faster or slower than the surgeon was expecting. The degradation rate is not easy to foresee.

Q4: What are the essential features of these biodegradable implants and what could be improved?

Again, being MRI friendly is an important feature.

Mechanical properties are also important and could be improved for some applications. I think it will be important for you to test the specimens along the time (cyclic tests). Check the ISO and ASTM standards for this kind of implants. This could be improved for some applications.

Accurate degradation rate and the degradation process – for small implants there's usually no problems, however, the bigger the implant, the bigger is the local acidic concentration. We need implants with more accurate degradation rate and the degradation process shouldn't induce local inflammatory reactions.

Q5: Is the price a key decision feature when choosing the type of implant to use? If you decide to use a metallic implant over a biodegradable one, do you take into account the cost of a second surgery to remove it?

Yes price is important, however, the most important is the overall price of the procedure (surgery). It also depends on the market you approach. A surgery within the sports medicine field is different when compared to a surgery within the traumatology field. Therefore, there are a lot of stakeholders and other features (e.g. hospital managers, surgeons, orthopedic specialty, imaging requirements, insurance companies, patient, etc.) that can influence the weight of the price when choosing the implant to use.

Q6: Are you familiar with chitosan? And with chitosan-based medical devices (e.g. wound dressings, bone substitutes, drug delivery systems, etc.)?

Yes I know the polymer and I know it has been used in tissue engineering, but I don't know any medical devices with chitosan in their composition.

(Brief introduction of Altakitin; Ceramed and its medical devices; and 3D dense chitosan-based specimens)

Q7: Among the different surgeries made nowadays for different types of bone fractures and/or ligament repairs, which ones do you think can benefit from these chitosan-based absorbable implants?

I would say applications that don't require big mechanical strengths but more important than that, the kind of applications that require post-surgery MRI, like knee ACL repair.

Q8: How many devices (screws, plates, pins, etc.) are usually required for a specific surgery? Do you know their size/measures and the materials they are made from? What are the brands that you are familiar with and their prices?

I know the main companies and I know that the required number of implants and their sizes depend on each surgery/application.

Q9: Imagine that tomorrow some company launches chitosan-based implants in the

market. Would you use/recommend them instead of the ones you are using today? Why?

Let me explain you how it works. Here in the US if you want to put a medical device in the market, or an implant in this case, you have to define very well the market you want to approach and the specific application. For example, you cannot simply launch an implant for orthopedic applications. You need to say and to show that your device is good (or better than the existing ones) for a specific procedure, like knee ligament repairs in sports medicine. You need to be specific!

One of the main mistakes of small companies trying to enter the medical devices market is this one. Instead of focusing on a specific niche market and sell their products with adequate marketing strategies for those users (because an orthopedic surgeon specialized in knee surgeries within sports medicine has different needs than an orthopedic surgeon specialized in hand or foot bone fracture repairs), they try to approach the orthopedic market in general which is usually a wrong strategy. What I would recommend to you is to focus on one application within the orthopedic field and focus your strategy on that market, instead of trying to develop screws, plates and pins with different sizes and/or properties for a big number of orthopedic applications.

Answering your question, if that company were able to show me the advantages of using its product instead of others, I would say yes. However, implants made of something that is extracted from seafood shells does not seem to be very appealing from a medical point of view, so try to focus your strategy on the properties/features of your final product (e.g. MRI friendly, degradation rate, no adverse reaction, good mechanical properties, etc.) and not in the material itself.

Thank you for your availability.

Interview with a bioengineering and orthopedic surgery professor

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Brief introduction of Altakitin; Ceramed and its medical devices; and 3D dense chitosan-based products

Q1: What are the main advantages and disadvantages of the bioabsorbable fixation implants when compared with the metallic ones?

Advantages: material degrades, so there is no need to remove devices after fracture heals.

Disadvantages: lower strength when compared with metallics, but may have adequate strength for certain low load-bearing applications.

Q2: Do you know the mechanical properties of these absorbable implants? Do they match the applications needs? And what is your opinion about their local foreign-body reactions, premature material breakdown and their acidic degradation process?

Yes, the mechanical properties and degradation products of biodegradable implants like sutures and molded devices are well documented. Certain polymer like Polyglycolic acid (PGA) showed effects of acidic degradable products. But slow absorbing polymers like polylactic acid or Copolymer of PGA and trimethylene carbonate based devices do not show the effect of their degradation products on tissues.

Q3: How many devices (screws, plates, pins, etc.) are usually required for a specific surgery? Do you know their size/measures and the materials they are made from? What are the brands that you are familiar with and their prices?

It is hard to answer this question. Depending on what fracture is being fixed, the plates come with different number of screws. The majority of implants are made of stainless steel, metallic alloys of titanium and cobalt-chromium and also pure titanium are currently used for fracture fixations.

Q4: Are you familiar with chitosan? And with chitosan-based medical devices (e.g. wound dressings, bone substitutes, drug delivery systems, etc.)?

Yes, we have done some work to fix fractures using a paste of chitosan and calcium sulfate. You can check: Jerome Saltarrelli Jr, Debi P. Mukherjee. In vivo testing of of a bone graft containing chitosan, calcium sulfate and osteoblasts in a paste form in a critical size defect model in rat. 2009 *J. Biomedical Science and Engineering*, 2, 24-29 Published on line.

Q5: Among the different surgeries made nowadays for different types of bone fractures and/or ligament repairs, which ones do you think can benefit from chitosan-based absorbable implants?

Chitosan-based wound closure devices like bandages are marketed currently. No devices made from 100% chitosan polymer is approved by FDA, as I know.

Q6: Imagine that in the future some company launches chitosan-based implants in the market. Would you use (or recommend) them instead of the ones made of synthetic polymers? Why?

I would like to see its use as a coating, meshes, or delivery vehicles.

Q7: In your opinion what could be the most promising application for chitosan-based implants, among the different orthopedic applications? Why?

Chitosan-based delivery system for cells, growth factors like BMPs and other osteogenic agents for fracture repair are promising candidates for future applications.

Thank you for your availability

Interview with a biological materials expert

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Brief introduction of Altakitin; Ceramed and its medical devices; and 3D dense chitosan-based products

Q1: Are you familiar with internal fixation devices, specially the biodegradable ones?

Yes, I'm familiar with some of them. I don´t know if you already have made some *in vivo* tests, or tests with cells, but with such a crosslinked specimen you have, you have to be sure that cells will adhere after some time, in order to replace the implant with new tissue. So, if you have a screw and you want it to degrade and at the same time be replaced by new tissue, you have to be sure that cells will adhere to your implant before it loses its properties.

For instance, we had some problems with our material because of that. It was so dense that after receiving feedback from medical doctors and other medical devices companies, we understood that we needed to sacrifice some mechanical properties in order to have better biological properties. Anyway, as soon as you start with these *in vivo* experiments you will see the difference between the amorphous and crystalline specimens.

Another important thing is the degradation rate of these chitosan-based implants. However, I think it's quite easy to tune it with ultrasounds (US). Basically, if you want the implant to degrade faster than it does, you treat it with US. Since you stimulate the bonds between the chains (and you break some of them, actually) the implant will degrade faster.

Q2: Do you know the mechanical properties of these implants? Do they match the applications needs? And what about their local foreign-body reactions, premature material breakdown and their acidic degradation process?

I don't know exactly the mechanical properties of the implants, but as I said before, according to the conversations I had with people from the field, sometimes is more important to sacrifice the mechanical properties for some applications, in order to match the biological requirements. And 'the stronger the better' is not always true. It depends a lot the kind of application you want. Usually, there is an optimal properties range for each application. And again, the cells stimulation around the tissue and the implant is also very important.

Regarding the problems you mentioned that are related with synthetic polymers, they don't

exist with chitosan. If you guarantee that the chitosan you produce/use doesn´t have remaining proteins, you won´t have foreign-body reactions. Actually, there are already some companies that are pushing chitosan-based products for medical applications because of its high biocompatibility.

Q3: Do you think chitosan can be an appealing alternative to use in this kind of devices instead of the polymers that are commonly used (synthetic polymers)? Why?

Yes, definitely. It has much better properties than the synthetic ones. Chitosan is a haemostatic material and it is antibacterial for gram-negative bacteria. It also has its interesting intrinsic properties.

Q4: Since you are also working with chitosan, what do you think that are the biggest advantages of this natural polymer? And disadvantages?

Basically the best thing is that there are no immune reactions. It seems to degrade very well inside the body and it can match a big range of properties. Chitosan is a natural structural polymer so if you can match the mechanical properties of some applications, you have everything!

The disadvantages are related with its fabrication, I guess. It seems to be more difficult to fabricate it than other polymers. Plus, chitosan is not a thermoplastic polymer so it is also difficult to manipulate, or shape it for some applications.

Q5: Can you tell me more about the 'shrilk' material you developed, regarding its properties and applications? Are you developing any kind of biomedical device using this material?

Well, there are parts of the study I cannot disclose but we are approaching both medical applications and consumer products applications. Regarding medical applications, we are interested in internal surgeries related with different kinds of hernia. One of our products can be a kind of a patch to help holding some organ and keep it in its correct place.

Q6: Do you already have a chitosan-based product/material in the market? What kind of process/product features is important to evaluate when you are thinking in a new business proposition (e.g. market value, competitiveness, etc.)?

No, not yet, but we are working on that.

Concerning the main features of a medical product/device, I would say its biocompatibility is very important and also you (as a company/provider) want to take care about how your product/material works inside the body environment. That's why I was saying that *in vivo*

experiments are so important. You want to make sure your medical device won't fail!

Q7: Regarding the main goal of my project again, do you have any recommendations concerning other potential applications for these specimens? And what do you think should be our next step?

Besides the screws, plates and everything you can make for different orthopedic applications, some kind of hard meshes where cells would be able to travel back and forth could also be interesting. There are also some spine applications that could benefit from your specimens (e.g. degradable cages).

Your material/specimen looks amazing but you still have to measure a lot of properties. As a next step I would say that after measuring the properties of your specimen when it is dried, you should put it in water (swelling properties) and follow exactly the same procedure and measure the same properties after 24h, one week, or even one month, depending on the requirements of your potential application. I mean, if you need a screw to hold something for several weeks, it cannot swell and lose its properties after 24 hours of being implanted. Then, the basic *in vivo* and *in vitro* experiments are also very important to perform.

I would recommend you to find some collaboration with surgeons in Portugal that could help you finding the best application and required properties. Here we have a lot of collaboration with MD (Medical Doctors) and hospitals and it has been very useful. I'm recommending you to start doing it in Portugal (or in Europe) since it might be easier for you (company, university, student). To do it here you would need to find a collaboration with an institute, with a grant, for instance, or try to find a way to compensate their feedback (nothing is for free!).

Thank you for your availability.

Interview with a tissue engineering expert

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Brief introduction of Altakitin; Ceramed and its medical devices; and 3D dense chitosan-based products

Q1: Are you familiar with internal fixation devices, specially the biodegradable ones?

Yes. Basically the PLGA and PLLA are the most used synthetic polymers and they are FDA approved. They have been used a lot in tissue engineering.

Q2: Do you know the mechanical properties of these implants? Do they match the applications needs? And what about their local foreign-body reactions, premature material breakdown and their acidic degradation process?

These materials are stiff. They usually have the required stiffness. However, they have some issues regarding their degradation process. The problem is that there are two different stages when characterizing the implants: before implantation and after implantation. Before implantation you have the *in vitro* studies, where you do experiments outside the body, but you try to bring the environment of the body to these experiments (media). These two synthetic polymers I have mentioned presented some *in vitro* mechanical and degradation properties that were very promising but, as far as I know, their *in vivo* properties were not that good. They lost their mechanical properties faster than expected and they were not attractive materials for the cells and they also had a high immune response.

About an year ago I was working in a cervical cage project and I remember we did some compressive tests to the materials (PLGA and PLLA) and as far as I remember they should tolerate about 40 MPa for the ultimate compressive strength. These cages have a kind of rectangle shape, with a rough surface and with a hollow in the middle. Their size was about 15 x 10 mm.

Q3: Do you think chitosan can be an appealing alternative to use in this kind of devices instead of the polymers that are commonly used (synthetic polymers)? Why?

I personally have worked with chitosan for drug delivery system and it has shown that it is

really attractive for cells. However, you have to answer a lot of questions before, in order to know if chitosan is better than other materials for bioabsorbable devices. For instance, are the mechanical properties more adequate than PLGA or PLLA implants? What about chitosan's degradation process? Can you control it? How?

Another point is that chitosan is a natural material, meaning that it comes from other species. Although you are using a deactivated natural material, since you remove the proteins, the source is still other animal. Therefore, you have a lot of people that still prefer to use the synthetic polymers. Another issue is the reproducibility of your material/implant. Can you produce thousand times the same material with exactly the same properties?

If you answer these questions you will have the answer to your question!

Q4: Since you are working with synthetic polymers, what do you think that are their biggest advantages over this natural polymer? And disadvantages?

I believe synthetic polymers are easier to manipulate in order to have the desired properties. On the other hand, I believe that they are less 'adorable' for cells, meaning that cells are more willing to attach to natural polymers than to synthetic ones. That's why we use proteins to treat the surface of some synthetic polymers to make the implants more attractive to the cells.

You also have to take into account the availability of your material and the costs. The costs of the material and the fabrication process! In the end, if you have a cheaper implant with at least as good biocompatible and biomechanical properties as the synthetic polymers ones, I would say you have a lot of advantages in using chitosan-based implants.

Q5: What kind of devices are you developing with the materials you are working with and what are the main challenges you have been facing?

I'm working with a novel synthetic polymer, called PGS and as far as I know it is FDA approved. I'm working in the soft tissue engineering field and I'm working on a project to replace heart valve tissue, especially for pediatric applications. Thus, we need a material with specific elastic properties like the one we are using.

Q6: Do you already have a product/material in the market? What kind of process/product features is important to evaluate when you are thinking in a new business proposition (e.g. market value, competitiveness, etc.)?

No, this product is not yet in the market. Although the material was FDA approved, the product itself was not.

Q7: Regarding the main goal of my project again, do you have any recommendations concerning other potential applications for these specimens? And what do you think should be our next step?

You need to know how these implants would react in an environment similar to the body, so you need to start with *in vitro* experiments. You need to know the degradation process. You should put them in the media and see how the geometry of your implant changes, as well as its mechanical properties changes over time. Then, you can also see how attractive your material for cells is. After knowing these properties you can start narrowing the potential applications for your chitosan specimens.

Thank you for your availability.

Interview with a medical devices expert

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Brief introduction of Altakitin; Ceramed and its medical devices; and 3D dense chitosan-based products

Q1: Are you familiar with internal fixation devices, specially the biodegradable ones?

Yes, I am familiar with internal fixation devices, but not the biodegradable ones. To my knowledge, internal fixation devices are always selected for superior mechanical properties, which in general, biodegradable materials lack. For example, in our company (aap Implantate AG) the LOQTEQ plate system is an example of an internal fixation system composed of a titanium alloy.

Q2: Do you know the mechanical properties of these implants? Do they match the applications needs? And what about their local foreign-body reactions, premature material breakdown and their acidic degradation process?

Referring to my previous answer, I don't know biodegradable fixators. The disadvantage besides 'weak' mechanical properties would be that degradation products are a serious issue regarding immunological responses. Also, premature material breakdown might result in fracture of the fixator and possibly destabilize the treated area within the patient, something which should be avoided at all costs.

Q3: Do you think chitosan can be an appealing alternative to use in this kind of devices instead of the polymers that are commonly used (synthetic polymers)? Why?

Chitosan could be an alternative in orthopedics applications, but preferably not in a load-bearing situation. Also, the degradation (if there is any) and the cytocompatibility should be investigated well *in vivo*.

Q4: Since you are familiar with biopolymers, in your opinion what are the biggest advantages and disadvantages of natural polymers (e.g. chitosan) over the synthetic polymers (e.g. PLLA, PLGA, etc.)?

Synthetic polymers are more tunable in physicochemical properties and have been FDA approved, in the case of PLGA. Natural polymers could be useful as well, if they are degradable and have no immunological problems.

Q5: What kind of devices are you developing with the materials you are working with and what are the main challenges you have been facing?

That is classified. However, we do face problems in the regulatory field regarding CE marking and/or FDA certification. Especially difficult are the technical questions if parameters should be tested *in vitro/in vivo* or if literature references are insufficient.

Q6: Do you already have a product/material in the market? What kind of process/product features is important to evaluate when you are thinking in a new business proposition (e.g. market value, competitiveness, etc.)?

Check www.emcm.com for our products. Preferably to think of a new product or concept would be the best choice. However, creating existing products with improved efficacy/properties or cheaper to manufacture would be a suitable candidate as well.

Q7: Regarding the main goal of my project again, do you have any recommendations concerning other potential applications for these specimens? And what do you think should be our next step?

Come up with a prototype, and try to standardize the process as much as possible.

Thank you for your availability.