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Development of multifunctional hybrid scaffolds for massive bone defects filling and regeneration: critical analysis of literature and new solutions

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Development of multifunctional hybrid scaffolds for massive bone defects filling and regeneration

"When something is important enough, you do it even if the odds are not in your favour"

Elon Musk

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Resumo

Representando menos de 0,2% de todos os casos de cancro, os cancros ósseos primários são extremamente incomuns e, na maioria das vezes, curáveis, no entanto, cerca de 50% destes tumores podem metastizar e, portanto, uma intervenção precoce é frequentemente necessária.

A ressecção de tumores ou de variantes destes geralmente leva à criação de grandes defeitos ósseos que constituem um problema reconstrutivo. Os procedimentos padrões usados atualmente para resolver este e outros problemas relacionados são soluções viáveis, no entanto, ainda longe de serem ideais, constatando-se a presença de diversos riscos e de um elevado rácio de insucesso.

Com os contínuos avanços nos demais conteúdos teóricos e em fabricação (nomeadamente impressão 3D), os substitutos de *scaffolds* ("andaimes") ósseos recorrendo a suportes biodegradáveis inteligentes têm vindo a revolucionar a engenharia de tecidos ósseos e a medicina regenerativa. Para além disso, apesar das mais diversas opções atualmente disponíveis, o consenso científico é que o *scaffold* ideal deverá ter uma composição híbrida.

Esta tese pretende fornecer uma revisão geral do estado da arte atual acerca dos principais conceitos associados a *scaffolds* 3D à base de quitosano. São analisados *scaffolds* porosos produzidos por técnicas convencionais, à base de hidrogel de quitosano reticulado quimicamente. Devido à sua crescente popularidade, o agente de reticulação genipina foi selecionado como estudo de caso. Segue-se uma análise relacionada com os métodos mais recentes de fabrico de *scaffolds* reforçados por técnicas de prototipagem rápida (PLA é posteriormente examinado como material de impressão). A incorporação de agentes bioativos e células é avaliada em ambas as opções. Os diferentes fatores que influenciam as propriedades de cada material, o desempenho geral da estrutura 3D e os métodos mais comuns de preparação de superfície são também tópicos discutidos. Para finalizar, destacam-se os aspetos ainda sujeitos a aperfeiçoamento e as perspetivas futuras para a tecnologia de *scaffolds*.

Palavras-chave: Entrega de fármacos; Hidrogel; Impressão 3D; Quitosano; *Scaffold*.

Abstract

Despite the growing number of survival cases, due to the increase in cases, the death rate from cancer-related diseases has been increasing over the years. Being less than 0.2% of all cancers, primary bone cancers are extremely unusual and often curable. However, about 50% of these tumors can metastasize, so early intervention is frequently necessary.

Tumor or tumor-like resection derived from osteosarcoma usually leads to the creation of large bone defects, which constitute a reconstructive problem. The current standard procedures used to resolve this and other related issues are viable solutions. However, they are far from being ideal, still carrying many risks, and often failing.

With the ongoing advances in a variety of theoretical subjects and in manufacturing (namely 3D printing), bone graft substitutes using smart biodegradable scaffolds are revolutionizing bone tissue engineering and regenerative medicine. In addition, despite the numerous options currently available, the scientific consensus is that the ideal bone graft should likely have a hybrid composition.

The goal of this thesis is to provide an overall review of the current state-of-the-art on the main concepts associated with chitosan-based 3D scaffolds. Porous scaffolds produced by conventional fabrication techniques, using chemically cross-linked chitosan hydrogel are analysed. Due to its growing popularity, cross-linking agent genipin was selected as a case study. Follows an analysis on the most recent methods of scaffold fabrication reinforced by rapid prototyping techniques (PLA is further examined as printing material). The incorporation of bioactive agents and cells is evaluated in both options.

The different factors that influence the properties of each material, the overall performance of the 3D structures, and the most common methods of surface modification are also discussed topics. To finalize, the key aspects that still need improvement and future perspectives for scaffold technology are highlighted.

Keywords: 3D printing; Chitosan; Drug delivery; Hydrogel; Scaffold.

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Acronyms

2PP	Two-Photon Polymerization
3DP	3D Printing
ADSC	Adipose-Derived Cell
AM	Additive Manufacturing
BMP	Bone Morphogenetic Protein
BTE	Bone Tissue Engineering
CAD	Computer-Aided Design
CAM	Computer-Aided Manufacturing
CS	Chitosan
DBM	Demineralized Bone Matrix
DD	Deacetylation
EBM	Electron Beam Melting
ESWT	Extracorporeal Shock Wave Therapy
FDM	Fused Deposition Modelling
GEN	Genipin
HMWC	High Molecular Weight Chitosan
iPSC	Induced Pluripotent Stem Cell
LIPUS	Low Intensity Pulsed Ultrasound
LMWC	Low Molecular Weight Chitosan
MJP	MultiJet Printing
MSC	Mesenchymal Stem Cell
MW	Molecular Weight
PCL	Poly(Caprolactone)
PDLA	Poly(D-Lactic Acid)
PDLLA	Poly(D, L-Lactic Acid)
PEG	Poly(Ethylene Glycol)
PEMF	Pulsed Electromagnetic Fields
PGA	Poly(Glycolic Acid)
PHEMA	Poly(2-Hydroxypropyl Methacrylate)
PLA	Poly(Lactic Acid)
PLLA	Poly(L-Lactic Acid)
PVA	Poly(Vinyl Alcohol)
RIA	Reamer-Irrigator-Aspirator
RP	Rapid Prototyping

SLA	Stereolithography
SLM	Selective Laser Melting
SLS	Selective Laser Sintering

Motivation and Objectives

Cancer is a malignant tumor/neoplasm that comprises a large group of diseases. It has one of the highest mortality rates worldwide and the second highest in the United States, right after heart-related diseases. However, unlike cardiovascular deaths, the death rate from cancer-related diseases has been increasing. This is due to an increase in occurrences derived from different factors including rapid growth and aging of the population (mostly in undeveloped countries where a large part of the world population resides), unhealthy lifestyles adopted by each individual (smoking, unhealthy or unbalanced diet, lack of exercise) and rising patient awareness [1]. Being less than 0.2% of all cancers (approximately 2900 cases/year in the United States), primary bone cancers are extremely unusual and often curable if found in early stages [2].

Despite bone cancer having a low incidence compared to tumors that affect vital organs (lung, chest, prostate, kidney, and most of the soft tissues), about 50% of these tumors have the ability to metastasize (secondary bone cancer). In this case, there's a spread of cancer cells from a primary site to a secondary site, such as bone, giving rise to problems that include infection, pain, anemia, small or massive fractures, and mobility problems [3]. Secondary bone cancer cannot be cured, but treatment can reduce symptoms and improve life quality. In some cases, such treatments can keep secondary bone cancer under control for many years [4,5]. Because of this, cancer surgery is most effective if implemented at an early stage, when the tumor is still localized.

The three most common types of bone cancer manifestations are osteosarcomas (35-36%), chondrosarcomas (20-30%), and Ewing sarcomas (16%) [3,4]. Their origin is mainly unknown (especially the Ewing sarcoma cases) and needs further investigation. Nevertheless, some of the known causes associated with the appearance of an undesirable condition include radiation treatment, chemical and genetic agents, viruses, and Paget's disease [6].

Normally, there is no need for medical intervention in the event of injuries or during bone development or continuous remodeling over an individual's lifetime. Bone tissue has the intrinsic ability to self-regenerate, recovering, in most cases, the pre-existing properties without scar formation [7]. However, in the case of one/multiple large or complex bone defects, possibly caused by surgery (malignant tumor resection), the regenerative process fails, leaving behind a wide range of complications that could affect one's lifestyle. To counter this and recover functionalization, the gap/gaps should be filled/regenerated in the shortest possible time. Traditional therapeutic approaches currently applied in the early phases, such as low-intensity pulsed ultrasound (LIPUS), extracorporeal shock wave therapy (ESWT), and pulsed electromagnetic fields (PEMF) often reveal ineffective, time-intensive often requiring more than just one intervention and ultimately unable to fulfill the task [4,8,9]. Furthermore, studies suggest that up to 10% of all bone fractures are related to impaired healing (taking place before or after possible initial treatments), giving rise to delayed union or complete non-union [10]. Some of the current limitations imposed by these procedures include a maximum of 10 mm of fracture size that should have good mechanical stability of the defect fixation and no indication of infection [7]. Because of all these limiting factors, in most cases, a different approach is required.

Bone grafts have been in use for many years, being, in fact, the current standard procedure in large fracture healing cases and the closest available option for what is considered "ideal". In recent years, due to the growing demand for more and better products, their research has undergone an exponential increase. With more than two million procedures worldwide, bone grafting is the second most common tissue transplantation, right after blood transfusion [11,12]. A bone graft is usually used in orthopedics, dentistry, and neurosurgery to enhance, repair, or replace bone tissue. More specifically, bone grafts are used when there is an inability of the bone tissue to regenerate autonomously, when the situation is too complex for traditional treatments, or after spinal fusions, removal of bone tumors, and congenital and degenerative diseases [12,13]. Despite the high number of successful cases, like any other intervention, bone grafts carry risks and can fail. These risks can be more accentuated according to the existence of

factors such as surgical errors, osteoporosis, smoking, periodontal disease, immune system flaws, and systemic conditions [14].

Taking into consideration the previous information, other options are currently being explored. In this work, the current concepts, materials, and methods related to the production of bone graft substitutes (particularly scaffolds) for massive bone defects filling and regeneration (necessary after osteosarcoma) are reviewed. This monograph intent is, however, to dwell into this review, mainly considering two main chitosan-based systems:

- Porous scaffolds produced via traditional fabrication techniques, using chemically cross-linked hydrogel (due to its growing popularity, cross-linking agent genipin was selected as a case study);
- Scaffold fabrication of chitosan-based hydrogels combined with rapid prototyping, with a focus on FDM printing (being by far the most used material in FDM, PLA was chosen for further analysis).

Six major parcels compose this work. The first one comprises the theoretical introduction corresponding to the current advances in the area, as well as the challenges that come with them and several other relevant aspects related to bone tissue engineering. The deacetylation process (and many of the related parameters) and functionalization of chitosan follows. In this research, the main conventional scaffold fabrication techniques and the implications that come with them are based on genipin. Therefore, before this matter, genipin-chitosan interaction is mentioned, where key concepts such as pH, temperature and concentration influence on the gelation process are discussed. After this, several rapid prototyping techniques are presented. Here, PLA and surface modification in FDM are considered topics. The ability of each system to incorporate bioactive agents or cells is also evaluated. To finalize, the key aspects that still need improvement and future perspectives for scaffold technology are highlighted.

1 Introduction

1.1 Massive bone defects and bone grafts

Bone is a highly and dynamic vascularized tissue with an intrinsic self-regenerating capacity, so in most cases, extensive medical intervention is not necessary, and the tissue can regain most of its previous properties autonomously (largely due to the presence and activity of bone cells such as osteoclasts and osteoblasts). However, in the event of a severe and large bone defect caused by the most common tumor, osteosarcoma, the body is unable to perform a proper and effective repair on its own. Therefore, in most cases, medical involvement and treatment are necessary [6,15,16].

Currently, there are some viable options for the treatment of different types of massive bone defects by using bone grafts or bone substitutes that are well tolerated, resist infection, and are rapidly vascularized. Nevertheless, these alternatives carry some disadvantages. An ideal bone graft should meet several characteristics, which include the presence of osteoinduction, osteoconduction, osteogenesis, osteointegration, and full vascularization [17,18]. Osteoinductivity is related to a graft that, resorting to growth factors such as bone morphogenetic proteins (BMP's), is capable of triggering differentiation of cells in the local or surrounding tissue to form osteoblastic phenotypes capable of forming bone tissue [19]. Osteoconductivity refers to the ability of the graft to allow cell-infiltration and to the support in the development of new bony tissue (by attachment of new osteoblasts and osteoprogenitor cells) on its surface [19,20]. Osteogenenicity is induced by osteoinductivity and refers to the process of natural bone growth and bone repair that should occur between the graft and the preexisting bone when in a proper environment [18,21]. Osteointegration or osseointegration describes the process and ability of a graft to bond to the bone surface without intervening soft tissue and in such a way that it cannot be detached without fracture [22]. Obtaining adequate vascular network formation is challenging but crucial since bone growth can only take place if a proper blood supply is present. Blood vessels are responsible for the supply of nutrients, cells, oxygen, growth factors, and elimination of waste products and toxins [19]. In addition to the previous requirements, such graft should exhibit biocompatibility, bioactivity, controlled kinetic biodegradation, non-immunogenicity, interconnectivity (between the pores and the oxygen for cells to "breathe"), and mechanical properties (strength) [23,24]. Other factors, such as geometry and size, also contribute to the selection of an appropriate bone graft [8]. Four different types of bone grafts exist depending on their sources:

- Autografts: bone tissue is transplanted from one site to another in the same patient;
- Allografts: bone tissue is transplanted between two genetically non-identical bodies of the same species, often from a cadaver;
- Xenografts: bone tissue is transplanted between two different species;
- Isografts: a subdivision of allografts in which bone tissue is transferred between two genetically identical specimens [25].

Only the autografts satisfy most of the characteristics previously referred [8,17,26]. These types of bone grafts minimize the transmission of infections and the probability of immunoreactions, so, at first glance, these materials are optimal. However, because of donor-site morbidity in roughly 20% of all cases, complications from further medical treatment and limited supply associated with the high volume of material needed, these grafts are increasingly being left behind [19]. An autograft is commonly harvested from the anterior and posterior iliac crests (the reason being the vast cancellous structure and cell volume) and more recently from the intramedullary femoral canal using the Reamer-Irrigator-Aspirator (RIA) system [16,27]. However, most of these procedures give rise to secondary problems such as pelvic fractures, deformity, scarring, non-unions, infections, inflammations, bleeding, and continual pain [8,25,26,28]. Nevertheless, they are still considered the "gold standard" grafts for large bone defects [8,16,25].

Allografts are the second most used bone-grafting approach and are very useful when autograft bone is inadequate. In addition to the previous disadvantages, in allografts, osteogenesis is low or not present, there is increased immunogenicity, no cellular component because of the devitalization process by freeze-drying or irradiation (to minimize the immune response) and they are mostly put aside because of disease transmission and limited osteoinductive properties [20,26,28]. Allografts are mainly used in the elderly population since it is the age group with the least quality supply of autografts [20]. The main advantage of using this type of allograft is the immediate availability of a graft with personalized size and shape [29]. Besides this, they can be acquired in a wide range of forms, such as fresh, demineralized, de-lipidized, sterilized, fresh-frozen, and freeze-dried [30]. Despite not ideal in their pure form, since allograft procedure only requires one surgical incision, plenty of studies dwell in the search for an allograft-based material that could compete with the current widely accepted autografts. One of the most common variations of allografts that have been demonstrated as a successful bone substitute with osteoinductive and osteoconductive (when combined with an adjunctive scaffold) properties is demineralized bone matrices (DBM's) [28]. Despite the identical properties to allografts, the conditions for this type of allograft to be used are very restrictive and complex. Most of the literature shows good results *in vitro* but a lack of results *in vivo*, so, until changes are made, the human clinical use of these grafts will remain unproven and impractical [31,32]. Along with DBM's, other preparations include cortical and corticocancellous grafts, osteochondral and whole-bone segments, and morselized and cancellous chips [16]. Isografts, a variation of allografts, have the highest probability of success since the rejection of the tissue should never occur. However, the conditions for this procedure to happen are very limited since it can only occur with identical (monozygotic) twins [33].

Xenografts, unlike allografts, are available on a large scale, have high porosity, which could promote tissue healing and good mechanical properties [8]. They are mainly harvested from porcine, bovine, or coral species, and because of that, zoonotic diseases, rejection of the transplanted tissue, non-compatibility, and loss of osteoconductive and osteoinductive properties are common problems. All these aspects converge in a graft that needs lifelong monitoring after surgical intervention with a low success rate and ethical and religious concerns attached [8,34].

1.2 Bone graft substitutes

An autograft is associated with extensive complications and expenditures, and so, other materials might be a viable alternative [35]. Bone tissue engineering (BTE) is a newly developing field that combines engineering, physics, biology, and material methods to repair, replace, maintain, or improve the function of a specific tissue [36]. BTE has been increasingly demonstrating great potential to overcome the drawbacks of conventional bone grafts by taking advantage of synthetically fabricated or naturally derived biomaterials that promote differentiation and proliferation of bone cells, generating new functional tissues instead of implanting non-living scaffolds [16,28,34]. Through this medicine branch, new approaches are being studied so that issues derived from conditions, such as osteosarcoma, can be overcome [37]. The classic BTE term is directly related to several different aspects that must be met, such as, a biocompatible scaffold that closely resembles the natural bone extracellular matrix and a sufficient vascularization to match the growing tissue [26]. Despite the advances made, many difficulties remain, and so, new and more viable options should be found and studied to satisfy the main requirements of BTE [24].

The main bone graft substitutes can be placed in one of four major classification groups: allograft-based, factor-based, cell-based, and natural or synthetic-based (ceramic, polymeric, metallic, and composites) [19]. In addition to these, depending on the situation, procedures/techniques such as megaprosthesis, masquelet induced membrane, ilizarov method, in-patient bioreactor, and 3D printing can also be applied [8].

As previously mentioned, one of the major difficulties linked to the success of bone grafts is the absence of processes such as osteoinduction and osteogenesis. To work around this problem, bone graft

substitutes (such as scaffolds) should have a 3D matrix, spatiotemporally controlled growth factors (and other bioactive molecules) that lead to bone regeneration in the contact zone between native bone and graft material, and structural support for cell colonization [31,38,39]. Regarding the scaffold composition, a set of characteristics must be present. Biodegradability is one of them; the scaffold should degrade over a certain period and at the same rate as tissue growth rate to maintain stable properties between the tissue and the scaffold. If this does not occur, then this arrangement works more as a permanent implant than a temporary implant where there is no replacement of the scaffold by the natural extracellular matrix [39,40]. Besides this, such design should have similar properties to the ones found in the bone tissue (to avoid stress shielding), demonstrate exceptional biocompatibility (migration, adhesion, proliferation, and differentiation of bone cells, as well as nontoxicity), minimal immunogenicity, good mechanical properties (weight-bearing and support for cell growth), intended geometry (porosity and pore sizes are key parameters to control cell infiltration and attachment) and ability to induce angiogenesis [39,41].

Biomolecules are organic molecules that comprise proteins, lipids, carbohydrates, and nucleic acids. Specifically, growth factors are a major key in bone repair and regeneration. These are mostly a subdivision of cytokines that stimulate the intracellular domain and, depending on the stimulus that they invoke, can have an inflammatory, proliferative, migratory, osteogenic, osteoinductive, or angiogenic influence [19,42,43]. Since these factors can be isolated and synthesized, an extensive range of possibilities is increasingly being opened, mainly in applications that envision incorporation with bone matrix (scaffold) for controlled drug release [19]. BMPs are the growth factors with the most effective osteoinductive effect [44]. Not devaluing the extensive advantages of these factors, some disadvantages must be considered. The risk of contamination, protein instability, low solubility, immunogenicity, and high manufacturing costs are some of them [45].

A continuing growing manner to improve and increase the applications of bone grafts substitutes centers on the use of stem cells and their regenerative value, which opens opportunities for bone grafts to be applied in specific relevant clinical cases. These stem cells include mesenchymal stem cells (MSCs) where pre-differentiation is not required and adipose-derived stem cells (ADSCs), embryonic stem cells (ESCs) and, more recently, induced pluripotent stem cells (iPSCs), these last three with poor differentiation potential and predifferentiation necessary [46]. These sources, if placed into the proper environment, can be converted, for example, into osteoblasts (bone), fibroblasts (skin, connective tissue), adipocytes (fat tissue), chondroblasts (cartilage), and myocytes (muscle). Nevertheless, many questions remain to be answered regarding how these cells intervene in bone formation [19,43,47].

Despite the use, over many years, of synthetic or natural biomaterials such as ceramics, polymers, metals, and composites, mostly for replacement purposes, only in more recent years has the regenerative goal been introduced. Ceramics, mainly calcium phosphates, have been extensively studied since hydroxyapatite makes up around 70% of the bone tissue (by weight), with collagen making the other 30% [39,48]. These materials allow the incorporation of biologically active ions or biomolecules in their structure. However, even though being the material that most closely resembles bone tissue, they are also the most susceptible to fracture, which, combined with their low versatility and customization options, makes them an unreliable alternative, especially when solo used [39]. The bioceramic glass variations are also a possibility to consider, but because of their stiffness and brittleness, they are normally also put aside. Polymers offer a wider and more controllable range of customization options for the desired scaffold, including porosity, degradation time, strength, and microstructure [49]. They can be from a natural or synthetic origin and typically include collagen, chitosan (CS), and the poly(α-hydroxy acids) based polymers such as poly(lactic acid) (PLA) [50]. Because of their close similarity to bone tissue, natural polymers are considered to have the greatest potential in tissue engineering. On the other hand, synthetic materials present the best control over the chemical, physical and mechanical properties of customization previously referred [49]. Another important strand to be considered are hydrogels, which are natural and/or synthetic polymers that form 3D networks chemically or physically cross-linked and can absorb water [51]. Since biodegradability is usually not present, metallic implants

tend to fall into the classification of permanent implants [39]. On top of this, metallic scaffolds may weaken osseointegration over the long-run, trigger infections, osteolysis, and implant wear [19].

Despite the numerous options already studied for bone tissue engineering, the most accepted conclusion is that the use of only one type of material will not match all the required specifications of an ideal scaffold. Therefore, composite biomaterials are increasingly becoming the target in bone graft substitutes research, where the advantages of each different material are combined [52]. These grafts combine the structural advantages of the scaffold with the biological elements to trigger cell proliferation, differentiation, and osteogenesis [12]. One of the most promising areas of research focuses on ceramic-polymer composites, where the toughness and compressive strength of polymers meets the mechanical integrity and bioactivity of the calcium phosphates. However, since polymers, in general, degrade faster than ceramics, it is important to find an approach that guarantees uniform resorption of the scaffold [53].

Another alternative is the combination of chitosan, which is a natural polymer with poor mechanical properties but vast antibacterial properties, with a polymeric material, such as PLA, which despite synthetic, is also a degradable polymer with great mechanical properties. Innumerous studies find that the combination of these and other materials greatly improves the overall performance of a scaffold compared to their solo use [54]. New methods are currently being explored in order to develop these systems. One of the most promising ones is rapid prototyping (RP). With this technique, the fabrication of well-defined and reproducible personalized scaffolds (based on the patient tissue defects) becomes feasible, mainly via synthetic polymers, and can further be combined with other components, such as ceramics, biomolecules, and specific cells [8]. The literature also shows that the cell delivery alone results in issues such as poor cell retention, low cell integration at the site of delivery and cell death. However, when combined with a biomaterial carrier, cell viability and differentiation are enhanced [55].

At the moment, the exceptional biological and mechanical properties of the natural bone are still to be mimicked. The field of regenerative medicine is still in its early stages of development and until the current limitations fade away and the ideal bone substitutes start to emerge, there is still plenty of research that needs to be made, whether on new alternatives or in the development and optimization of the ones that already exist.

2 Chitosan-based hydrogels

Chitosan has been formulated in the form of microspheres, nanofibers, porous scaffolds, and hydrogels [56]. Hydrogels are cross-linked 3D polymeric networks that, because of the existence of hydrophilic functional groups, are known for their unique ability to retain more than 10% of their total weight or volume in water [57]. Due to their similarity to the organic tissue, they are currently used in a wide range of biomedical and bio-engineering applications such as tissue engineering, wound dressings, and drug delivery [58]. A robust gel containing 0.1-10% polymer by weight will exhibit a highly porous structure with ideal properties for a constant and continuous release and diffusion of nutrients, biomolecules, and oxygen over a certain period (a couple of days up to several months), while still providing a suitable environment for cell infiltration and interconnectivity with low mechanical irritation on the surrounding tissue [59,60]. Because of this, the existing disadvantages associated with hydrogels are mainly belittled, and solutions are studied. Taking into account the hydrophilic nature of chitosan, the first main barrier is the fact that most drugs used in the field are hydrophobic. Also, the mechanical properties (namely tensile strength) of hydrogels are not high enough compared to the values expected in BTE, occasionally causing a premature release of the drug components before reaching the destination [59]. The most common natural polymer types are proteins and polysaccharides, such as collagen, elastin, fibrin, silk, gelatin, alginate, hyaluronic acid, and chitosan. Equivalently, the most used synthetic polymer types are poly(2-hydroxypropyl methacrylate) (PHEMA), poly(vinyl alcohol) (PVA), poly(ethylene glycol) (PEG), poly(caprolactone) (PCL), poly(glycolic acid) (PGA) and PLA [60,61].

Being biocompatible, biodegradable, stable, antibacterial, non-allergenic, and non-toxic, CS is one of the biopolymers that has been attracting the most interest in the biomedical and biotechnological fields. Its unique properties of high surface area, tensile strength, conductivity, and porosity are also of great relevance [62–65]. CS can be obtained from chitin, which, after cellulose, is the most abundant natural polymer. However, because of the highly restricted solubilization conditions (in water and organic solvents), the interest in this material for bio-applications is mainly lost [66]. Chitin can mostly be obtained from waste carapaces of crustacean (crab and shrimp), but also insects, fungus, mollusks, green algae, and yeast [67,68].

2.1 Deacetylation process and parameter optimization

By removing the acetyl groups in chitin (by chemical hydrolysis or enzymatic methods), during deacetylation, CS can be obtained (CS is chitin with at least a 50% deacetylation degree) [69]. The exposure of amine functional groups (NH_2) allows CS to interact with drugs, polymers, cells, and nanoparticles, becoming a convenient link in pharmaceutical applications [70]. Typically, the degree of deacetylation (DD) of CS can be classified as low (55%-70% degree), medium (70%-85%), high (85%-95%), and ultra-high (95%-100%). The increase in DD percentage translates into a slower degradation rate, conferring CS the ability to last months *in vivo* and allowing plenty of time for tissue reparation and regeneration [67,71,72]. DD of commercial CS usually ranges from 70-95%, which is more than enough for most applications [64]. Je *et al.* compared 50% and 90% deacetylated chitosan's, reporting an overall improvement in functional properties with the 90% deacetylated version [73]. To measure the DD, plenty of methods are available, including UV-spectrophotometry, infrared and near-infrared spectroscopy, enzymatic determination, and others, from which nuclear magnetic resonance is by far the most widely used [74,75].

The temperature, molecular weight (MW), pH, pressure, exposure duration and concentration to/of the basic solution, distribution of acetyl groups, industrial source of chitin (purity of the raw material), and the use of solvents (ethanol, amyl alcohol, n-butyl alcohol, and others) are all parameters that need to be accounted since the variation of each one provides different physical and chemical properties to CS [65,66,71,76]. Knowing that the glycosidic bonds on chitin are easily affected by acidic hydrolysis

(which can cause unwanted chain termination), the chemical hydrolysis process that enables deacetylation is usually base-catalyzed (Figure 2.1) [77,78].

Sodium hydroxide (NaOH) is frequently used as an alkali agent. Since the dissolution of NaOH is an exothermic reaction and deacetylation an endothermic reaction, NaOH works as a catalyst, promoting heat release and, in consequence, increasing the temperature of the solution and promoting the formation of higher DD CS. The released heat alone is not sufficient for deacetylation to take place. However, it improves the efficiency of the reaction and reduces energy intake [65,79]. An increase in DD means that more amine (NH_2) groups are exposed, and more intra- and intermolecular hydrogen bonds can occur. Additionally, chains lose acetyl groups and become more compact, which translates into higher crystallinity and better flexibility. This improves CS stability and leads to a decrease in solubility and hydrophilicity, reducing the polymer degradation rate (less swelling and enzyme penetration) [67,79–84]. The DD increase is usually correlated to the loss of MW, yet numerous studies report that this connection is not significant to the point of conditioning the degradation of the polymer [80,84–86]. This means that, contrary to DD, the higher the temperature and pressure of the reaction, the lower the MW [87]. Regardless of DD, an increase in MW, just like with deacetylation, increases the crystalline profile and viscosity, improves the stability, and decreases the solubility (solvent has more difficulty surrounding the molecules), reducing the degradation rate [87]. As the reaction develops, the chains break, and the MW decreases, reverting to the previous events. MW can be classified as low MW chitosan (LMWC) from 5-10 kDa (average) or as high MW chitosan (HMWC) from 10 kDa and up [72,88]. Despite the low solubility and toxicity associated with HMWC, when linked to a high DD, this CS version delivers small and stable complexes and enhanced delivery efficiency [89]. On the other hand, LMWC is water-soluble and is linked to antimicrobial, antitumor, and antifungal activity [90]. In concept, the ideal MW should be one that shares the best of both worlds, meaning that it should have an intermediate value, avoiding the high viscosity from HMWC (preventing the incorporation and retention into a matrix) and the low viscosity derived from LHWC (which leads to leakage).

All the previously stated correlations are well established. However, in practice, they are not always true since more factors need to be considered [71,85]. For example, Tomihata *et al.* and Ahmad *et al.* described an increase in hydrophilicity when DD values were brought to around 50% (increase in swelling behaviour and decrease in mechanical strength compared to DD < 50%), and a decrease when DD values were higher (decrease in swelling behaviour and increase in mechanical strength compared to DD ≈ 50%) [80,91]. When DD is low, the hydrophilic nature of the amine groups dominates, and water uptake increases. However, with the increase in DD to the intended value (one that ensures the desirable amount of amine groups), the hydrogen bonds start to overshadow the previous situation, and stability overtakes. The study conducted by Freier *et al.* found that for 50% DD, degradation was maximum (as so was solubility), decreasing as it approached the 0% and 100% DD values, indicating that more variables must be taken into consideration in the hydration process (no linear graph, meaning more than 2 variables) [92]. Another study reported no distinct relation between DD and hydrophilicity [67]. Nguyen *et al.* reported that DD and MW do not have an impact on the degradation rate for temperatures below 28°C [84]. Despite being closely related to better properties in the field, a higher DD or MW is not always the better option. As Tangsadthakun *et al.* also showed, lower MW CS promoted and stimulated cell proliferation in connective tissue fibroblasts more efficiently than its higher version [93]. With so many factors, it is easy to recognize that small variations in each of them are the reason for such results [67,80].

The presence of the amine (NH_2) and hydroxyl (OH) functional groups in CS makes it possible for further chemical and physical modifications, with a direct impact on many activities, such as antibacterial, immune-related, and lipid-lowering [79,94]. To explore these features, and mainly because of the crystalline structure limiting its applications, CS must be dissolved in an aqueous solution, specifically, an acidic one with pH inferior to 6-6.3. Acetic and hydrochloric acids are commonly used, but formic, oxalic, and lactic acids can also be convenient [69,71,94–96]. Higher acid concentration and lower pH generally translates into higher protonation of the amine groups on the C-2 position, forming cationic amine groups (NH_3^+) and increasing the dissolution [72]. After this step, a viscous solution takes

form, giving CS the ability to function as a suspending agent, stabilizer, or thickener [87,97]. In addition, solubility, swelling, durability, porosity, mechanical strength, and chemical stability (including long-term stability) of CS can all be subject to further manipulation [86,98].

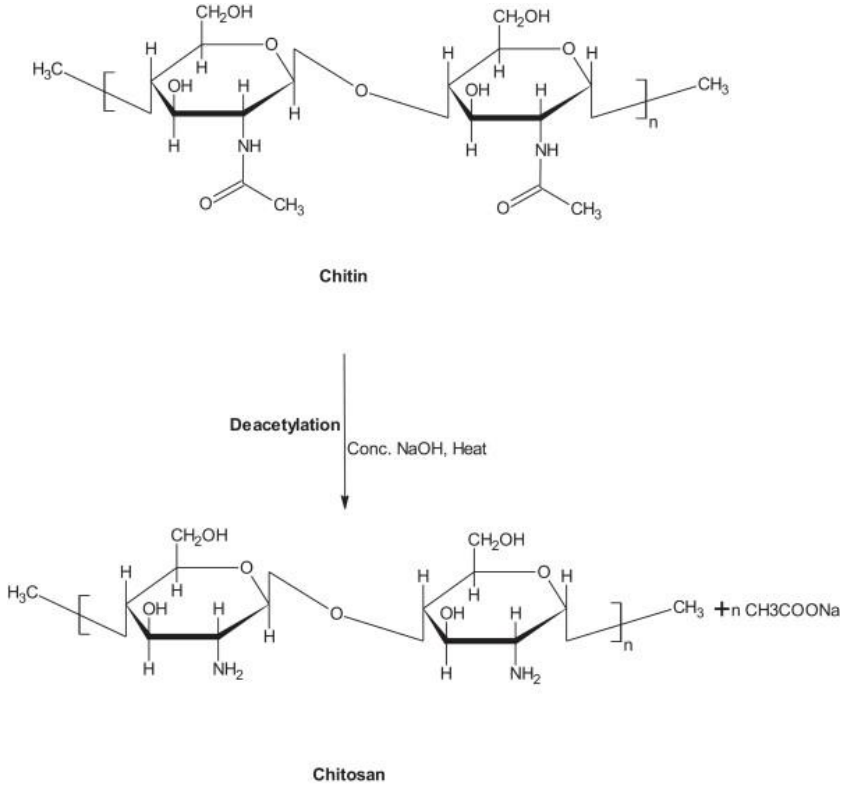

Figure 2.1 – Deacetylation process of chitin to chitosan [63].

2.2 Methods of preparation of chitosan-based hydrogels

Chitosan is not frequently used in drug delivery systems due to its hydrophilic nature, low chemical stability and mechanical strength, uncontrollable physiological degradability, and prolonged gelation time. This implies that CS must be cross-linked, which will improve its sorption properties (smaller particles and a larger number of linkages) [99,100]. Some of the most relevant derivatives include carboxyalkyl and hydroxyalkyl CS, quaternized CS, thiolated CS, sugar-nearing CS, cyclodextrin-linked CS, and acid-modified CS, each one translating into a different and specific change in native CS properties [101,102].

The presence of reactive functional groups (amine and hydroxyl) in chitosan's backbone and its derivatives allow its chemical and/or physical modification/cross-linking. By taking these procedures, water can penetrate the material structure without interfering with the strong interactions responsible for maintaining the packed configuration, making cross-linked CS swell without solubilizing when immersed in aqueous solutions [65,103].

Chemical cross-linking can be done mainly through cross-linkers or photopolymerization. In the first method, chemical agents with two or more functional groups create irreversible intermolecular covalent bonds between chains to produce a hydrophilic 3D network with improved solubility and mucoadhesion [64,103]. Adding to the primary bonding (covalent interactions), which increases with cross-link density, hydrogen and hydrophobic interactions also need to be considered [104,105]. Agents/cross-linkers such as glutaraldehyde, formaldehyde, vanillin, genipin, and others are mainly used and are typically more stable and stronger than the physical ones, exhibiting resistance to environment fluctuations [98,106,107]. Some cross-linkers have some drawbacks for biomedical

applications. For example, glutaraldehyde is known to be related to cytotoxicity and neurotoxicity in certain concentrations, thus requiring a purification step to eliminate the toxic unreacted cross-linkers. However, even if purified, some studies report the prevalence of toxicity. Therefore, less toxic cross-linkers such as GEN are an attractive alternative [95,104,107]. Photopolymerization also works with covalent bonding, but instead of cross-linkers, it utilizes photoinitiators and visible or UV irradiation to trigger radical polymerization and achieve cross-linking [107,108]. This method can be useful not only for drug delivery but also in tissue engineering for scaffold preparation with multiple geometries and rapid entrapment of cells. Another advantage of using chitosan-based photopolymerization is the possibility for solubilization at neutral pH and in-situ hydrogel formation, opening possibilities for many applications [107,109].

Physical modification is mainly associated with reversible ionic cross-linking or with polyelectrolyte complexes. The types of interactions include van der Walls forces, ionic interactions, stereo complex formation, hydrogen bonding, and others, all in general, less stable than chemical reactions [108,110]. Because CS is a positively charged polyelectrolyte, ionic cross-linking takes advantage of anions and uses them as cross-linkers to produce nanoparticles. These ions include calcium chloride and sodium tripolyphosphate. They are based on ionic interactions between the chains and have a clear preparation with high biocompatibility. However, being mechanically weaker and with less stable networks, they can give rise to materials with low durability, which are more exposed to environmental conditions than covalently cross-linked modifications (can cause instability) [98,108,111–113]. Polyelectrolyte complexes are composed of two opposite charged polymeric solutions (the electrolyte and a larger molecule) that form spontaneous ionic interactions when mixed [108,114]. Similar to ionic cross-linking, polyelectrolyte complexes resort to reversible covalent cross-linking and are sensitive to the environment (temperature, pH, ionic strength, and others) [108]. The most commonly used negative charged natural polymers include alginate, heparin, pectin, dextran, chondroitin sulfate, and hyaluronic acid. Synthetic polymers, such as polylactic, polyphosphoric, and polyacrylic acids, can also be used [101,104,107,115].

Another modification route is self-assembly, which uses specific moieties to hydrophobically graft CS [114]. These moieties are biocompatible groups or molecules with hydrophobic nature, making them the perfect candidate to encapsulate poorly water-soluble drugs (or be the drug itself) and associate them with CS [64,116]. Their combination gives rise to amphiphilic copolymers that tend to form micelles or nanoparticles when dispersed in water. These core-shell structures constitute a potential drug delivery system, providing the core-attached compound with protection from external conditions and controlled release. The moieties are usually molecules of cholesterol, lactose, acyl, alkyl, steroids, fatty acids, azide and cholic, 5β-cholanic, and deoxycholic acids [64,107,114,117,118]. Typically, self-assembly derivatives are based on chemical modifications; however, by adding a neutralization step, physical CS thermogels, often used for injectable formulations, can also be obtained [116,119].

3 Hydrogel scaffolds

3.1 Genipin as cross-linking agent – case study

Genipin (GEN) is a bifunctional aglycone and organic cross-linking agent used in combination with collagen, proteins, gelatin, and chitosan, which is soluble in water, ethanol, and methanol [62,110,120,121]. It is a white crystalline powder derived from geniposide, which in turn can be isolated from *Gardenia jasminoides Ellis* and *Genipina americana* fruit extract. The process (Figure 3.1) is based on enzymatic hydrolyzation (through β-glucosidase), produced by microbiological components, such as intestinal bacteria (oriental traditional method) or *Penicillium nigricans* (mass production method) [58,62,120,122].

Evidenced by the increase in published works, GEN biochemical interest in regenerative medicine has been increasingly growing and is now the most attractive non-toxic cross-linker [120]. In addition to the biochemical significance, biocompatibility, stability, and well-defined chemistry, GEN is 5000 to 10000 times less cytotoxic in comparison to the most popular cross-linkers, glutaraldehyde and

formaldehyde [58,62]. Besides this, GEN products also exhibit increased stability and mechanical strength and a slower degradation rate, which directly influences the drug release period [95,107,123]. Nevertheless, due to its lower cross-linking reactivity, this chemical compound is commonly left out, a situation that is progressively changing as new scientific papers work to find the ideal processing parameters for improvement of the gelation times [124]. This is an important aspect to take into consideration for *in vivo* applications since slow gelation can cause the hydrogel to leak (loss of drug delivery components). In contrast, an excessively short gelation period can lead to incompatibility problems between the tissue and the hydrogel [125]. Due to the previously stated advantages, GEN is a great candidate for cross-linking processes. Furthermore, it reacts with materials with primary amine groups. Genipin-chitosan hydrogel applications include wound dressing, encapsulation of biological cells and other products, carriers for controlled drug delivery, and fabrication of synthetic tissues and cartilage substitutes [58,62,120].

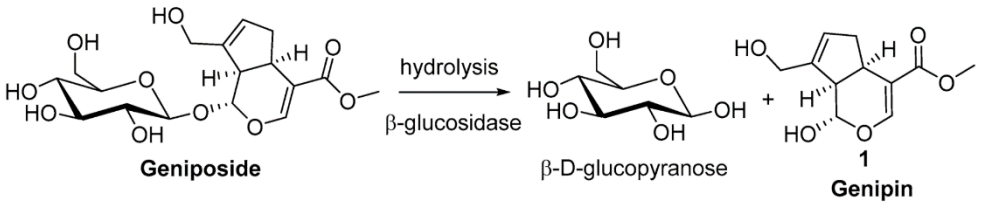

Figure 3.1 – Transformation scheme of geniposide into genipin [126].

Genipin application as an agent has been studied continuously for over two decades, but the reaction mechanism with CS is still not fully understood and needs further investigation [58,127,128]. Nevertheless, the genipin-chitosan reaction can be described through two cross-linking events [58,120,129,130]. The first and faster one [Figure 3.2 (1)] occurs under neutral or mildly acidic conditions and is based on the ring-opening reaction of GEN. It starts with a nucleophilic attack on the C-3 genipin carbon atom by a primary amine group associated with a CS molecule. This action culminates in dihydropyran ring-opening and replacement of the oxygen atom in the ring by a tertiary amine, forming a heterocyclic compound. Short chains of cross-linking bridges are formed, which can also occur in neutral conditions. The second and slower reaction [Figure 3.2 (2)] involves the replacement of the ester group of GEN (releasing methane as a by-product) by a secondary amide linkage, associated with a second CS molecule (via S_N2 nucleophilic substitution; involves the release of a methanol group) [58,62,97,120,126,127,131,132]. The completion of both events means that a bifunctional cross-link has formed between the two CS molecules. Despite being the most accepted mechanism, several scientific studies have investigated the subject, having obtained results that, although similar, add new details and alternative reactions to the previous explanation [126,127,131].

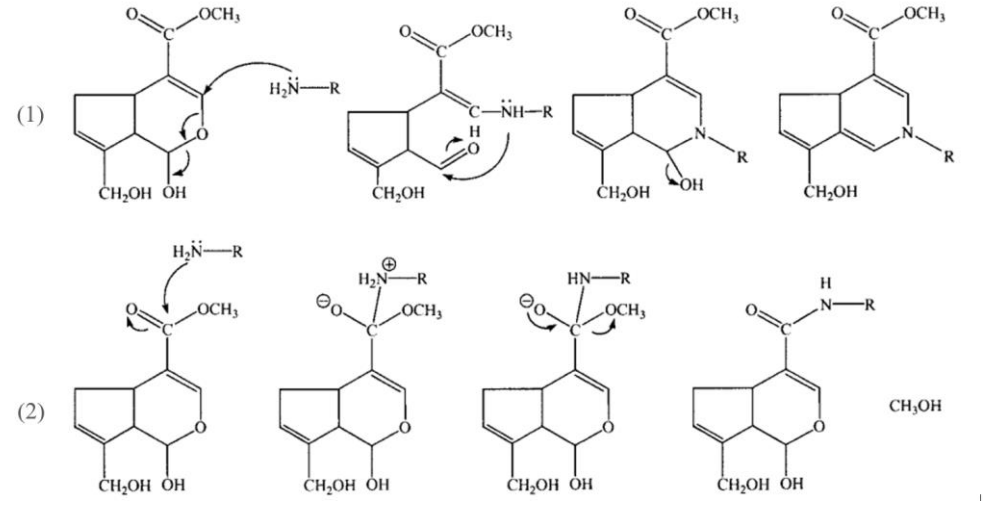

Figure 3.2 – Genipin-chitosan cross-linking reaction schemes (1) and (2). Adapted from Butler *et al*. [97].

3.1.1 pH influence on the gelation process

Due to the ring-opening polymerization and being pH-dependent, the cross-linking degree and mechanism pathway will be greatly affected by pH variations, having a direct effect on the preparation and utilization of chitosan-based gels in a vast range of applications [58,133]. For example, it is important to understand the influence of pH in cross-link density since an increase in this parameter means stiffer samples and an improvement in cell bioadhesion and viability on the hydrogel surface [134].

Under basic conditions, the replacement of the oxygen atom and the ring-opening reaction is given through a nucleophilic attack by hydroxyl ions in an aqueous solution. If the base solution is strong enough (pH>10), this could lead to GEN self-polymerization before the reaction with the amino groups, resulting in the emergence of long polymerized GEN bridges linking the CS chains. Also, in these strong conditions, the ester groups in GEN hydrolyse. For these reasons, the stronger the base conditions, the lower is the cross-link density, meaning an increase in swelling behaviour [113,132,133,135,136]. Despite improving the nucleophilic replacement of the ester groups in GEN, an acidic environment can also cause the protonation of the CS amine groups, inhibiting the nucleophilic attack of the GEN C-3 carbon (GEN reaction becomes unfavourable) [97,133,135]. Furthermore, it was found that, in these conditions, the reaction with the ester molecules in GEN was less predominant than the ring-opening reaction [133]. Therefore, neutral conditions (mainly around 7.4) are considered ideal for forming stronger, tougher, and stiffer genipin-cross-linked CS gels exhibiting high cross-link density with high viscosity (low free volume), low degree of swelling, low degradability, enhanced elasticity, and thermal stability [120,128,133,137]. Moura *et al.* report that when under physiological pH, even the pure and low cross-linked CS samples gelled (by an increase of temperature), while at pH 5 no gelation is observed, not even under high temperatures [132]. Recalling the chapter 2.1, although CS is insoluble at neutral pH, after it is dissolved in acid, the reaction between GEN and CS becomes possible.

The color of the cross-linked genipin-chitosan system is directly related to the cross-link density and varies from yellow to brown [133,138]. At the start of the genipin-chitosan reaction, when cross-linking is still insignificant, the solution should be clear, colorless, and viscous [133,139]. As the cross-linking reaction starts, a slightly yellow coloration (typical in strongly acidic conditions) appears and is followed by a green/bluish tone [97,139]. At ideal conditions, the genipin-chitosan cross-linking produces a stable and fluorescent dark-blue hydrogel (this color is also closely related to the spontaneous radical-induced polymerization of GEN when exposed to air) [97,121,125,129,139]. Despite being an indicator of effective cross-linked CS, the blue color could condition the scaffold color and appearance, possibly limiting the use of GEN (mainly if stored under environmental conditions due to the oxygen radicals) [86,140,141]. Finally, if prepared in a basic environment, the genipin-chitosan solution will produce a brownish color [130,133].

To change the pH into the desirable values associated with high cross-linking, a step of neutralization is often introduced. This step is carried by a basic solution, usually NaOH, which will increase the pH of the solvent [100,129,130,132,133,138,142]. This way, CS is stabilized and prevented from solubilizing, which means better physical, thermal and mechanical properties [143]. To prevent polymer precipitation, the rapid addition of this solution should be avoided [130].

3.1.2 Temperature influence on the gelation process

The temperature (mainly curing temperature) can also have an important role in dictating the gelation process. Many studies reveal a decrease in gelation time for higher temperatures (irreversible cross-linking processes prevails over side reactions) [86,129,135,138,144]. By evaluating the color of the formed gels, Chen *et al*. tested genipin-chitosan gelation under 4°C, 20°C, and 37°C at different incubation times. The dark-blue colored hydrogels started to emerge after 12 hours for the highest temperature, while at the lowest temperature, after 48 hours, only a slightly yellow color non-gelled mixture was observed, meaning almost no occurrence of genipin cross-linking [138]. Dimita *et al.* found the same situation while using temperatures up to 50°C [129]. Cho *et al*. report enhancement of genipin-chitosan

reaction for temperatures in the 60-85°C range [144]. Szymańska studied the thermal degradation of CS and found that its physicochemical properties are maintained for temperatures inferior to the glass transition. For the most part, the biomedical applications of chitosan-based materials do not require temperatures above 100°C. If excessively high, the temperatures could lead to thermal degradation, meaning that a middle point, where the low temperatures associated with high gelation time are also avoided, should be preferred [86]. The selected drug delivery components also need to be accounted for, seeing that they can reduce the glass transition temperature [86,102]. However, as will be shown later, the selected temperatures to induce gelation can vary, and in some cases, the curing step might not be used.

3.1.3 Concentration influence on the gelation process

In resemblance to temperature, an increase in cross-linker concentration (and in the genipin-chitosan ratio) improves to a certain degree, the gelation process (mainly the duration) and the associated mechanical properties. Multiple studies report a 0.025% GEN concentration (by weight) as sufficient for full CS cross-linking. However, a lower percentage is related to solubilization in acidic and neutral conditions leading to a decrease in cross-link density. Because of this, and to guarantee that all the cross-linking sites are saturated, the elected percentages are typically higher [62]. Multiple studies describe the production of genipin-chitosan hydrogels with 0.1% GEN concentration [130,145,146]. Maria *et al.* found that an increase up to 0.2% in GEN concentration did not lead to any decrease in cell viability [100]. Moura *et al*., by increasing the GEN concentration from 0 to 0.15%, also found a significant reduction in gelation time [132]. Another study compared a 0.3% formulation with a 0.7% one and found that the 0.3% option improved the degradation time (decrease in swelling degree). In contrast, the more concentrated option facilitated water penetration in the implant [147]. However, despite the benefits of a higher GEN concentration, above a critical percentage, the produced gels become brittle and stiff with poor mechanical stability [130,138].

3.1.4 Fluorescence imaging in genipin-chitosan hydrogels

One of the particularities from the genipin-chitosan cross-link reaction is the manifestation of fluorescence via fluorophores, which opens the possibility for structural visualization and a better understanding of the biodegradation and distribution of the produced gels (real-time monitorization) [58,62,125]. The fluorescence intensity is a great indication of how efficient the cross-linking reaction was, increasing or decreasing as the cross-linking density increases or decreases, respectively [58,148]. After the initial growth and reaching the peak point, the fluorescence intensity tends to decrease, which is possibly caused by the methanol-hydrogel reaction [58,138].

This property can be visualized and studied through a variety of techniques, including fluorescence, confocal, multiphoton, and transmission electron microscopies [149,150]. By comparing the fluorescence through some of these different methods, Hwang *et al*. were able to extract different information about the optical spectral and structural properties of the genipin-collagen cross-link reaction [151]. Also, it is important to understand that the optimal fluorescence emission is highly dependent on the excitation wavelength. Genipin-amino cross-link products absorb at the mid-UV (250-300 nm), near-UV (320-370 nm), and visible wavelength range (400-600 nm), while the fluorescent wavelength range comprises the 380-700 nm region [138,151]. Chen *et al*. studied genipin-chitosan fluorescence using an excitation wavelength of 369 nm to obtain the 380-700 nm emission spectra, through which the maximum fluorescence wavelength was given at 470 nm [138]. The study by Hwang *et al*. (genipin-collagen) also describes an emission maximum at 464 nm when using 300-400 nm excitation wavelengths [151]. For image acquisition, Matcham *et al.* (genipin-chitosan + PVP) resorted to a 485 nm excitation wavelength to produce a 520 nm emission one [58].

3.2 Properties and fabrication techniques

In order to allow controlled cell infiltration and attachment, incorporation of drug delivery systems, neovascularization, cell migration and proliferation, bone ingrowth and nutrient, oxygen and waste transport, the polymeric scaffold should have a large internal surface area with appropriate pore distribution, large pore sizes with high interconnectivity and high porosity [36,102,152]. In contrast, when decreased, the previous properties lead to a reduction in free space, density, and pore thickness, leading to the production of a scaffold with better mechanical strength. Because of this, depending on whether the intended scaffold application requires high levels of mechanical strength or not, an equilibrium in porosity properties should be found [152]. In addition, such scaffold should closely resemble the porosity of an adult human bone, which is composed of 5-10% porosity in the outer shell (cortical bone) and 75-85% porosity in the inner shell (cancellous bone) [153].

The ideal pore size and porosity for some types of cells is still yet to be defined, which hinders the manufacturing and development of scaffolds. According to past studies, the minimum pore size, allowing enough space for cell adhesion and ingrowth and nutrient, gas, and waste transport, should be approximately 100 μm [154–156]. Based on the papers reviewed by Velasco *et al*., who did an overview on pore sizes past works, 150-600 μm (with porous volume of 75-80%) [157], 300-1200 μm (with porous volume 70-80%) [158] and 300 μm or above (porosity < 90%) [155] have all been recommended values, improving osteogenesis, blood vessel formation and bone tissue ingrowth [154,159]. Microporosity (pore sizes < 10 μm) is also reported to affect surface topography and is usually linked to a scaffold with the ability to be impregnated by biological fluids, exhibiting increased protein adsorption and ionic solubility [71,160]. The differences in reported porosities are, once again, due to the dissimilar parameters used, the complexity of the processes involved, and the drug delivery systems selected (which depend on the intended application for the scaffold) [159]. The ideal scaffold is probably a hierarchical macro/microporous structure, combining the best of both worlds [161]. Finally, the development of the scaffold also facilitates the production of structures with different geometries, allows radiolucency studies, and increases stability upon storage [159].

The most commonly used techniques present the ability to produce scaffolds with pore sizes in the order of 100-500 μm with porosity up to 90%, but can also be set to produce microporosity [159]. These are: solvent casting/particulate leaching, gas and dense gas foaming, phase-separation/lyophilization (freeze-drying) and phase-separation/freeze-gelation [36,124,162]. A survey on chitosan-based systems fabricated by these methods for BTE is provided in Table 7.1. The main disadvantage of using these procedures is the formation of a weak and randomly organized internal structure, which compromises the control over physical and mechanical properties [159]. In contrast, RP techniques provide relatively good mechanical strength, and their properties can be easily modified. Thus, the assembly of hydrogel and synthetic polymer via RP techniques to form a composite scaffold system might be a wise way of maintaining the advantages of each component while avoiding their drawbacks. Rogina *et al*. describe a three-component system produced through freeze-gelation containing chitosan, hydroxyapatite and PLA (printed via FDM). The addition of hydroxyapatite proved to contribute to a decrease in the scaffold degradation time (relevant for long-period applications), and to an increase in the proliferation of MSC's [163]. Another investigation done by Zakaria reports a surface entrapment of chitosan on 3D printed PLA scaffold for bone regeneration. The findings indicate high porosity, uniform distribution chitosan and controlled, and repetitive architecture on entrapped (up to 8 μm in depth) 3D printed scaffold [164].

3.2.1 Phase-separation options

3.2.1.1 Phase-separation and lyophilization

This technique is widely known for being fairly easy to combine with other manufacturing procedures [165]. Mainly depending on the neutralization stage (usually with NaOH), two paths can be

chosen to obtain a porous scaffold. Either the neutralization is initiated before the freezing step (1) or after the freeze-drying phase (2) [54,100,152].

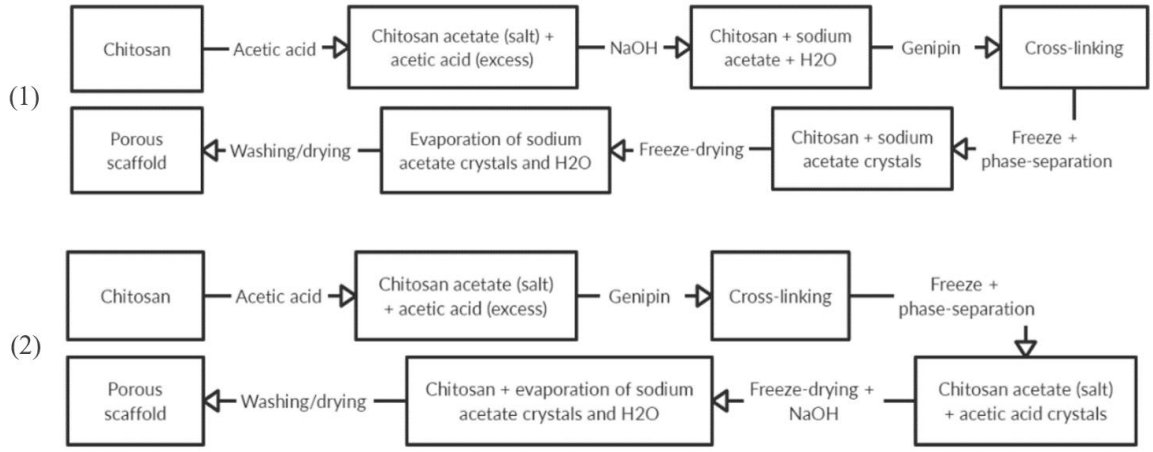

Figure 3.3 – Sequence of events (1) and (2) in the phase-separation/lyophilization technique.

Some aspects should be considered in both sequences of events. In the first option (1), a curing step (described in detail in chapter 3.1.2) is often applied for a certain period of time before the start of phase-separation, increasing the efficiency of the cross-linking process. If applied in the second case (2), since the neutralization only occurs later in the process, the rise in temperatures caused by curing would destroy the overall porosity of the scaffold. The prepared genipin-chitosan samples are placed in a container/mould of choice and are subsequently frozen, typically at -20°C [56,100,163,166]. The freeze-drying is done in a lyophilizer at low pressure and low negative temperatures in order for the structure to be preserved while the solvent is being removed (it prevents the rise in temperature and recombination of the phase-separated solution or remelting of the frozen mould) [100,152,166]. Besides the cross-linking process itself, the freezing temperature (with direct impact on the cooling rate), polymer and cross-linking agent concentrations and thermal gradients can also influence the porosity specifications [96,166]. As an example, a lower freezing temperature leads to lower porosity, pore size and swelling degree, which translates into a slower drug release (faster colling, meaning smaller pores) [96]. A decrease in polymer concentration means increased pore sizes, affecting the efficiency of these systems for drug delivery purposes [62]. Since the removal of the residual solvent is not 100% efficient, easily identified by the characteristic smell, another lyophilization step can be conducted [166].

Overall, phase-separation and freeze-drying are relatively simple to carry out, without the requirement of high temperatures and leaching. However, high energy consumption, high costs, small pore sizes, lack of porosity uniformity (due to freezing variations throughout the scaffold structure), and time-consuming steps are some of the drawbacks associated with these techniques [166,167]. In addition, one of the major challenges of this process is the development of surface skin in the scaffold-air interface. This condition is caused when the used temperatures are not low enough, and the formed structure cannot withstand the interfacial tension from the solvent evaporation (prevents oxygen diffusion and nutrient exchange) [152]. Through constant addition of freezing agent to prevent temperature increase, the apparatus designed and used by Ming-Hua Ho *et al.* is one way to prevent surface skin, assuring that the drying temperature is kept low [166].

3.2.1.2 Phase-separation and freeze-gelation

Although freeze-drying can avoid the loss of structural integrity, it is highly time and energy consuming and does not usually prevents the formation of surface skin [166]. To circumvent these issues, freeze-gelation can be implemented instead of freeze-drying. After the freeze and phase-separation phases, the structure is immersed in gelation solution (at -20°C) composed of NaOH (provides pH increase for gelation) and ethanol (lowers the freezing point) (Figure 3.4) [152,162,163,166,168].

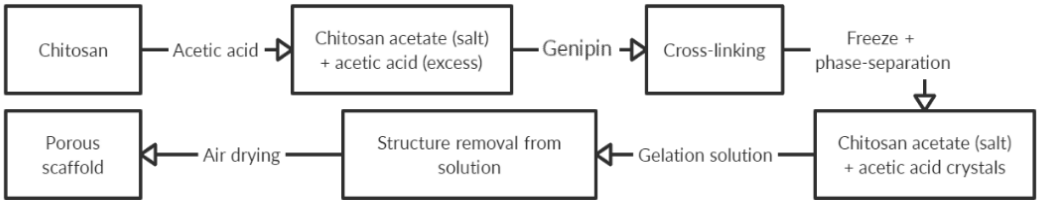

Figure 3.4 – Sequence of events in the phase-separation/freeze-gelation technique.

By using the freeze-gelation method, the lyophilization step can be discarded. Because the gelation process occurs below the freezing point of the CS solution, the structure created from the phase separation phase is kept. Compared to freeze-drying, freeze-gelation also retains fewer quantities of residual solvent and is easier to scale up [166]. Since the initial freezing step is the same as the one in chapter 3.2.1.1, the relation between freeze temperature and porosity properties also applies in this situation.

3.2.2 Gas foaming options

3.2.2.1 Conventional gas foaming

Gas foaming can be conducted through conventional or dense gas methods. The first one is based on the introduction of a foaming or blowing agent (typically sodium or ammonium bicarbonate) on the polymer solution (Figure 3.5) [152]. After almost all the solvent has evaporated, the semi-solidified polymer/salt complex is introduced to a warm or hot bath (for temperature increase) where the resulting sample is chemically decomposed, releasing inert gases (for example, CO_2, NH_2, and N_2) [156,169,170]. The simultaneous process of gas foaming and leaching out of ammonium bicarbonate particulates from the solidifying polymer matrix leads to the formation of scaffolds with up to 90% porosity and good pore interconnectivity in the interior regions of the structure [156,171,172]. Because it does not require the use of organic solvents, the possibility of toxic effects is minimized [166,173]. However, surface skin phenomena and the need for surfactants for foam stabilization are major barriers in this technique [170,174]. By using conventional gas foaming, research groups report the fabrication of porous scaffolds with pore dimensions values of ≈ 30 μm [124], 200-300 μm [156], 300-400 μm [173]. The main factors controlling porosity properties are temperature, pressure and the amount of foaming agent added to the polymer gel paste. [156,174].

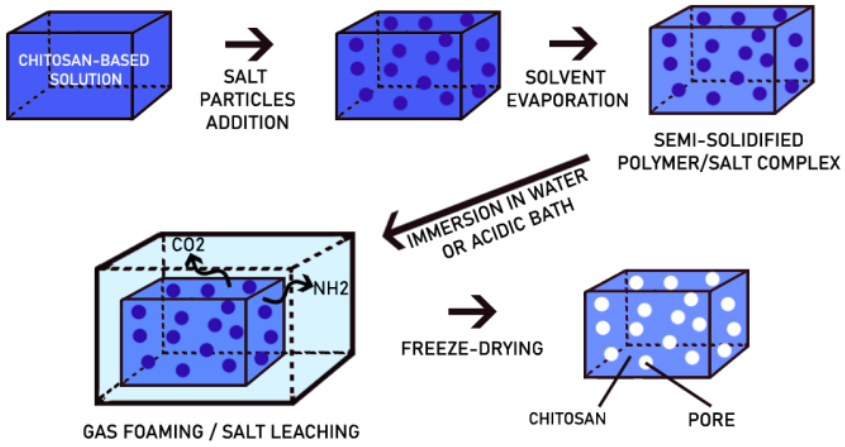

Figure 3.5 – Scheme of the sequence of events in conventional gas foaming.

3.2.2.2 Dense gas foaming

The second method of gas foaming involves high-pressure processing, where a gas is introduced (typically CO_2) at supercritical state to the polymer solution. By applying high pressure, the gas is able to diffuse into the polymer, originating a single-phase polymer/gas solution [174]. The depressurization step that follows gives rise to nucleation (a consequence of thermodynamic instability) and growth of gas bubbles, which give place to pore formation and shaping of the final porous structure [152]. This technique grants control over porosity and pore size and is also suitable for the incorporation of heat-sensitive biological agents inside the scaffold [175]. In the case of hydrophobic polymers, this operation can be conducted without a solvent. However, the same does not apply to hydrophilic/crystalline polymers, such as CS, where the low solubility of CO_2 prevents it from solubilizing in the polymeric solution [170]. One solution is to resort to supercritical CO_2-water emulsion, where co-solvents will help with the CO_2 solubilization process [170,176]. Another solution based on the same concept avoids the use of solvent but introduces a non-biodegradable surfactant [170]. One author describes the production of highly interconnected CS porous scaffolds by using supercritical CO_2-water emulsion with biodegradable surfactants [176]. One of the most promising options, which also avoids surfactants and surface skin, is described by introducing the dense gas at high pressure (≈ 60 bar) to an aqueous phase (37 °C) formed by the polymer and its cross-linker (Figure 3.6) [124,170,177].

Lower temperatures (lead to a decrease in CO_2 solubility) and a faster depressurisation step (excessive speed can lead to non-homogeneous pores) result in the formation of smaller pore diameters. The use of higher pressures also creates smaller pores and increases porosity [124,152]. This method avoids the use of high temperatures and the need for aqueous/polymer interfaces [174]. However, similarly to the conventional process, the lack of pore interconnectivity (only 10-30%), specifically on the surface, and the formation of surface skin can still be observed [172,178]. Pore sizes are also limited [175]. Even so, by varying the processing parameters, highly interconnected pores of 5 μm [177], 30-40 μm [124], 100 μm [178] and 50-200 μm [170] have been reported. In their study, Chengdong *et al.* were able to successfully produce genipin-chitosan homogenous and porous scaffolds without surface skin formation [124].

Gas foaming can avoid the use of organic solvents and surfactants. The porosity properties vary according to the rate of nucleation and gas diffusion [152]. Also, to surpass the different obstacles, both gas foaming techniques are often combined with other processes, mainly particulate leaching [170,172,173].

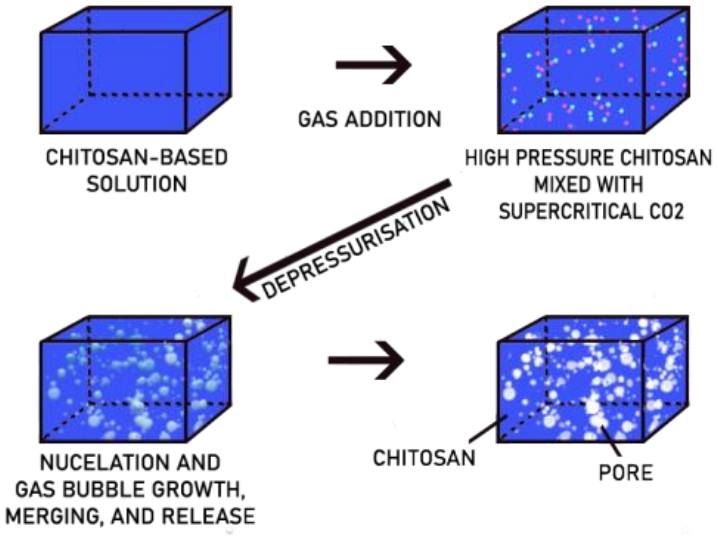

Figure 3.6 – Scheme of the sequence of events in dense gas foaming.

3.2.3 Solvent casting and particulate leaching

This relatively simple technique is the most used for porous scaffold manufacturing [179]. It starts by dissolving the polymer in an organic solvent. The suitable sacrificial porogens (salts, gelatin, sugars,

and paraffin) are then added to a mould with the polymer solution [152]. Following solvent removal/evaporation by temperature or pressure action, a porogen-polymer network is formed. The porogens are leached (typically with water), and the spaces originally taken by porogens turn into pores (Figure 3.7) [174,179,180]. In general, this process presents good control over pore interconnectivity and size, enabling highly porous structures with a large range of pore sizes [165,181,182]. However, the use of organic solvents (which could lead to lower cell viability), poor control over the orientation, the possibility of deficient pore shape, and a loss of interconnectivity originated from the dispersion of porogens in the polymer solution are all disadvantages that must be accounted for [180,182]. Pore sizes from 10-100 μm [178] up to 500 μm and 93% porosity [174] have been reported. It was also found that a high interconnectivity can be achieved by implementing 70 wt% of porogen in the polymer solution [179]. An increase in porogen amount, size and shape means larger pore sizes and thinner pore walls [152,174]. Despite the extensive work conducted with this method with different materials, the specific use of CS found in literature is scarce, and more research needs to be conducted.

One of the most frequently used variations of this technique is the combination of phase separation and freeze-drying. In this case, two levels of porosity with a large range of pore sizes can be created [152,174,183].

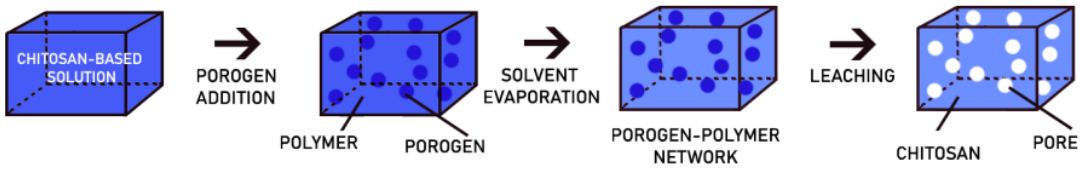

Figure 3.7 – Scheme of the sequence of events in solvent casting/particulate leaching.

4 Scaffolds - Rapid Prototyping

Although hydrogel scaffolds with high porosity and pore size are advantageous for applications involving cell adhesion and ingrowth, these structures have a lack of mechanical properties (mainly compressive strength), consistency, and reproducibility. Also, despite contributing to an increase in mechanical properties, the addition of a higher concentration of cross-linking agent is still not sufficient to withstand the mechanical pressures encountered *in vivo*. The conventional methods (chapters 3.2.1, 3.2.2, and 3.2.3) of scaffold fabrication are incapable of precisely controlling pore size, pore interconnectivity, pore spatial distribution, pore geometry, and incorporation of multiple cell types [184]. Furthermore, cell exposure to carcinogens and toxins derived from organic solvent residues is a challenging problem [185]. However, the main drawback of procedures that make use of steps that require exposure to harsh conditions or the introduction of organic solvents is the reduced bioactivity caused by the poor incorporation of bioactive or heat-sensitive biomolecules in the scaffold [186,187]. To maintain mechanical integrity and still be able to provide the necessary bioactivity described in the chapter 3.2, the hydrogels can be combined with rapid prototyping (RP) techniques.

RP uses a computer-aided design (CAD) and computer-aided manufacturing (CAM) in order to design and rapidly produce customized structures with complex and defined geometry [188]. The technology makes use of additive manufacturing (AM), where, instead of removing material, complex structures are produced via layer-by-layer overlapping. RP techniques are either based on top-down or bottom-up approaches. The first one provides an extensive choice of processing materials and makes use of AM to build 3D scaffolds with the desired structure for tissue applications. This process further involves the seeding of living cells into the struts. Bottom-up options are more recent and are related to the assembly of small, non-diffusion limited, and cell-laden modules for constructing 3D engineering tissues via 3D bioprinting. However, these options are slow and are associated with low mechanical properties, which are more suitable for soft tissue engineering [48,189,190].

Three main technologies make up the RP systems based on top-down approaches: solid-based (comprises fused deposition modelling), liquid-based (comprises stereolithography and two-photon polymerization), and powder-based technologies (includes selective laser sintering and three-dimensional printing). Most of these printing methods have resolutions of 50-500 μm [184,191].

4.1 Additive Manufacturing techniques

4.1.1 Stereolithography

Stereolithography (SLA) fabricates complex structures by using a layer-by-layer approach with a photo-curable liquid polymer as the main raw material. Here, an UV laser is used to induce spatially controlled solidification of liquid-based resins. This technique can be classified into two main types: bottom-up (can create softer printed structures) and top-down (safer and more reliable), based on build platform movement and laser motion [191]. Basically, the excitation of molecules from predetermined sites on the material surface by laser light (where a single UV-photon is sufficient to start the process) causes photopolymerization of the resin and further solidification of the material while leaving the remaining areas in liquid form. Once this layer is solidified, the built layer is recoated with new liquid resin, and the lifting platform moves for solidification of the subsequent layer. The 3D structure is obtained through repetition of this process and removal of the excess resin [192,193].

SLA is fast and it provides easy removal of support materials, mainly by heating. It is fast and delivers high resolution (greater than 50 μm), high accuracy, freedom of design, and excellent surface finish. It is also easy to remove support materials and avoids the nozzle clogging problem found in other techniques. Fabrication of highly porous and interconnected scaffolds have also been reported by using this method [194,195]. However, SLA has a limited option of materials and is a slow process with a lack of monolithic mechanical structure [184,192,196].

By taking advantage of micro-stereolithography (μSLA), which follows the same principle as the SLA approach, resolutions up to 1 μm can be reached, enabling the formation of well-defined complex structures. This is mainly done by focusing the laser beam more precisely [196]. Besides, this technique has demonstrated to have superior cell proliferation [197].

4.1.2 Two-photon polymerization

Two-photon polymerization (2PP or TPP) is also a resin-based option. However, contrary to SLA, it does not rely on a single photon absorption to initiate photopolymerization. Instead, the photosensitive material is exposed to near-infrared (NIR) femtosecond laser pulses, which provides the necessary intensity to cause the absorption of two photons and initiate photopolymerization [184,190,198].

2PP is simple, fast, and does not require a controlled environment [184]. One of the advantages of 2PP over SLA is that, since most used resins are transparent to NIR, the laser light is not absorbed by the resin in the first few micrometers. This means that, in 2PP, polymerization is induced along a volume of material, allowing the manufacturing of a wide range of complex 3D structures. Plus, the excitation region created by the two-photon is smaller than the one originated by the single-photon, meaning better resolution. In fact, 2PP is the technique that has the highest resolution of all the RP procedures, with values reaching 0.1 μm [190,198]. However, 2PP restrictions associated with scalability are a drawback and can compromise the use of this technique over SLA [199].

4.1.3 Selective laser sintering

Similar to SLA and 2PP, selective laser sintering (SLS) also uses laser technology. Here, continuous lasers, such as CO_2 lasers, heat powders of polymers, ceramics, or metals to near their melting point. This procedure allows the formation of a single solid object. The powder layer has a thickness of 100-200 μm and is spread on the powder bed by a cylindrical roller. After laser scanning, the sintering layer is lowered, making space for the next layer of powder. This process is repeated until the final structure is produced. The attained resolution is mainly dictated by the size of the particles used, laser beam

diameter, and heat transfer in the powder bed and can be limited by the unwanted fusion of surrounding powder particles [190,192,196].

The final parts are accurate, lightweight, highly durable, and are both heat and chemical resistant. Besides, since the non-sintered powders act as support, this process does not require the use of extra support structures, and so, complex structures with overhanging regions can be obtained. These same powders need to be removed, manually or with brushing and powder blasting, which despite avoiding the need for organic solvents, presents a disadvantage. Also, the high processing temperatures (> 37 °C) and the high associated costs are some additional drawbacks of this method [184,199,200]. Nevertheless, plenty of works can be found in the literature regarding the fabrication of SLS porous scaffolds and their relation to each other [201–205].

Selective Laser Melting (SLM) is a similar option to SLS, mainly involving pure materials. However, this time, the laser is used to achieve full melting of the fine powders in an inert gas environment [196]. By doing this, instead of just fusing the layers, these are homogeneously mixed with each other, forming a solid and dense material, without the need for binders or post-processing procedures, usually applied in SLS [196,206].

A variation of these methods is electron beam melting (EBM), which works on the same principle as SLS/SLM but with an electron beam as the power source to sinter or fuse the materials. Contrary to the previous techniques, EBM operates under very low pressures in order to avoid oxidation issues. Furthermore, it has higher power efficiency and higher scanning speed [207,208]. Porous titanium alloy scaffolds produced using this method have successfully been tested and described as compatible with the structural properties found in human bone [209,210].

4.1.4 Three-Dimensional printing

3D printing (3DP) is also a powder-based technique and works through the injection of a liquid binder from the printer head onto a specific region on the powder bed (containing a thin layer of polymer, ceramic, metallic or composite powder) according to the software cross-sectional model. This is followed by the drying of the binder and by the lowering of the workstation, making space for the next layer of powder. This process is repeated, leading to layer-by-layer formation until the final structure is produced [192,196,211].

3DP is low cost, fast, does not need the use of toxic components and provides rough surface fabrication, important for cell interaction [184]. It also allows the fabrication of highly porous structures with complete pore interconnectivity (these aspects can be independently controlled) [179]. Ceramic materials can be produced through this technique since they are often sensitive to the high temperatures typically found in other options [191,212]. Similar to the SLS/SLM processes, this technique does not require the use of temporary support structures and allows for overhanging regions. However, post-processing is required to remove the unprocessed powder, which is currently difficult to do with porous structures. The achievable resolution is not the best either, with values of approximately 200-300 μm with limited pore size [184,190,213].

4.1.5 MultiJet printing

MultiJet printing (MJP) is a deposition-based technology that uses a 2-axis (x,y) moving inkjet head to create subsequent layers of photocurable material on a build tray. This material is deposited in droplet form and is then hardened (polymerized) by UV light exposure. Finally, the tray moves down a layer in the Z direction, and the process is repeated until the entire workpiece is finished [190,214,215].

The existence of a high number of nozzles gives the possibility of printing different materials at the same time. By taking advantage of this particularity, this is the only process that allows the printing of multi-color structures [215]. MJP can provide complex geometries with a very high-quality resolution of up to 16 μm [215,216]. Wax material, which is used to fill voids and give support for overhanging features, can be easily removed without damaging the model and conferring smoothness to the surface [191,217]. However, both the printer and the materials used in it are expensive. This is mainly due to a limitation

in jet-compatible materials since the physical properties of the used material influence the behavior of the droplets and the liquid jet [190,214].

4.1.6 Fused deposition modelling

The most commonly used AM technique is fused deposition modelling (FDM) based on a single (Figure 4.1) or dual nozzle method (allows for multi-material printing) [218]. These systems operate at the range of 150-250°C in the x, y and z axes and use a pinch roller or screw feed mechanism, where a thin thermoplastic filament is continuously melted and extruded through a nozzle tip. The extruded polymer filament is horizontally deposited layer-by-layer and immediately hardens, in a specific pattern, until the structure is completed [175,219]. To assure good interlayer adhesion, the previously deposited layer must be at a temperature just below the solidification point of the material [196]. Compared to other printing techniques, FDM is a low-cost, multi-functional, and simple printing technology. Besides, this process does not require the use of organic solvents [192]. However, poor surface finish, nozzle clogging, relatively long building time, lack of suitable materials, and high-temperature processing are some of the drawbacks that still need improvement [175,220]. The limited resolution of approximately 250 μm and the accuracy provided by the jet size are also issues that highlight the value of using a composite scaffold, meaning that FDM is not adequate for the fabrication of microstructures [161,178]. In fact, FDM is usually indicated for basic proof-of-concept models and simple prototyping. A solution to this problem is the incorporation of hydrogels in the printed structure. As previously mentioned, hydrogels can easily exhibit a highly porous structure while still maintaining bioactivity.

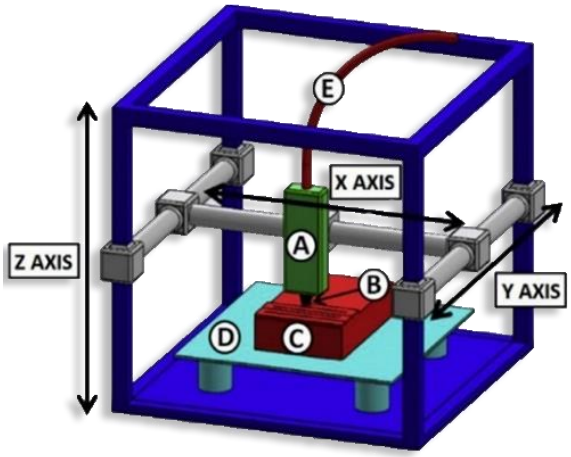

Figure 4.1 – Single nozzle FDM scheme: (A) extruder; (B) nozzle; (C) printed part; (D) printed bed/hot plate; (E) filament. Adapted with permission from Mazzanti *et al.* [221] under the copyright creative commons attribution license.

Also, despite the wide range of materials that can be used, these need to be thermoplastic, where the applied temperatures present a challenge for the direct incorporation of biological materials during the fabrication [190]. The poor adjacent layer fusion caused by the rapid cooling of the extruded material can be solved by promoting cross-linking amid layers (via gamma-irradiation post-printing) or by inducing covalent bonding (via Diels-Alder reaction) [217].

The design and synthesis of the polymeric scaffold produced through FDM manufacturing can be set to meet the necessary requirements of the cancellous bone (Young's Modulus of 0.02-2 GPa and compressive strength of 1-20 MPa). However, the literature reports Young's Modulus and compressive strength values of 1-30 GPa and 100-230 MPa for cortical bone, which are much higher than what is conceivable with such technique. For these cases, bioceramic scaffolds produced through other techniques, such as MJP, are currently more reliable [37,159,222,223].

The thermal and mechanical stresses to which the material is subjected upon extrusion lead to a variation in MW, degradation time, structural integrity, and mechanical properties. To create a proper scaffold design for future *in vivo* applications it is essential to understand these behaviors and their influence on the material response [224,225]. As for now, the loss of mechanical properties remains a barrier to the effective use of FDM [223,226]. For example, a drastic decrease in PLA Young's modulus can be explained by mass loss during medium treatment leading to thin struts. Nevertheless, given the right conditions, PLA scaffolds are still able to preserve their structural integrity for months or even years, making them an excellent option for load-bearing applications [227]. These are further explored in the next chapter.

Besides the quality of the raw material, the instrumentation also plays an important role. The main parameters that can be manipulated for process optimization, according to the intended scaffold, are the printing speed/pressure, nozzle diameter, hot-end temperature (extrusion temperature), flow rate, drop position, distance between points, layer thickness and printing orientation [178,228]. Considering the high number of different parameters, reaching optimal printing conditions usually requires a "trial and error" approach with multiple recalibrations, which is far from being ideal [229,230].

4.1.6.1 Poly(lactic acid)

Poly(lactic acid) is an aliphatic polyester and the most widely used synthetic material for BTE, drug delivery, and absorbable implants [174]. This biodegradable and bioabsorbable polymer with thermoplastic properties also presents excellent biocompatibility, thermal stability and plasticity, low viscosity, and processability [226].

PLA can be synthesized from L-and/or D-lactic acids; however, since L-lactic acids are involved in the cellular metabolism and in the minimization of the risk of adverse reactions, they are more attractive for biomedical use than D-lactic acids [224]. Depending on the envisioned application, PLA can be processed using two primary techniques: direct polycondensation and ring-opening polymerization (ROP). The first requires longer reaction times and allows to obtain PLA with low MW, meaning weak mechanical properties and increased degradation rates. In contrast, besides requiring milder and shorter reaction conditions and times without the production of undesirable by-products, by using a metal catalyst, ROP allows to obtain PLA with high MW (>50000 Da), good mechanical properties, and decreased degradation rates, widely useful for long-term applications [223,231–233]. Through the ring-opening polymerization of D-lactide and L-lactide isomers, semi-crystalline PLA stereoisomers poly(D-lactic acid) (PDLA) and poly(L-lactic acid) (PLLA), respectively, can be obtained [234]. The copolymer poly(D, L-lactic acid) (PDLLA) (usually amorphous), created from the racemic mixture of L-and D-lactic acid molecules, can also be produced [224,233]. Commercial PLA is obtained from the combination of PLLA and PDLA or copolymer PDLLA, where a PLA with PLLA content superior to 90% will favor a crystalline profile. On the contrary, by minimizing the optical purity through PLLA reduction, the crystallinity of PLA can be decreased (loss of thermal stability) [235] (optical purity variation is the most popular option to tune into the desirable specifications). A higher contribution of PDLLA will also cause a decrease in mechanical properties and overall thermal stability [233]. Depending on the crystallinity, a variety of properties (for instance, hardness, tensile strength, stiffness, melting temperature, and elastic modulus) can be modified [224].

Changing the L/D enantiomer ratio and sequence, MW, temperature, pressure, annealing time, and path of synthesis also leads to a variety of physiochemical properties and parameters (mainly degradation and crystallization kinetics) that can be tailored for each specific application [224,226,233,236].

Since the main purpose of PLA in this review is to provide load-bearing traits to the composite scaffold, the semicrystalline PLA option is more attractive when compared to the amorphous one. The glass transition and melting temperatures (T_g and T_m, respectively) of high semicrystalline MW PLA ranges from 45-60 °C [224] and 150-184°C [224,233,236], respectively. Other physical properties are summarized in Table 4.1.

Table 4.1 – Physical properties of semicrystalline PLA [159,237].*

Density – ρ (g/cm^3)	**Tensile (Yield) Strength – σ_y (MPa)**	**Young's Modulus – E (GPa)**	**Ultimate Strain – ε (%)**	**Flexural Modulus – E_f (GPa)**
1.36	50-70	3-3.5	4	5

*The values here presented are an approximation since these properties strongly depend on a wide range of parameters.

The values presented in Table 4.1 strongly depend on the composition of stereoisomers and degree of orientation. Furthermore, unlike thermal properties, mechanical properties are highly dependent on MW. These and other variations are thoroughly described in the work done by Farah *et al.* [237].

Despite the many advantages associated with PLA, this material also presents some major drawbacks, including low degradation rate (excessive for many applications), moderate hydrophobicity (contact angle approximately 80°), meaning poor cell affinity, brittleness (applications requiring higher stress and plastic deformation are limited) and lack of reactive side-chain groups (makes it hard for surface and bulk modifications) [237,238].

For *in vivo* application, the degradation of PLA is mainly conducted through hydrolysis and enzymatic action with the creation of non-toxic products that are removed via natural metabolic pathways [226]. Besides the factors mentioned above, the degradation process is further dependent on water diffusion rate into the polymer, pH (low pH values usually promotes faster hydrolysis), sterilization process (decreases MW), catalyst (which can also cause the appearance of metal impurities), intended material application (degradation will be enhanced in high-stress conditions; if the PLA structure is implanted in a region of low vascularization, the degradation process will be enhanced by the acidic environment originated from the failing of removal of by-products) and fabrication route (fused deposition modelling, for example, causes the loss of MW) [224,237,239]. Modifications on PLA can have a major influence on biodegradation and brittleness, mainly through copolymerization with other lactone-type monomers or hydrophilic macromonomers and through blending with other materials (mainly with bio and non-biodegradable polymers). Wang *et al*. provide extensive information on these procedures [240]. The formation of stereocomplexes (through blending) can also be used for property adjustment [224]. For example, the stereocomplex created from the combination of PLLA with PDLA induces an increase in crystallinity, glass transition, and melting temperatures, and thermal stability [232]. With this, one can better control the PLA degradation rate and the reparation/regeneration rate of the damaged bone tissues [241].

4.1.6.2 Scaffolds' surface modification and sterilization

Hydrophobicity and lack of reactive side-chain groups (low surface free energy, electricity, and roughness are also contributing issues) are typical challenges of most synthetic biodegradable polymers, including polyesters, namely PLA, with poor biological recognition on the surface [240,242]. These are associated with low cell affinity, adhesion, and proliferation, and so, often, scaffolds must undergo surface modification, making it possible to bind cell-recognition ligands. Table 4.2 summarizes the most common surface modification options.

Many of the procedures derived from the options shown in Table 4.2 are currently not optimal for 3D structures, mostly due to reducing the mechanical resistance and promoting uncontrollable surface roughness, and so, until all challenges are overcome, plenty of work still needs to be done [235]. Besides, most of the literature focuses its attention on plasma treatments, which are widely described as being non-permanent procedures, and so, other, better alternatives, should be studied [243,244]. It is important to note that the selection of the modification process should take into account the biomedical application and request [242].

Despite not explored in this work, the sterilization process can also have serious implications in the final scaffold (in a good or bad way) and must also be addressed before *in vivo* implantation. For

example, the plasma sterilization technique can improve the properties of the material surface, increasing cell-material interactions. In contrast, and using PLA as example of a degradable polymer, treatments involving high temperatures can lead to unwanted effects on material mechanical strength and MW. The scaffold must be able to withstand the sterilization process while still maintaining its properties. The most common options, as well as their advantages and disadvantages, are well stipulated in the literature [85,224,237,245–247].

Table 4.2 – Most common surface modification procedures of polymeric scaffolds [178,235,240,242,248].

Treatment	Technique	Mechanism	Benefits	Limitations
Surface coating (physical)	Surface coating	Immobilization of growth and attachment factors	Simple, effective; use of biocompatible and cell affinity natural biofunctional materials for coating	Time-consuming and expensive; passive adsorption can compromise the configuration of the adsorbed molecules; coating is easily removed with water or body fluids
Alkaline hydrolysis treatment (chemical)	Random, graft and block copolymerization	Copolymerization of different monomers	Simple, convenient; resulting groups can be used to conjugate the bioactive molecules	Hydrolysis changes surface morphology and bulk mechanical properties; residual alkali is not easily removed
Plasma modification	Low-temperature plasma	Surface etching (topological modification)	Gas mixture, temperature, and pressure can be tuned according to the desired surface; can avoid significant alteration of physical/chemical properties and morphology structure	Plasma on the surface can migrate to the inside, lowering the functional groups on the surface and minimizing cell attachment efficiency; low penetration; mostly indicated for 2D films; irregularity of the modified surface; the need for a vacuum environment
	Biomolecule anchoring	Introduction of functional groups that promote anchoring of bioactive molecules	Enhanced growth-factor preservation; specific methods can prevent plasma from affecting bulk properties	Special plasma generator and gas are needed; expensive

5 Intelligent delivery

5.1 Drug and biomolecule loading

Drugs and biomolecules can be immobilized on the scaffold surface by means of covalent linking, using reactive functional groups, or via physical adsorption, using noncovalent conjugation, and so, surface modification of the polymeric 3D printed structure is usually needed. However, when incorporated in hydrogels, this process is no longer necessary. As referred in chapter 2, being rough and soft materials with the appropriate physicochemical properties, hydrogels already have the ability to absorb physiological fluids and to promote cell adhesion, proliferation, migration, and infiltration, without the need for further intervention [249–253].

The simple immersion of the 3D printed structure in a hydrogel solution carrying bioactive factors followed by cross-linking is enough to create the composite scaffold with the immobilized bioactive

agents [254]. Chemical cross-linking through cross-linkers, however, is usually followed by a curing step, where if not low enough, the temperature can lead to the denaturation of the biomolecules (where mainly proteins and nucleic acids have their molecular structure modified) [255,256]. Because of this, photopolymerization or physical cross-linking is usually preferred, allowing the loading of the components under mild conditions while the solution undergoes gelation. Also, by varying the density and nature of the cross-links between the hydrogel chains, one can control the diffusion of the bioactive agents through the matrix and the degradation rate of the hydrogel itself [257]. Since the hydrogel is combined with a 3D printed structure that confers mechanical support, the weakening of the mechanical properties of hydrogels, caused by the physical modification, does not lead to the instability that is usually encountered in these cases.

Besides the chemical and physical properties of the gel, the loading methods are another aspect that needs to be considered. The bioactive agents (drugs/biomolecules) can be loaded into the hydrogel using permeation (diffusion), entrapment, or tethering (covalent bonding) processes, where their diffusion is mainly limited by their size with respect to the distance between cross-linked polymer elements (further depending on the dosage, release profile and molecular characteristics of these same agents). These methods are analysed in the following table.

Table 5.1 – Standard drug/biomolecule loading strategies for hydrogels [102,258,259].

Treatment	Description	Benefits	Limitations
Permeation	Hydrogel is combined with loaded solution medium; loading elements slowly diffuse into the gel; suitable for small molecules	Easiest method; high loading efficiencies for hydrophilic drugs; small chance of bioactive agent deactivation	No *in situ* gelation possible; extensive loading time; high bursting degree
Entrapment	Hydrogel is combined with loaded solution medium by addition of cross-linking or complexation agents; suitable for small molecules, larger drugs, bioligands and hydrophobic/hydrophilic drugs; average bursting degree	Allows *in situ* gelation; drug stability is preserved; effective for small molecules, proteins, peptides, micro/nanospheres;	Does not allow for control over amount of drug loaded; occurrence of toxic material leaching; *in situ* loading can lead to unwanted reactions on fragile biomolecules
Tethering	Bioactive agents are covalently immobilized within hydrogel networks; suitable for small molecules, peptides and proteins; better for hydrophilic drugs	Allows *in situ* gelation; no bursting degree; degradation rate of gel self-adjusts to the rate of cell infiltration	Can cause bioactive agent deactivation;

As the hydrogel degrades with time (the action of body fluids and cells causes decreasing mechanical properties, which in turn lead to process acceleration), the loaded components will be gradually released and delivered to the surrounding tissues [260]. An orchestrated performance of each of these biomolecules is required for successful development of bone tissue within the scaffold [53].

Wang *et al*, for example, by making use of surface coating and entrapment, allowed improved chitosan retention on the PLA structure. Here, process reverse was initiated by dipping the scaffold in excess of nonsolvent solution for biomacromolecule surface trapping. Chitosan was homogeneously mixed with acetic acid and followed by acetone addition. After immersion in the solution, the scaffold

was transferred to a NaOH bath for neutralization and finally vacuum-dried. This approach is also simple and cost-effective [261].

A deeper understanding of drug and biomolecule loading, and controlled-release formulations, including the respective release mechanisms from hydrogel devices, can be attained by analysing the works of Lin *et al.* and Peppas *et al.* [262,263].

5.2 Cell seeding

Besides the incorporation of the previous therapeutics, the increasing trend is to produce a scaffold that can also perform as a carrier for cell delivery purposes. The reason for this is that, by having control over the release of biomolecules, one can also control the proliferation and differentiation of the seeded cells, further improving tissue regeneration [264]. Cell seeding procedures used for the repopulation of the scaffolds can be static (do not involve any type of mechanical stimuli) or dynamic/active (involves mechanical stimuli) [55]. All the methods need to be safe, reproducible, provide minimal seeding time and damage to the scaffold and guarantee high cellular retention, cellular viability preservation, and spatially uniform distribution of cells. [265,266]. The most popular methods include:

- **Static seeding:** concentrated cell suspension is directly poured onto the scaffold surface (via pipetting) and allowed to infiltrate the scaffold (via passive diffusion); it is by far the most popular method. Cells are not exposed to large mechanical forces, meaning they do not risk being damaged. However, it can lead to reduced cell seeding efficiency, nonhomogeneous cell distribution, especially in larger structures [267–269];
- **Rotational seeding:** a graft is usually placed in a spinner flask containing cell suspension, but a rotating vessel can also be used. The spinner-flask option induces a turbulent and unsteady flow of the media using a magnetic bar (for magnetic stirring) or an impeller blade. This medium is continuously stirred around immobile constructs, which provokes the transport of the cells to and into the scaffold. Gas exchange is given by surface aeration. Rotating vessel options induce laminar flow by making use of gravitational, rotational, and viscous drag forces. Due to these forces, the scaffold remains at a mobile suspended state through the loaded medium, and the mechanical stress, turbulence, and fluid shear are minimized. Gas exchange takes place at the bases of the container or through an inner cylinder. Rotational seeding, in general, increases cell-scaffold interaction and seeding efficiency. Slow speed and poor surface seeding are some of the drawbacks [265,270–273]. A variation from rotational seeding is centrifugal seeding, which uses the same notions but applies much higher rotational velocities. This option has demonstrated its potential to enhance cell proliferation and distribution. However, the vast parameters to be considered need to be synchronized one by one according to the objectives, which is challenging. Besides, the high speeds used in this process can lead to cell disruption [265,274];
- **Perfusion seeding:** the physiological conditions are replicated by the bioreactor (by means of pumping the medium through its chamber), providing the conditioning of scaffolds, and so, the increase in survival and growth of the cells. Perfusion seeding can be divided into two situations: direct perfusion, where the loaded medium flows through the 3D structure (provides better loading of the central region of the scaffold when compared to rotational seeding), or indirect perfusion, where the flow surrounding the scaffold is improved. Benefits of using these techniques include better cellular differentiation, deposition, and retention of the cells. Besides the environment given by the bioreactor, this system also removes biomechanical stresses from the cells and greatly improves the quality of tissue formation after *in vivo* implantation, compared to static strategies. Limitations include long processing times, the complexity of the bioreactor systems, and the need for optimization [265,271,275,276];

- **Magnetic seeding:** takes advantage of magnetic microparticles and combines them with the cells in solution, which become controllable when subjected to an adjacent magnetic force. This magnetization can be conducted by incorporating the magnetic nanoparticles within the cell (requires the use of magnetic cationic liposomes) or by attaching these same nanoparticles to receptors on the surface of the cell (requires the use of superparamagnetic, monosized polymer particles). By applying this process, cells can be easily attracted to each other, and promoting cell infiltration becomes unchallenging and efficient. In addition, excellent control over the exerted stress and good reproducibility can be achieved. The main downside of this technique is its toxicity, but reports describing no toxicity can also be found in the literature. This and other aspects force this method to be further tested before any *in vivo* application becomes a reality [265,273,276–278];
- **Vacuum seeding:** simple procedure that utilizes pressure differential, generated by a pump or syringe, to coerce the cell loaded solution (deposited onto the scaffolds) through the targeted structure, leaving the cells trapped inside. Some of the limitations include the high complexity of the bioreactor and limitations related to the size of the scaffold [265,279]. Previous works provide a deeper understanding of this method [280–282].

Despite the advantages of static seeding techniques, compared to the dynamic ones, these are usually less efficient and with an inferior homogeneous distribution of the cells. Dynamic options also confer higher quantities of cells across the scaffold. However, the drawbacks of each dynamic procedure, combined with the high complexity and prolonged seeding times, are some of the problems that still need to be addressed in these procedures [267].

The main challenge, caused by hydrostatic forces, is the poor propagation of the cells into the interior of the structures [265]. Because of this, and in order to produce a scaffold with an even density of cells throughout the entire structure, instead of seeding the cells after the scaffold is produced, the cells can be loaded into the pre-gel solution, where they are physically isolated from the surrounding conditions while still keeping their properties (mainly due to the high-water content environment) [55,265,283]. To prevent cell damage, just like with drugs and biomolecules, the cross-linking scheme must be carefully selected. In these cases, cross-linking that implies temperature intervention or hypertonic conditions (associated with ionic cross-linking, where an excess of negative charged ions being linked with the positively charged chitosan, can lead to the coagulation of the chitosan solution) presents a challenge, and so, photopolymerization is mostly preferred [260,284].

6 Conclusions

Traditional methods of bone grafting, such as autografts and allografts, are outdated, and new alternatives are rapidly gaining popularity. In this context, bone graft substitutes based on scaffolds are a promising approach for combating osteosarcoma related complications and are set to eventually take out the current standard options. BTE research has vastly advanced in recent years and is focused on developing and improving new materials and manufacturing techniques that emulate the physiological conditions as closely as possible. Scaffolds have been minutely analysed with consideration for these aspects; however, the majority of the studied scaffolds are still in their early stages of research, still needing long testing periods before clinical use. In fact, despite some successful *in vitro* reports, the literature still lacks in *in vivo* reports, and less invasive options must be studied to advance tissue repair and regeneration. However, success in the area of synthetic bone graft substitutes has been difficult by the unpredictability of the biological responses to these materials.

Due to their similarity to organic tissue, hydrogels are currently used in a wide range of biomedical and bio-engineering applications. More specifically, chitosan hydrogels have been gaining scientific attention. The unique properties found in chitosan, including, tensile strength, conductivity, porosity, and easy manipulation, are all of great interest to formulate hydrogels for BTE.

The author of this review hopes to provide the readers with a deeper knowledge of the BTE approaches and its state-of-the-art research. This work was focused on the analysis, description, optimization, and comparison of two major hydrogel-based procedures of scaffold manufacturing for BTE applications.

The first considered option dwells on the possibility of producing a porous scaffold via conventional manufacturing of chitosan-based hydrogels. Despite the many benefits associated with these scaffolds, due to the weak and randomly organized internal structure, as well as low consistency and reproducibility, they have their physical and mechanical properties compromised. Another complication is the use of organic/toxic solvents. However, the real issue of using these techniques comes from reduced bioactivity, which is affected by some of the processes used to induce porosity. Chemical cross-linking, for example, often implies the use of a curing step, which may cause the denaturation of the components. Because of this, most of the works found in the literature dwell on this method with the single intention of studying cell-based systems. The cross-linking agent genipin was selected as a case study, and its influence on the gelation process was assessed (pH, temperature, and concentration effect on genipin-chitosan gelation were also investigated). Genipin products are non-toxic and exhibit increased stability and mechanical strength. Furthermore, the genipin-chitosan cross-link reaction manifests fluorescence, which opens the possibility for structural visualization and a better understanding of the biodegradation and distribution of the produced gels (real-time monitorization). Another aspect that should equally be considered is the environment surrounding the hydrogel, which should be enhanced and better controlled to deliver the physiological and nutritional needs for increased cell activity.

To dodge the disadvantages related to the previous approach, the possibility of combining the hydrogel with a printed structure obtained through rapid prototyping is considered. However, the incorporation of these agents can only be done during post-processing situations where no destructive stages are used, and the problems of poor incorporation of bioactive or heat-sensitive biomolecules are overcome. Cell seeding processes also need to be carefully selected. By combining the targeted cells with the pre-gel solution and using photopolymerization or physical cross-linking alternatives, where the loading of the components is conducted under mild conditions, the mechanical properties of the scaffolds can be better preserved. Despite the advancements in the area, the ideal cell seeding procedure is yet to be formulated, meaning that more effort should be dedicated to the developing of new strategies that contribute to control the incorporation and release of such agents. In addition, the scaffold should closely mimic the porosity of the natural bone, which does not have a homogeneous distribution (cortical bone has much less porosity than cancellous bone). In most of the presented techniques, a uniform homogenous structure is produced, meaning that the scaffolds also require modification. Besides, the ideal pore size and porosity for particular types of cells is still yet to be defined, which hinders the manufacturing and development of scaffolds.

While rapid prototyping is gaining popularity, many challenges remain. Considering the high number of different and significant parameters, reaching optimal printing conditions usually requires a "trial and error" approach with multiple *in vitro* or *in vivo* recalibrations, which is far from being ideal. Limitations related to materials, design, processing time and temperature, scalability, costs, resolution, accuracy, and post-processing procedures are still an issue in most 3D printing alternatives. For example, despite the reports on 3D manufacturing at scales down to the nanometers, further research still needs to be done until these values become normalized. Until then, these techniques will only be used reliably for scales larger than 100 μm. Furthermore, with the increasing commercialization of the intervening polymers, such as PLA, most studies dismiss the importance of the crystallization process and put most of the effort in the processing phase of the scaffold, limiting the possible applications for BTE.

Taking everything into account, the design and implementation of BTE scaffolds remains a challenge. However, the use of chitosan-based hydrogels combined with other materials and biological systems holds great potential for future BTE studies. The porous composite system produced via conventional manufacturing here reviewed could be used for cell-based systems, where cell adhesion, proliferation, migration, and infiltration are of interest. However, if the objective is to also incorporate drug/biomolecule systems for *in vivo* applications, the method that combines the hydrogel with a printed structure obtained through rapid prototyping should be preferred.

7 Future perspectives

The combination of synthetic polymers, such as PLA, with chitosan-based hydrogels is not new. However, the incorporation of these gels in a custom-made scaffold produced through rapid prototyping techniques is relatively recent, and the studies found in the literature are still scarce.

Despite possessing osteoconductive properties, currently used implants have demonstrated reduced biologic activity. Because of this, future works should dedicate time to improving the activity of these implants by using bioactive agents (which leads to the improvement of osteoinduction and osteogenesis).

To elevate the scientific progress of these systems, some considerations should be taken. The current limitations associated with RP techniques (post-processing, resolution, accuracy, and others) should be improved without increasing the cost or efficiency of these systems. As progress is made in the field, the fact that most RP options are only used reliably at scales larger than 100 μm, will be a problem of the past, and nanometer resolutions will become simple and easy to obtain.

As for now, the combination with bioactive agents needs to be made in post-processing conditions, which translates into poor propagation of the cells into the interior of the structures. Cell seeding processes also need improvement. One of the most promising areas being developed is based on microneedles. This method uses direct bulk seeding to dodge the problems related to poor cell seeding.

Problems related to prolonged seeding times and high complexity are matters that need further improvement. Major research should be invested in developing the current and new manufacturing techniques that provide the possibility for the incorporation of bioactive agents in the scaffold. A new range of biomaterials, suitable for BTE applications and more resistant to the harsh manufacturing conditions (such as high temperature and hypertonic conditions), should also be explored. Furthermore, a new method for optimizing the scaffold manufacturing process must be investigated so that the current "trial and error" approach is finished. This, however, is particularly complicated since the parameters that determine the scaffold properties are complex and often lack information in the literature, where the attention is mainly poured into biological studies. The fabrication of undesired homogeneous structures also needs to be addressed since the scaffold should have heterogeneous characteristics. This can be done, for example, by creating a deeper interaction between CAD and RP systems.

3D Bioprinting techniques are one of the most recent technologies with great potential for tissue engineering that have been reported to be suitable for the incorporation of bioactive agents during the fabrication process. Bioprinting makes use of a bio-ink (hydrogel pre-solution loaded with the previous components) as the material source to produce a scaffold with some of the properties that are difficult to control in normal scaffold manufacturing techniques, including control over construct microstructure and spatial content, porosity, and cell distribution. However, bioprinting is slow and is associated with low mechanical properties, which are more suitable for soft tissue engineering, meaning that, until these systems can be applied to hard tissue, more research needs to be done.

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Supporting Information

Table 7.1 – Survey on chitosan-based systems fabricated by conventional methods for BTE.

Chitosan DD (%)	Chitosan MW (kDA)	Preparation method	Material	Pore size [μm]	Porosity (%)	Delivery system	Ref.
87	200	Phase separation; freeze-drying	CH; GEN	—	—	TSA cells; FBS; penicillin/streptomycin (pen-strep)	[100]
85	255	Phase-separation; freeze-gelation	CH; gelatin; GA	200 – 400	—	Not applicable	[285]
85	>310	Phase-separation; freeze-extraction / freeze-gelation / freeze-drying	CH; PCL	10 – 100	—	Not applicable	[162]
> 85	—	Phase-separation; freeze-gelation	CH; nano β-TCP; GEN or TPP	171 – 199	68 – 86	hMSCs; FBS; pen-strep	[168]
92.8	389	Phase-separation; freeze-drying	CH; Col; nHAp; PLA	60 – 150	96.79 – 98.53	Osteoblasts; pen-strep; FBS	[54]
—	Medium	Dense gas foaming (CO_2)	CH; GEN	32	—	Human skin fibroblast cells GM3348; FBS; pen-strep	[124]
86	1000	Phase-separation; freeze-drying; particulate leaching (NaCl)	CH	7 – 30; 200 – 500	—	Fibroblast cells NIH-3T3; FBS; L-glutamine	[181]

Table 7.1 – (Cont.)

Chitosan DD (%)	Chitosan MW (kDA)	Preparation method	Material	Pore size [μm]	Porosity (%)	Delivery system	Ref.
75 – 85	50 – 190	Conventional gas foaming ($NaHCO_3$); phase-separation; freeze-drying	CH; agarose; nHAp	10 – 70; 150 – 400	50 – 70	Mouse calvarial preosteoblast cell line; FBS; pen-strep	[286]
90	—	Phase-separation; freeze-drying	CH; GEN	20 – 160	76.50 – 92.14	Diclofenac sodium	[96]
75 – 85	Low	Phase-separation; freeze-gelation	CH; GEN; silica	10 – 100	95	Not applicable	[287]
89	—	Phase-separation; freeze-drying	CH; gelatin; GEN	—	—	Osteoblast-like cells; FBS	[288]
—	Medium	Phase-separation; freeze-drying; particulate-leaching (NaCl)	CH; 46S6 bioactive glass	—	90	Not applicable	[289]
95 – 98	100 – 300	3D-printing (FDM); phase-separation; freeze-gelation	CH; HA; PLA	960	61	hMSCs; FGF2; FBS; pen strep	[163]
95	150 – 200	3D-printing (modified FDM); phase-separation; freeze-gelation	CH; raffinose	3.5 – 20	—	Fibroblasts (C84)	[290]

Table 7.1 – (Cont.)

Chitosan DD (%)	Chitosan MW (kDA)	Preparation method	Material	Pore size [μm]	Porosity (%)	Delivery system	Ref.
75 – 85	Low	Phase-separation; freeze-drying	CH; β-GP; GO	—	50 – 60	rBMSCs; MG-63 cells; mMSCs (C3H10T1/2); FBS	[154]
75 – 85	Medium	Phase-separation; freeze-drying; microwave-assisted gas foaming ($NaHCO_3$)	CH; glyoxal	102 – 728	—	MG-63 cell line; FBS; pen-strep; L-glutamine	[143]
—	—	Phase-separation; freeze-drying; freeze-gelation; freeze-extraction	CH; PLLA; PLGA; alginate	60 – 150	> 80	Rat osteosarcoma cells (ROS 17/2.8)	[166]
87.4	466	Phase-separation; Freeze-drying	CH; collagen; GEN	200 – 500	—	BDCs; ADCs; FBS; amphotericin B; pen-strep	[123]
—	—	Conventional gas foaming ($NaHCO_3$); phase-separation; freeze-drying	CH; β-TCP	10 – 1000	90	MG-63 cell line	[171]
—	—	Sintering; phase-separation; freeze-drying	CH; HA; β-TCP; Polyurethane; GA	300 – 600	—	MG-63 osteoblast cells	[291]
—	800	Phase-separation; freeze-drying	CH; HA; gelatin; GA	300 – 500	85.2 – 95.8	Rat calvarial osteoblasts	[292]

Table 7.1 – (Cont.)

Chitosan DD (%)	Chitosan MW (kDA)	Preparation method	Material	Pore size [μm]	Porosity (%)	Delivery system	Ref.
> 90	100 – 300	Particulate leaching (NaCl); phase-separation; freeze-drying	CH; gelatin; EDC/NHS; GA	280 – 290	> 95	Not applicable	[183]
90	6	Phase-separation; freeze-drying	CH; gelatin; HA; GA	35 – 150	65 – 72	Human umbilical cord MSCs; FBS; pen-strep	[293]
≥ 85	—	Phase-separation; freeze-frying	CH; CBS; GEN	50 – 100	—	MEFs (3T3); FBS; pen-strep; fungizone antimycotic solution	[294]
78	900	Phase-separation; Freeze-drying	CH; gelatin; GA; PLLA	30 – 100	95 – 98	Not applicable	[295]

GA, glutaraldehyde; PCL, polycaprolactone; TCP, tricalcium phosphate; TPP, tripolyphosphate nHAp, nanohydroxyapatite; HA, hydroxyapatite; GP, glycerophosphate; GO, graphene oxide; PLGA, poly(lactic-co-glycolic acid); EDC, N-(3- dimethylaminopropyl)-N′-ethylcarbodiimide hydrocholoride; NHS, N-hydroxysuccinimide; TSA, mammary adenocarcinoma; hMSCs, human mesenchymal stem cells; FBS, fetal bovine serum; FGF2, fibroblast growth factor 2; rBMSCs, rat bone marrow-derived mesenchymal stem cells; mMSCs, mouse mesenchymal stem cells; BDCs, bone marrow-derived cells; ADCs, adipose tissue-derived cells; MEFs, mouse embryonic fibroblast cells.