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Faculdade de Farmácia

Management of bone diseases: looking at scaffold-based strategies for drug delivery

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Monografia orientada pela Professora Doutora Ana Francisca Bettencourt,
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Resumo

O tecido ósseo caracteriza-se por uma capacidade autorregenerativa ímpar, completa e contínua. Porém, quando defeitos ósseos causados por distúrbios significativos na homeostase tecidual tomam grandes proporções, este processo autorregenerativo torna-se insuficiente. Além disso a população está cada vez mais envelhecida, sedentária e obesa constituindo sérios fatores de risco para o desenvolvimento de doenças ósseas associadas à insuficiência autorregenerativa e a morbilidade e mortalidade elevadas. Assim, torna-se imperativo descobrir abordagens estratégicas que visem a substituição, remodelação e regeneração óssea. Os "scaffolds" ósseos têm sido usados com sucesso como enxertos ósseos sintéticos há muitos anos. Contudo, estratégias recentes de engenharia de tecido ósseo pretendem explorar a sua multifuncionalidade através da veiculação de fármacos. As doenças ósseas podem ser particularmente difíceis de tratar devido à não-vascularização da cartilagem adjacente, pelo que a administração direcionada de fármacos ao osso pode apresentar vários benefícios. Os mais conhecidos são as concentrações elevadas locais de fármaco, a diminuição do "uptake" sistémico e consequente minimização de efeitos adversos enquanto se assegura um espaço para crescimento de tecido ósseo novo e saudável. Apesar da evolução científica, poucos estudos incidem no uso de "scaffolds" ósseos como sistemas de veiculação de fármacos. Consequentemente, este trabalho tem como objetivo efetuar uma revisão da literatura sobre o uso de "scaffolds" ósseos como sistemas de veiculação de fármacos e assim reforçar o seu potencial para aplicações clínicas. Primeiramente, são apresentadas as propriedades que tornam um "scaffold" ósseo ideal para a veiculação de fármacos. Seguidamente, são descritos os distintos grupos de biomateriais, os diferentes processos de fabrico e de incorporação de fármacos. Posteriormente, é discutido o interesse terapêutico dos "scaffolds" como sistemas de veiculação de fármacos em cinco doenças ósseas – osteomielite, osteoporose, osteoartrite, osteossarcoma e metástases ósseas. Por fim, são examinados os desafios desta possível abordagem terapêutica e as perspetivas futuras. Embora os "scaffolds" ósseos apresentem benefícios terapêuticos ao agirem como sistemas de veiculação de fármacos, é necessário efetuar mais investigações pré-clínicas e clínicas para fortalecer a sua compreensão e permitir a tradução de evidência experimental na prática clínica. O descompasso entre a evolução científica e a regulamentação permanece como um dos principais desafios futuros.

Palavras-chave: scaffolds; veiculação farmacológica; regeneração óssea

Abstract

The bone tissue is peerlessly characterized for its complete and continuous self-regeneration capacity. However, large-scale bone defects caused by major disturbances in the tissue's homeostasis may overpower this self-regenerative process. Furthermore, the ageing population, the increment in obesity incidence, and the sedentary lifestyles are serious risk factors for the development of bone diseases which are associated with failure of the self-regenerative process, high morbidity and mortality rates. Thus, there is an ever-growing need for strategic approaches targeting bone replacement, its remodelling, and its regeneration. Bone scaffolds have successfully been used as synthetic bone grafts for many years yet, recent bone tissue engineering strategies draw the attention to explore their multifunctionality by investigating them as drug delivery systems. Considering the fact that bone illnesses can be substantially challenging to treat due to the avascular nature of the surrounding cartilage, targeted drug delivery to the bone can comprise many benefits. The most well-known are the local high drug concentrations, the diminutive systemic uptake, and subsequent adverse effects minimization whilst a space that secures new, healthy bone tissue growth is maintained. Despite this promising scientific progress, studies underlining bone scaffolds' use as local drug delivery systems are not abundant. In light of this, the present work aims to do a literature review of bone scaffolds' use for drug delivery purposes and thus bring to the fore their potential for clinical applications. Firstly, the properties of an ideal drug-loaded bone scaffold are presented. Next, distinct biomaterial groups alongside the different manufacturing technologies as well as drug-loading methods are described. Then, scaffolds' therapeutic interest as drug delivery systems is discussed for five bone disorders – osteomyelitis, osteoporosis, osteoarthritis, osteosarcoma, and cancer bone metastasis. Finally, the challenges of this possible therapeutic approach and future perspectives are examined. Albeit bone scaffolds show therapeutic benefits by acting as drug delivery systems, further pre-clinical and clinical assessments are needed to strengthen their understanding and enable research evidence translation into clinical practice. The mismatch between scientific evolution and regulatory frameworks remains one of the major future challenges.

Keywords: scaffolds; drug delivery; bone regeneration

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List of abbreviations

BBS: Bovine Bone Substitutes

BTE: Bone Tissue Engineering

CMC-MC-P: Carboxymethyl Chitosan (CMC)-Methylcellulose (MC)-Pluronic (P)

COL: Collagen

CPC: Calcium Phosphate Cement

DDS: Drug Delivery Systems

ECM: Extracellular matrix

FDM: Fused Deposition Modelling

HA: Hydroxyapatite

ISO: International Organization of Standardization

MBG: Mesoporous Bioactive Glass

MIC: Minimum Inhibitory Concentration

MRSA: Methicillin Resistant *Staphylococcus aureus*

MTD: Maximum Tolerated Dose

NanoSiHA: Nanocrystalline silicon substituted hydroxyapatite

nHA: Nanohydroxyapatite

NSAID: Nonsteroidal Anti-inflammatory Drugs

PCEC: Poly(ε-caprolactone)–poly(ethylene glycol)–poly(ε-caprolactone)

PCL: Polycaprolactone

PEG: Polyethylene glycol

PLA: Polylactic acid

PLGA: Poly (DL-lactic-co-glycolic acid)

PLLA: Poly L-lactic acid

PLGA-PEG-PLGA: Poly(D, L-lactide-co-glycolic acid)-poly(ethylene glycol)-poly(D, L-lactide-co-glycolic acid)

PMMA: Poly(methyl methacrylate)

PU: Polyurethane

PVA: Polyvinyl alcohol

RANKL: Nuclear Factor kappa-B ligand

RP: Rapid Prototyping

SC/PL: Solvent Casting/Particulate Leaching

SDF-1**α**: Stromal Cell-Derived Factor-1**α**

SFFT: Solid Free-form Fabrication Techniques

SiHA: Crystalline silicon substituted hydroxyapatite

TCP: Tricalcium Phosphate

TGF-**β**: **β**-Transforming Growth Factor

VEGF: Vascular Endothelial Growth Factor

ZIF8: Zeolitic imidazolate framework-8

3D: Three-dimensional

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1. Introduction

The bone tissue plays a pivotal role in the well-functioning of the body since it provides structural support but also contributes to metabolic activities e.g., calcium and phosphate storage and blood cell production (1). Additionally, the osseous tissue has the unparalleled feature of self-regeneration in a complete, effective and continuous manner (1,2). However, this self-healing process cannot take place alone for large-scale bone defects caused by trauma, infection, tumour resection or in any cases in which the regenerative process is undermined (1,3–5). Besides this important factor, one must consider the demographic and public health issues such as the ageing population, the increment in obesity incidence and the sedentary lifestyles. These are serious risk factors for the development of bone diseases which are associated with failure of the self-regenerative process and also high morbidity and mortality rates. Therefore, the need for efficient bone replacement and regeneration strategies to contradict the negative impact on the health and quality of life emerges (5,6).

At the moment, the gold-standard for bone tissue regeneration enhancement is autologous bone grafting (i.e., bone tissue transplantation from one part to another in the patient's own body) (3,5,7–9). The reason behind resides in its remarkable capacity of inducing cell differentiation as well as cell growth along with its histocompatibility and non-immunogenic character (3,5,9). Regardless, there are a myriad of hindrances and these are mainly related to the fact that a surgical procedure is required: high harvesting cost, post-operative mobility difficulties and a higher risk of infection (3,8,10). Another significant shortcoming is the limited availability of autogenous grafts (5,9,10). Bone allografting (i.e., bone tissue transplantation from cadaveric or living donors) is an alternative to this drawback however, immune rejection is much more likely to occur and there is the possibility of disease transmission from the donor to the patient (3,8–10).

From this perspective, Bone Tissue Engineering (BTE) has been fundamental to investigate safer, more cost-effective and easier to handle alternatives (9). Bone scaffolds are being extensively studied and are becoming a very promising option for synthetic bone grafting. They act as a temporary platform that provides structural support and enables cell and nutrients diffusion for new bone tissue growth (4,5). Designing a scaffold for bone repair and reconstruction does not come as a simple task since a defined set of structural (e.g., porosity and mechanical properties) and biological (e.g., biocompatibility and biodegradability) criteria must be met (5,7). Formerly, the primary concerns were to obtain scaffolds that would be

bioinert and able to fill void space while offering mechanical support (7). Currently, researchers have demonstrated interest in improving scaffolds' functionality by impregnating growth factors or synthetic drugs granting a synergistic therapeutic effect for bone disorders (3,9).

Bone scaffolds' applications as local drug delivery systems (DDS) is a novel BTE strategy and a quite interesting one. Bone diseases treatments are frequently challenging owing to the avascular environment of the adjoining cartilage (11). Drug delivery to the target area through bone scaffolds allows local high drug concentrations, minimizes systemic uptake and therefore drug toxicity while maintaining the space to ensure new healthy bone formation (1,9,11). Other benefits that come with this approach are the sustained drug delivery and the facilitated delivery of hydrophobic drugs. Moreover, refining drug delivery efficiency enables not only a reduction in dose frequency but also in the number of therapeutic agents which in turn leads to better patient compliance (11). Notwithstanding, this strategy also includes challenges related to drug release kinetics, material(s) and manufacturing process eligibility, the need for more pre-clinical and clinical research and regulatory aspects that can delay its use in clinical practice (1,12).

The aim of this thesis is to review and highlight bone scaffolds' potential for clinical applications as local drug delivery systems in bone diseases. Firstly, the properties for ideal drug-loaded bone scaffolds are explained. Secondly, different biomaterial types and their advantages along with their disadvantages followed by distinct manufacturing and drug-loading methods for scaffold fabrication are presented. Thirdly, numerous studies revealing bone scaffolds' interest for clinical use as local DDS in distinct bone illnesses are discussed. Lastly, challenges of this possible therapeutic approach and future perspectives are examined.

2. Methodology

The present work consists in a review of the literature regarding the scientific progress of scaffolds as local drug delivery systems and their potential for clinical applications in bone disorders.

Accordingly, the research was carried out through four main databases - *PubMed*, *Science Direct*, *Nature* and *Google Scholar* – from January to June 2021 using keywords such as "scaffold(s)", "bone", "treatment" and "local drug delivery systems". Depending on the chapter that was being written, some of those terms were combined with more precise ones e.g., "properties", "manufacturing/fabrication technology", "biomaterials", "osteomyelitis", "osteoporosis", "osteoarthritis", "osteosarcoma" and "cancer bone metastasis".

Inclusion and exclusion criteria established three main requirements. Firstly, only articles published between 2012 and 2021 were considered. However, there were two exceptions to this criterion. The first one was due to the reference of a minimum inhibitory concentration (MIC) value that had not been obtained over experimentation in the study hence, it was necessary to include the original article which was from 2004. The second exception was due to the definition of the concept of "biocompatible" which was retrieved from a 2008 article. Secondly, all article types inserted – reviews, mini-reviews, book chapters and research articles (which in turn included *in vitro*, *in vivo* and case series studies) – where written in English. This condition also applied to the sites consulted – clinicaltrials.gov and worldwide.espacenet.com. Thirdly, for the purposes of this work, the concept of "drug" was defined as any substance capable of generating a therapeutic effect whether it being a small drug biomolecule, a growth-factor or a synthetic drug. However, foremost focus was on finding studies that used a synthetic drug as the therapeutic agent.

3. Drug-loaded Bone Scaffolds

Bone scaffold is a three-dimensional (3D) temporary mechanical structure designed to attain one main goal: be able to mimic the extracellular matrix (ECM) of the bone tissue and subsequently, allow a favourable setting for bone remodelling and regeneration processes to occur without major complications (5,13). Complementarily, the scaffold should be positioned in a way that hampers disturbing cells invasion (5).

Drug-loaded bone scaffolds come forward as a very promising BTE approach because they combine two fundamental aspects that are crucial to surpass a major challenge – the singular anatomical features of the bone tissue (1). The scaffold itself acts as a proper structural tool to attain bone repair and remodelling. Drug-loaded bone scaffolds act as local DDS and therefore are capable of specifically and on-site targeting the bone tissue which allows additional treatment of the injured area and healing process enhancement (1,14).

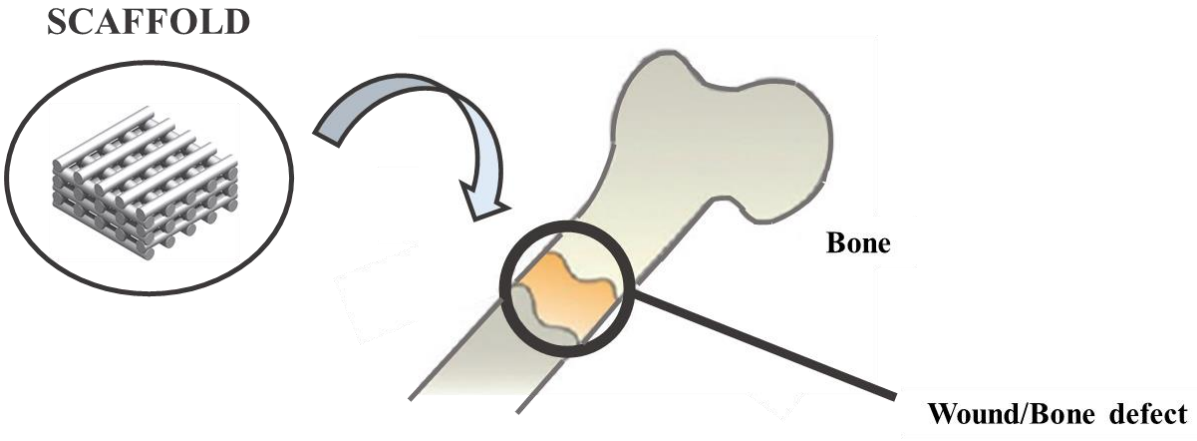

Figure 1 – Illustration of scaffold implantation for bone defect repair.

3.1 Main Properties

In order to be a propitious tool for bone tissue regeneration, a scaffold must fulfil crucial biological, mechanical and physical/geometrical requirements (15,16).

Biocompatibility, biofunctionality and biodegradability

A scaffold that can generate a precise biological response in the tissue where it is implanted must be biocompatible, defined in terms of "the ability of a material to perform with

an appropriate host response in a specific situation" (17). Thus, a biocompatible scaffold must be safe for human use as well as biofunctional, which means able to re-establish the functions of the replaced tissue. Scaffold's "safety" means the capability of supporting normal cellular activity without developing any sort of toxicity (5). Moreover, safety is also related with the scaffold's biodegradation due to the biologic environment (16). As the scaffold degradation occurs, it is awaited an increment in the scaffold's void space (to enable new tissue growth) but also innocuity of its resulting products i.e., so that they can be metabolized and eliminated from the body without or with paltry interference with other organs (12,16). Another key point of biodegradability is that the degradation rate should closely coincide with the tissue growth rate with the aim of maintaining steady properties in the tissue-scaffold environment during the healing and/or regenerative process (5,16).

Osteoconductivity; osteoinductivity; osteogenicity and osteointegrity

In addition to biocompatibility and biodegradability, bone scaffolds need to meet four specific biological requirements (9,16):

i) **Osteoconductivity** corresponding to the ability of enabling cell adherence, expansion and ECM deposition on the scaffold's surface and pores (16,18);

ii) **Osteoinductivity** which is associated to the capability to prompt new bone formation through biomolecular or mechanical stimuli recruiting precursor cells and enabling osteogenic differentiation to originate a certain phenotype or specific lineages (16);

iii) **Osteogenicity** which is associated to the ability to act as osteoblasts' or mesenchymal cells' reservoir because these cells can form and mineralize the ECM of new osseous tissue (16);

iv) **Osteointegrity** corresponding to the scaffold's ability of forming strong bonds with the adjoining bone tissue thus allowing material continuity and appropriate transition load (16,18).

Mechanical properties, wettability, pore size and porosity

When designing a bone scaffold, one must also bear in mind its mechanical properties e.g., elastic modulus, tensile strength, fracture toughness, fatigue and elongation percentage, for the purpose of obtaining mechanical compatibility and integrity and avoid bone loss or stress shielding which can be connected to the use of bone grafts since the implant's stiffness is different from the bone (5,12,16,18). The scaffold's mechanical strength is essential to hold the

structure at the implantation site, which is highly important because the scaffold's mechanical support must be secured until the regeneration process is completed (16,18).

The scaffold's wettability is also important since an adequate wettability heightens protein adhesion and therefore, cell attachment (15).

Lastly, the scaffold's pore size and porosity are very determinant physical/geometrical properties for cell proliferation and differentiation (16). This is due to the fact that a porous structure allows nutrients and oxygen diffusion from the adjoining tissue and excretion of metabolic products from the newly formed tissue. However, a high porosity alone is not sufficient to obtain bone regeneration. The scaffold's pores need to be highly interconnected to ensure cell migration and proliferation along with vascular ingrowth (12,16). The overall porosity and the pore size must confer to the scaffold a large internal surface area. The surface/volume ratio of porous scaffolds relies on the size of its pores. On one hand, a large surface area is indispensable for cell adhesion and proliferation; on the other hand, a large pore volume is necessary to contain and subsequently deliver a cell population capable of triggering healing and/or regeneration processes (16). Regarding drug-loaded bone scaffolds, it is worthy of note that the porosity also plays an important role in drug diffusion and later delivery (13).

The scaffold's design must consider all of the enounced desirable properties altogether since they can influence each other and lead to a compromise e.g., an increment in porosity leads to a decline in mechanical properties and increases the complexity of the scaffold's manufacturing process (16).

In terms of drug-loading, it is additionally required that the scaffold has a maximum loading capacity to enable local release in a controlled and sustainable manner (1,19,20). For that, the drug needs to preferably be homogenously distributed throughout the scaffold and show a stable character alongside binding affinity (19,20). Furthermore, the drug-loaded scaffold should bear and shield the drugs from environmental decomposition factors (1).

Figure 2 summarizes the requirements that an ideal drug-loaded bone scaffold should meet.

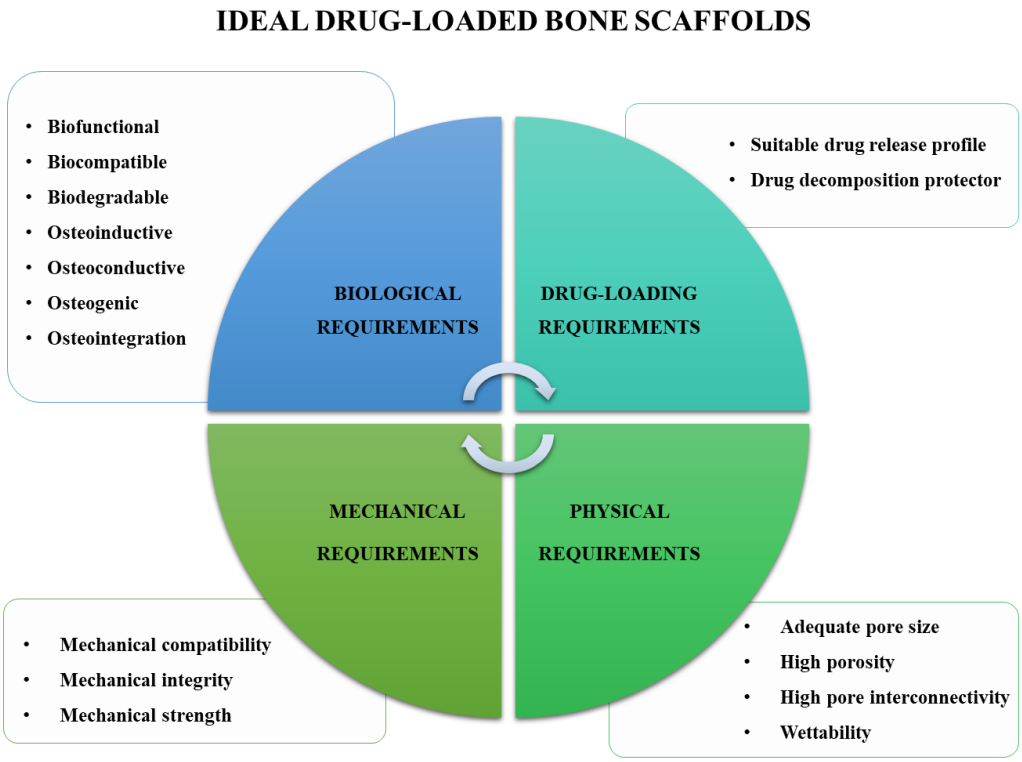

Figure 2 – Main properties that an ideal drug-loaded bone scaffold should present (1,5,12,13, 15-20)**.**

3.2 Biomaterials for Scaffold Fabrication

The selection of the biomaterial(s) for the scaffold's fabrication is pivotal because it will influence the implant's mechanical and biological properties as well as the opted fabrication process (1,15,16,21,22). Other factors that can influence the biomaterial's choice are its processability, malleability, scalability and injectability (5).

The purpose of a biomaterial is to interact with its surrounding biological environment in order to assess, treat, magnify, or replace any tissue, organ or function of the body without causing harm, nor being damaged during the process (16).

According to their chemical composition, the biomaterials can be divided into four main groups: metals, ceramics, polymers (natural and synthetic) and composites (1,15,16,21,22).

Metallic biomaterials have remarkable mechanical properties e.g., mechanical strength, and are capable of promoting osteointegration (1,15,22). Even though these biomaterials are usually corrosion resistant, their use may comprise a risk of corrosion which can lead to ion or

particle leaching and posterior toxicity (5,22,23). Metals can be biodegradable which is the case for magnesium, but the vast majority is bioinert such as titanium and its alloys (22,23).

Ceramics can be divided in two large groups: calcium phosphates and bioglasses. The principal advantage of calcium phosphates is their high chemical but also structural similarity to the mineral content of the bone which enables strong binding between the osseous tissue and the biomaterial's interface (16). This enables great osteoconductivity, osteoinductivity and osteogenicity (1,16,21,23). Furthermore, they can be used as coating components for mechanical strength enhancement purposes (in polymeric scaffolds) or bioactivity inducement (in metallic scaffolds) (16). It is also noteworthy that these materials have been used in drug delivery systems (15). Compounds like hydroxyapatite (HA) and tricalcium phosphate (TCP) are inserted in this group (1,16,22). Bioglasses interest, such as silicate, is mainly due to their strong binding to the bone tissue and osteoinductive ability. In comparison to calcium phosphates, they advantageously have a faster degradation rate (16). The main disadvantage of ceramics is their brittleness (23).

Polymers can also be divided in two major groups - natural and synthetic – and can be used in drug delivery systems (1,15,16,18,21,22). Natural polymers are very similar to ECM and therefore are biodegradable, bioactive and do not demand harsh chemicals for processing (18,21). Notwithstanding, their mechanical properties can be inadequate which affects the scaffold's stability and their processing is not only difficult but also expensive (18). In contrast, synthetic polymers have appropriate mechanical properties, low production costs and their properties can be tailored during the manufacturing process (18,21,23). Nonetheless, they lack similarity with the ECM and therefore biological properties, such as bioactivity, are inferior to natural polymers. Additionally, they lose mechanical strength once degradation starts (for biodegradable polymers) or may have effects of long-term permanence in the body (for non-biodegradable polymers) (18). Besides, the resulting acidic degradation may cause adverse tissue reactions (16,18,22,23). Amongst natural polymers, the most widely used for scaffolds' fabrication are collagen, hyaluronic acid and chitosan. Examples of synthetic polymers are poly(methyl methacrylate) (PMMA), polyurethane (PU), polylactic acid (PLA), polyethylene glycol (PEG), polycaprolactone (PCL), poly-L-lactide acid (PLLA) and poly(DL-lactic-co-glycolic acid) (PLGA) (1,15,16,21–23).

Composites combine two or more biomaterials that differ in composition or morphology (22,23). The principal objective is the improvement in the mechanical, physical, chemical

and/or biological scaffold's characteristics. For example, by merging ceramics to polymers the upcoming expected result would be a multifunctional composite. The ceramics would confer much better osteoinductive properties while polymers would allow to obtain higher processability, degradability rates and control over geometric characteristics (1,16,21,23).

All material groups present different advantages and disadvantages yet, they share one common major limitation: inadequate vascularization (15).

Table 1 summarizes the advantages, the disadvantages and supplies examples for each biomaterial class.

Table 1 - Advantages and disadvantages of different biomaterials used in scaffolds fabrication. (1,5,15,16,18,21–23)

Class of biomaterial	Examples	Advantages	Disadvantages
Metals	Iron (Fe) Magnesium (Mg) Titanium (Ti) and alloys	Outstanding mechanical properties Osteointegration capacity Corrosion resistant (e.g., titanium) Biodegradability (e.g., magnesium)	Risk of corrosion and toxic ion release Vast majority is non-biodegradable/bioinert Inadequate vascularization Elevated stiffness compared to the bone (can cause tissue deterioration)
Ceramics	Calcium phosphates Hydroxyapatite Tricalcium phosphate Bioactive glasses Silicate Borate	Bioresorbable Osteoconductive, osteoinductive and osteogenic Corrosion resistant Bioactive glasses present a faster degradation rate	Brittleness Poor load-bearing function Inadequate vascularization
Polymers	Natural Collagen Hyaluronic acid Chitosan Synthetic PMMA, PU, PLA, PEG, PCL, PLLA, PLGA	Biodegradable and bioresorbable Natural polymers do not demand harsh chemicals for processing and are bioactive Synthetic polymers have better mechanical properties, are less expensive and their properties can be tailored during processing	Natural polymers lack good mechanical properties Synthetic polymers degradation originates acidic products Inadequate vascularization

Table 1 - continue

Class of biomaterial	Examples	Advantages	Disadvantages
Composites	Natural-Synthetic Polymers Ceramics-Polymers	Combination of advantages of different biomaterials	The processing needs to assure the combined advantages are adequately expressed Inadequate vascularization

PMMA: Poly(methyl methacrylate); **PU**: Polyurethane; **PLA:** Polylactic acid; **PEG:** Polyethylene glycol; **PCL:** Polycaprolactone; **PLLA:** Poly L-lactic acid; **PLGA:** Poly (DL-lactic-co-glycolic acid).

3.3 Manufacturing Technology of Scaffolds and Drug-loading

Bone tissue engineering of scaffolds includes a large set of different strategies that allow their production in order to achieve specific properties.

In general, the fabrication processes of scaffolds can be classified in two main groups: conventional and additive manufacturing methods, each one presenting a diverse set of strategies. Additive manufacturing methods are also designated by rapid prototyping (RP) or solid free-form fabrication techniques (SFFT). A schematic illustration of the different manufacturing technologies that can be applied for scaffold construction (12,13,16,18,24) is summarized in Figure 3 and will be detailed next.

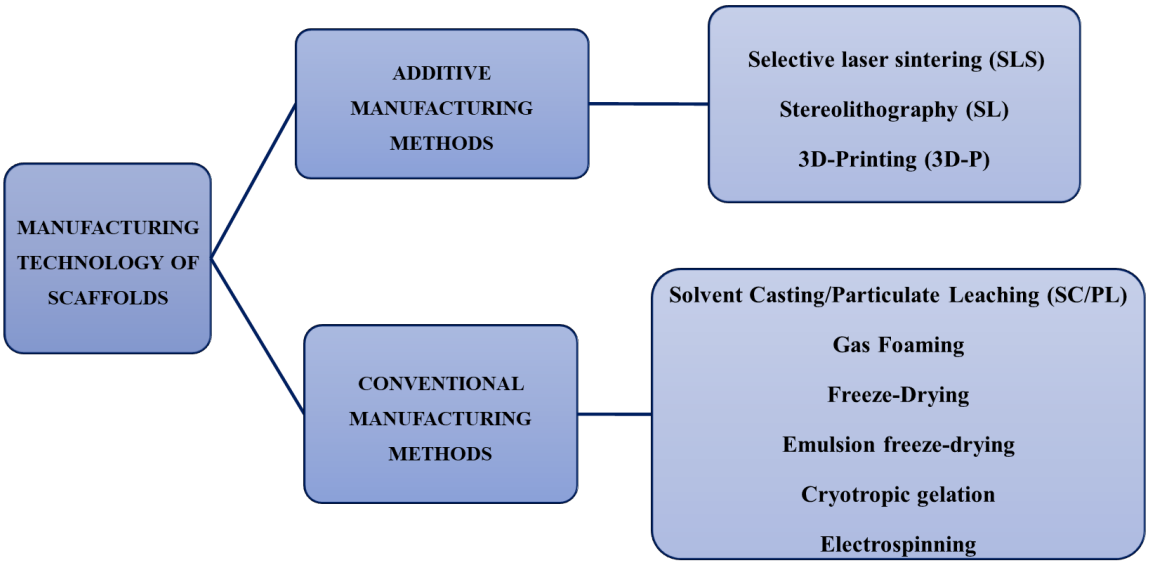

Figure 3 – Conventional and additive manufacturing methods for scaffold fabrication (12,13,16,18,24)**.**

Conventional Manufacturing methods

The biomaterials usually used for scaffold fabrication as drug delivery systems are mainly polymers, therefore the following described manufacturing methods are directed towards polymer-based scaffold fabrication techniques.

Solvent casting/particulate leaching (SC/PL) consists in dissolving a polymer in an organic solvent, mixing it with water soluble salt particles (e.g., sodium chloride or sodium citrate) and casting the resulting mixture into a mould (12,18,24). The solvent is then removed by evaporation or lyophilization and a porous structure is formed by leaching out the salt particles into water. The principal advantages of this method are its simplicity and its cost-effective equipment. However, it only permits to obtain simple scaffold shapes like flat sheets and tubes and the solvent remnants may cause toxicity (12,24).

To surpass this problem, the gas foaming method was developed (12,24). This strategy uses relatively inert gases (e.g., carbon dioxide or nitrogen) to apply pressure to moulded polymers until saturation is reached and the material becomes full of gas bubbles that will grow and assemble leading to the formation of porous sponge-like scaffolds. The major drawback is the lack of pore interconnectivity (12,18,24).

Freeze-drying methodology has also been used for scaffolds' production (13,24). In this case, a synthetic polymer is initially dissolved in a solvent and later cooled down below its freezing point so the solvent's solidification can take place and afterwards its evaporation. This process avoids high temperatures that can deteriorate biological activity of incorporated factors and the pore size can be facilely controlled by adjusting the temperature. Nevertheless, it is a time and energy consuming process, the solvents used present toxicity and the size pore production can be irregular (12). Further, emulsion freeze-drying method combines emulsification with freeze-drying. In this technique, a polymer solution in an organic solvent and water are homogenized. Then, the formed emulsion is quickly cooled down (to conserve the liquid structure) and the solvent and water are removed by freeze-drying (18,24).

Cryotropic gelation is another technique that can be useful to incorporate drugs, in particular growth factors, into scaffolds due to the low processing temperatures (13). It consists in freezing a polymer suspended in water or other solvent to a subfreezing temperature thus allowing for phase separation to occur between the solvent and the precursor solution. The water/solvent crystallizes and the precursor solution undergoes polymerization to form a gel. Once unfreezing occurs, the water/solvent crystals inside the polymerized gel are liquefied,

removed and a porous structure arises. Although it enables pore size control, pore interconnectivity is not assured and it compels additional procedures and moulds to define the outer shape of the scaffold (13).

Electrospinning methodology is many-sided and employs a high, steady electric field applied to a polymer in a solution or viscous state with the aim of diminishing surface tension and then creating fibres in a micrometre to nanometre range (12,13,24). The prime disadvantages are use of organic solvents and the challenging maintenance of structural characteristics after fabrication at a macroscopic scale (12,13).

Additive Manufacturing methods

In recent years, more advanced techniques based on a RP strategy have emerged (12,13,18). For the purpose of this thesis, only 3D-Printing techniques (also known as 3D-Plotting) will be detailed. These methods are defined and explained according to the International Organization of Standardization (ISO) committee ISO/TC 261 document, with the aim of creating a standardized and classified set on additive manufacturing methods (25). However, there is still a lack of harmonization between different authors for scaffolds' production processes definition and description.

3D-Printing is a modern technology that has numerous benefits: it can operate at room temperature which makes it ideal for pharmaceutical agents' incorporation; it is time, cost and set-up effective; finally, it enables a mass and high degree of customized, anatomically shaped scaffolds (12,13). For example, one 3D-Printing technique is based on the distribution of a liquid binder solution (responsible for particle bonding) onto a powder layer through inkjet print heads repeatedly until the formation of a powder bed (12). This process can be direct (also known as Direct-Writing or Robocasting) – the actual scaffold is printed – or indirect – a primary template is produced to work as mould for a secondary one (12,13). The direct process has a better control over the scaffold's architecture, but limits the pore size range available. The indirect method enables a much more versatile pore size range without affecting the printing resolution or the pore's interconnectivity although it restrains the scaffold's shape feature or design due to demoulding hindrance (12,13). Another common 3D-Printing method is Fused Deposition Modelling (FDM) which is an extrusion-based technology (26). This technique involves a thermoplastic material (e.g., polyvinyl alcohol (PVA) or PLA) usually in the filament form in which a pinch roller feed mechanism is needed to push the filament through one or more heated nozzles (24,26). The molten material is deposited onto a platform and creates a

two-dimensional layer. This repeated process leads to successive two-dimensional layers deposition, one on top of another, forming a three-dimensional scaffold (26)**.** This solvent-free method may result in highly porous scaffolds with good mechanical strength but it requires high processing temperature and limits the available material range (24).

Drug-loading methods

There are numerous strategies for drug-loading, namely (1,27):

i) the drug can be directly mixed with the scaffold's material(s) before production;

ii) one can previously incorporate the drug in a drug carrier such as micro/nanoparticles which will be added during the scaffold's production;

iii) optionally coat the scaffold in a polymer or composite solution;

iv) or simply adsorb or impregnate the drug after the scaffold's fabrication.

The selected method must consider factors such as the manufacturing technology of the scaffold, drug stability and its sought-after release rate, and the final application (27). After the fabrication process, it is necessary to determine important parameters that will indicate if the drug-loading was successful likewise drug-loading efficacy, drug release kinetics and mechanism, drug activity and drug stability (1).

Sterilization methods

Another noteworthy step during scaffold's manufacturing process that is rarely referred in the literature is the application of sterilization methods to eliminate possible microorganisms' contamination. Currently, there are not specified sterilization procedures for scaffolds. Thus, conventional methods applied to other dosage forms can be used such as heat treatment or gamma irradiation. Despite the chosen method, it cannot interfere with the scaffold's structural properties or its biocompatibility (8).

4. Interest of bone scaffolds as drug delivery systems

Bone disorders can be difficult to treat even when using standardized treatments due to the avascular environment of the surrounding cartilage which hampers drug delivery to the targeted site (11). Aiming a solution to this problem, drug-loaded bone scaffolds come forward as potentially powerful drug delivery systems. They can locally deliver elevated drug amounts in a controlled manner with limited adverse effects and a slight systemic uptake, which increases not only drug efficacy but also its safe use (1). Nevertheless, there are still some obstacles to overcome: difficult implantation in the osseous tissue, risk of infection and inflammation in the adjoining tissue and possible troubled drug penetration into the targeted site (1).

Bone scaffolds' possible employment as bone-targeted drug delivery systems in clinical applications is vast and the most frequent loaded drugs include antibiotics, bisphosphonates, anti-inflammatory and chemotherapeutic agents (1,14).

Figure 4 displays the bone pathologies addressed in the following subsections alongside the study types included for each one.

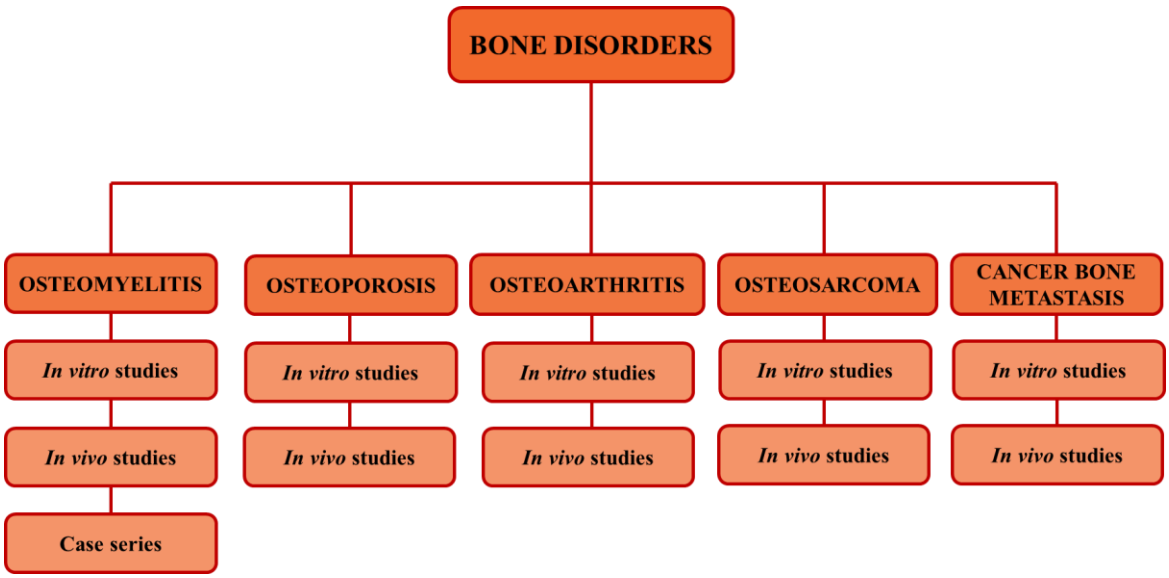

Figure 4 – Summary of the five bone pathologies and different study types included about drug-loaded bone scaffolds' use for treatment purposes.

4.1 Osteomyelitis

Osteomyelitis is a progressive inflammatory condition of the bone which develops most commonly through a bacterial infection caused by the microorganism *Staphylococcus aureus* (1,28). This disease has multiple infection origins e.g., prosthetic-joint infection, post-traumatic infection, vertebral osteomyelitis and diabetic foot infection. It can present itself essentially in two forms: acute and chronic (1). In both cases, the invasion by the bacteria causes an immunologic response that leads to the accumulation of fluid, fibrin, degranulated platelets, red blood cells extraversion and inflammatory cells. This process heightens the intraosseous pressure and consequently the vascularization of the bone becomes defective causing poor blood supply as well as bone necrosis (1,29). Thus, antibiotic delivery through oral or intravenous administration turns practically inefficient (1). In order to accentuate the antibiotics concentration at the infection site as well as to diminish adverse side effects, local antibiotic delivery through scaffolds can be an auspicious therapeutic strategy.

Using diverse manufacturing technologies as well as distinct biomaterials and antibiotics, researchers have tried to understand if scaffolds could be an asset in treating osteomyelitis. Numerous *in vitro* studies along with *in vivo* experiments have shown promising results.

In vitro studies

Different *in vitro* studies (Table 2) showed the high potential of scaffolds with multiple advantages. Dorati *et al.* succeeded in the preparation of highly porous and stable composite scaffolds capable of sustaining a high drug content as well as to rehydrate very rapidly. Moreover, a high gentamicin concentration was locally attainable in only 4 hours and its amount was always found to be superior to the MIC value – 0,002 mg/mL (30). Furthermore, the antibacterial activity against *E. coli* remained for 24 hours. The scaffolds also contained bovine bone substitutes (BBS) which stimulated cell adhesion without interfering with gentamicin's release. Thus, this study points out the advantageous employment of freeze-dried mouldable composite scaffolds loaded with gentamicin, since they present anti-osteomyelitis properties and can be immediately used in surgical procedures (30).

Other studies highlight the importance of using the scaffold material's properties to enhance bacterial eradication (31). For instance, chitosan scaffolds loaded with vancomycin encapsulated in zeolitic imidazolate framework-8 (ZIF8) nanocrystals had a pH-dependent release. It was noticed that the highest antibiotic amount delivered through the scaffold as well

as the more intense bacterial activity reduction occurred at pH = 5,4 which mimicked the environment of an inflamed tissue. These effects are due to chitosan's properties, but also to those of zinc atoms. Chitosan presents a swelling pH-dependent behaviour: there's an electrostatic repulsion between its protonated amino groups which is intensified in acidic conditions causing the polymer network to expand. Consequently, the ZIF8 dissolution speeds and leads to a better leaching out of zinc atoms which in turn present antibacterial properties due to the interaction between its positive charge and the negative charge of substances in the surfaces of bacteria (31). Another study highlighted the advantage of using chitosan as a scaffold material, since it showed that the concentration-dependent toxic cellular effects of the selected antibiotic – moxifloxacin – could be neutralized by this polymer. The authors attributed this result to chitosan's protective properties against oxidative cellular stress (32).

Scaffolds can also be practical to improve antibiotic stability. For example, for the purpose of surmounting the heat lability of rifampicin, an antibiotic used in osteomyelitis treatment, Lee *et al.* experimented to incorporate it in a PCL scaffold using 3D-printing (33) . This decision was due to the fact that PCL has a low melting point (60 º C) and therefore can be printed at low temperatures. Plus, PCL is biodegradable which means that no additional surgical procedure is required to remove it after its implantation. This was a very useful strategy since the scaffolds presented many desirable physical characteristics for the control rate and duration of antibiotics e.g., homogeneous porosity but also a steady release profile adequate for infection treatment. Moreover, no cell toxicity was observed and cell proliferation was not affected by the rifampicin-loaded-PCL scaffolds. Interestingly, the authors also used PMMA, which is non-biodegradable, and calcium phosphate cement (CPC) scaffolds for comparison and concluded that these did not have any advantageous properties to be applied as local drug delivery systems (33). However, recent case series with different osteomyelitis causes and target bones showed quite great outcomes using CPC as a scaffold material, even when comorbidities were present. This difference may be due to the antibiotic used, since in those clinical experiments gentamicin instead of rifampicin was loaded into the scaffolds. On the other hand, the authors also reported many disadvantages in exploiting PMMA as a scaffold material, including its non-biodegradability (34–36).

Emerging strategies are related to the scaffolds' multifunctionalities aiming multiple roles. For example, a recent study focused on the potential of magnetic properties of iron oxide nanoparticles incorporated in a 3D-printed PLA scaffold and loaded with minocycline (37) . The results led to the conclusion that the construct's magnetic properties could be preserved for

a long-term cell culture period and that the presence of a magnetic field in the bone tissue induced osteogenesis and bone regeneration. Moreover, the antibiotic was a great contributor to the activity against *S. aureus* and its loading into the platform did not interfere with iron oxide nanoparticles magneticity nor with cell proliferation (37).

There is also research about scaffolds' capacity of targeting specifically infected bone tissue by resistant strains. Padrão *et al.* reported efficiency against methicillin-resistant *Staphylococcus aureus* (MRSA) using vancomycin loaded in heparinized nanohydroxyapatite/collagen scaffolds (39). Indeed, the influence of temperature in antibiotic release and in scaffold's physical properties was observed. The constructs were produced at two different temperatures: 830 ºC and 1050 ºC. The ones that were fabricated at 1050 ºC presented higher macroporosity and grain size, equal microporosity, but lower nanoporosity than the ones generated at 830 º C. Higher compressive strength was also observed in 1050 ºC produced scaffolds. Although the scaffolds prepared at a lower temperature led to a higher antibiotic release, which was attributed to their larger surface area, both types led to a vancomycin release profile as follows: high initial burst followed by a 19 day-sustained release where vancomycin concentration was always superior to MIC for MRSA – 2 **μ**g/mL (38). The constructs produced at 830 ºC had lower mechanical resistance which interfered negatively with cell viability. Thus, the authors concluded that the scaffolds prepared at 1050 ºC were more suitable and stable for clinical application (39).

It is also important to understand the correlation of scaffolds' physical properties, such as porosity, with its drug release profile. Beenken and co-workers assessed two scaffold formulations with the same material components – hydroxyapatite and polyurethane – ratios but with distinct overall porosity and surface area (40). Each formulation was loaded with three different antibiotics: vancomycin, daptomycin and gentamicin. Their results showed that scaffolds with higher porosity led to increased antibiotic uptake along with its extended release. Furthermore, using the scaffolds with higher porosity, the authors determined drug saturation with the three previously mentioned antibiotics. Drug saturation was attained for all antibiotics in a concentration-dependent manner however, the concentration exposure needed for vancomycin (100 mg/L) was much lower than the one for gentamicin and daptomycin (200 mg/mL). The authors pointed out the fact that these last results only allowed relative conclusions since the concentrations referred had been defined by the volume of buffer used for antibiotic elution and frequency of sampling (40)**.**

Table 2 - Examples of drug-loaded scaffolds for osteomyelitis treatment (*in vitro* studies)

Scaffold Material(s)	Manufacturing Technology	Antibiotic(s)	Bacterial strain	Cell line	Main findings	Ref.
Chitosan	Freeze-Drying	Gentamicin (4 mg/mL)	*E. coli*	Human adult dermal fibroblasts	• Suitable properties for filler in bone surgery; • Stable; • Possible immediate use; • Anti-osteomyelitis properties.	(30)
Chitosan	Wet Spinning	Vancomycin (25 mg)	*S. aureus*	MC3T3-E1 pre-osteoblasts	• Considerable reduction of *S. aureus*; • pH-dependent drug release; • Good support for cell proliferation.	(31)
PCL	3D-Printing	Rifampicin (mixed with PCL powder in 4 different ratios – 20:1; 10:1; 5:1; 2,5:1)	*E. coli* *S. aureus*	Human osteoblasts	Growth inhibition activity against *E. coli* and *S. aureus.*	(33)
Heparinized nHA/COL	Polymer sponge Replication method	Vancomycin (50 mg/mL)	*S. aureus*	L929 fibroblasts	• Mechanical strength increment with porous macrostructure maintained; • High vancomycin concentration release (above MIC); • No cytotoxic effect.	(39)
Calcium phosphate Chitosan	*In situ* precipitation method	Moxifloxacin (scaffolds were physically mixed with this antibiotic at a ratio of 3:1)	MRSA ATCC 33591	Osteoblast-like MG-63 cells	Scaffolds with higher chitosan content presented good cell viability.	(32)

Table 2 - continue

Scaffold Material(s)	Manufacturing Technology	Antibiotic(s)	Bacterial strain	Cell line	Main findings	Ref.
Chitosan	3D-Printing	Minocycline (0,5 mg/mL)	*S. aureus*	Osteoblast-like MG-63 cells hBMSCs	• Magnetic properties; • Remarkable bioactivity; • Cytocompatible.	(37)
HA PU	Multistep approach (involving a salt leaching step)	**Vancomycin** Daptomycin Gentamicin (1.0 mg/mL; 10,0 mg/mL; 100,0mg/mL for each antibiotic)	*S. aureus*	N.A.	Scaffolds with higher porosity led to increased antibiotic uptake and its extended release.	(40)

Ref.: Reference; ***E.coli*:** *Escherichia coli*; ***S.aureus*:** *Staphylococcus aureus*; **nHA:** Nanohydroxyapatite; **COL:** Collagen; **MRSA:** Methicillin-resistant *Staphylococcus aureus*; **hBMSCs:** human bone marrow derived mesenchymal stromal cells; **HA:** Hydroxyapatite; **PU:** Polyurethane; **N.A.:** Non applicable.

In vivo studies

Distinct *in vivo* experiments (Table 3) corroborate the findings observed in the *in vitro* studies previously mentioned, even when using different antibiotics. For example, after *in vitro* assays, chitosan-calcium phosphate composite moxifloxacin-loaded scaffolds showed to be useful in preventing post-operative osteomyelitis in a rabbit model while exhibiting osteogenic and osteoconductive properties (32).

After performing the *in vitro* elution assay described earlier, Beenken *et al.* wondered if the antibiotic concentration was sufficient to prevent infection in a contaminated bone defect (40). For this purpose, they only used vancomycin because it provides a broader therapeutic range. The results showed that vancomycin-loading into the scaffolds in concentrations ≥ 50 mg/mL was sufficient to prevent or at least limit infection development in bone defects and that it did not interfere with the scaffolds' bone regenerative properties. However, the study presented some limitations. It could not prove that lower antibiotic concentrations could completely eradicate all viable bacteria and the experiment time period was 84 days therefore, new bone tissue formation and scaffolds resorption was not completely assessed in every case (40).

Another experiment in a rabbit model using levofloxacin-loaded (5 mg) mesoporous silica microspheres incorporated in a nanohydroxyapatite/polyurethane composite scaffold

demonstrated efficiency in treating chronic osteomyelitis with bone defects. The construct obtained was highly porous which is a major factor for new bone formation and permitted not only cell adhesion as its proliferation. Furthermore, it was demonstrated that the scaffolds could not only degrade at the end of 12 weeks but also that its resulting products contributed to new bone formation (41).

In the previously described studies in this segment, the authors used composite scaffolds. However, similar outcomes can also be obtained when using scaffolds fabricated with a single biomaterial. For instance, Bhattacharya *et al*. tested their ceftriaxone-sulbactam loaded hydroxyapatite scaffold in a rabbit model (42). The 42-days experiment showed a release superior to MIC of ceftriaxone-sulbactam against *S. aureus* compared to parenteral antibiotic administration. The histological findings indicated that bone healing and remodelling had occurred, which the authors attributed to the scaffold material, and that the infection was under control due to the antibiotics (42).

Table 3 - Examples of drug-loaded scaffolds for osteomyelitis treatment (*in vivo* studies)

Scaffold Material(s)	Manufacturing Technology	Antibiotic(s)	Bacterial strain	Animal model (Bone)	Main findings	Ref.
HA	Multistep approach	Ceftriaxone–sulbactam (500 mg/mL; the antibiotics' ratio was 2 sulbactam:1 ceftriaxone)	*S. aureus*	24 New Zealand white rabbits (1.5–2 kg b.wt., 8 months to 1 year old) as per Norden model **(Tibia)**	• Moderately high drug adsorption efficiency; • Efficient drug release up to 42 days; • Bone healing and remodelling.	(42)
nHA PU	*In situ* Foaming Method	Levofloxacin (1 mg; 5 mg)	*S. aureus*	60 New Zealand White rabbits (wt.: 2–4 kg) by the Norden method **(Tibia)**	• Higher efficiency for bone defects' treatment when using scaffolds loaded with 5 mg of levofloxacin; • High porosity; • Biodegradability (12 weeks) that contributes for bone formation; • Mechanical support for bone repair.	(41)

Table 3 - continue

Scaffold Material(s)	Manufacturing Technology	Antibiotic(s)	Bacterial strain	Animal model (Bone)	Main findings	Ref.
Calcium phosphate Chitosan	*In situ* precipitation method	Moxifloxacin (scaffolds were physically mixed with this antibiotic at a ratio of 3:1)	MRSA ATCC 33591	12 white New Zealand male rabbits (wt.: 3–3.5 K g) **(Tibia)**	● Promising results in osteomyelitis prevention; ● Bacterial load significantly reduced as well as inflammation and bone necrosis.	(32)
HA PU	Multistep approach (involving a salt leaching step)	Vancomycin (25 mg/mL; 50 mg/mL; 75 mg/mL; **100 mg/mL**)	*S. aureus*	Rabbit model of post-surgical osteomyelitis **(Radius)**	● Effective loading with several antibiotics; ● Infection prevention or its development limitation without compromising the scaffold's bone regenerative properties.	(40)

Ref.: Reference; **HA**: hydroxyapatite*;* ***S.aureus*:** *Staphylococcus aureus*; **nHA:** Nanohydroxyapatite; **PU:** Polyurethane; **MRSA:** Methicillin-resistant *Staphylococcus aureus*.

Case series

After *in vitro* and *in vivo* assays, it is important to conduct human studies aiming the translation of pre-clinical experimental evidence to clinical use. Nowadays, there have been few human studies reported, using a small number of patients (case series) as indicated in Table 4.

After performing the *in vivo* experiment, Bhattacharya *et al*. assayed their scaffolds in patients who suffered from osteomyelitis and that had had recurrent surgical interventions prior to the study (42). The results obtained were consistent with the ones observed in the *in vivo* experiment: the antibiotic-hydroxyapatite-loaded scaffolds biodegraded and were successful to contribute to bone repair without leading to new osteolysis, periosteal changes or adverse reactions, independently of the bone site of infection (tibia, mastoid bone or mandible). However, in one case there was partial extrusion of the implant only 5 weeks after the procedure took place even though osteomyelitis was healed. The authors attributed this occurrence to the loss of contact with healthy living bone (42).

McNally *et al.* presented a prospective series of 100 cases with infection including 105 bones where a single-stage protocol for chronic osteomyelitis was conducted. All patients were examined at a range of 12 to 34 months, except for one patient who died of a drug overdose five months into the study (35). Cerement G, which is a composite material of calcium sulphate and hydroxyapatite, loaded with gentamicin, was utilized as a dry dead cavity filler. The authors established two outcomes. The primary one consisted in annihilation of infection at a minimum of one year after surgery. It was considered that treatment had failed whenever there was a recurrent infection with positive cultures from further radiologically guided aspiration or biopsy; recurrent sinus formation; further surgery performed for infection or any patient in need of antibiotic treatment for continuing symptoms. Secondary outcomes were death, need for re-operation, pathological fracture at the site of operation procedure and disruption of wound recovery. Chronic osteomyelitis was eradicated in 96% cases, which means recurrence of infection only occurred in four patients. At final follow-up, all four patients stayed infection-free at 13, 16, 17 and 20 months after revision surgery. The death of three patients was determined as unrelated to the study. In total, 94 patients had normal wound healing and six patients had a white wound injury drainage, most likely due to dissolved calcium sulphate remnant. However, there were no cases of recurrent infection in early wound drainage. In total, 11 patients showed minor extraosseous outflow of cerement G into the adjoining soft tissue but there was no correlation found with the volume of the material used, the site of infection or the soft-tissue cover. The patients were asymptomatic and in 90,9% cases the material was reabsorbed. This event occurred 9 times in the first 50 patients, on the other hand in the second 50 it only happened twice – which the authors attributed to the improvement of material handling and wound closure (35).

A more recent study reported the potential of cerement G as an endcap in amputation stumps (34). A series of 13 patients presented lower limb amputation for chronic medullary osteomyelitis. Cerement G was used as an endcap at the time of the primary amputation through an injection into the medullary canal of the stump. The main goals were to control haemorrhage while delivering locally to the bone high doses of antibiotic to cure any residual infection as well as for prophylaxis purposes. Out of the 13 patients included, 12 had below-knee amputation and 1 an above knee amputation. In all cases, an amputation procedure was necessary because of chronic osteomyelitis with the fault of limb salvage operation. In addition, the infection was present in more than 6 months and there were several former debridement approaches along with systemic antibiotic therapy. Still, bone and soft tissue infection remained

untreated. The cerement G strategy didn't lead to any leaking or surgical site infection during the minimum follow up of 12 months. No haemorrhage, hematoma or seroma were observed neither osteomyelitis stump relapse or need for revision surgery. Hence, the healing process was satisfactory and led to successful prosthetic fitting (34).

Niazi *et al.* carried out a multicentre study and performed a retrospective review of patients who had a foot ulcer concomitantly with osteomyelitis (36). The therapeutic strategy was found effective when osteomyelitis was eradicated along with the standardization of inflammatory parameters and ulcer healing/stable ulcer. On the other hand, it was considered unsuccessful when eradication of infection had failed or recurrence of osteomyelitis/ulcer at the identical area appeared within 4 months of intervention. The study involved 70 patients with diabetic feet. All patients had foot ulcerations. Cerement G was employed as an adjuvant for dead space management. A white discharge was found in some cases however, it didn't raise any disturbance. The standard time for ulcer healing was 12 weeks. Patients were followed up in a meantime of 10 months. During that time, one patient died due to unrelated causes, eradication of osteomyelitis was attained in 90% cases and ulcer healing in 81% cases. Treatment was found unsuccessful in 10% cases. Thus, the authors considered local antibiotic delivery systems could be useful against residual biofilms and allow diffusion of the drug in areas with insufficient blood supply (36).

Although literature reports of scaffolds' application as DDS in osteomyelitis treatment are particularly vast for *in vitro* assays and that *in vivo* assays show a lot of potential, clinical studies with this purpose are very scarce. Indeed, the few clinical studies presented above have some limitations, which in some reports were also pointed out by the authors themselves, such as: a small number of patients involved, lack of control groups and a curt follow-up time period (34–36). Additionally, most of the clinical results were obtained with cerement G which is a biocomposite material loaded with gentamicin as the antibiotic; it would be interesting to find out if similar outcomes are observed when different material(s) and/or antibiotics are applied.

Table 4 - Examples of drug-loaded scaffolds for osteomyelitis treatment (case series)

Scaffold Material(s)	Antibiotic(s)	Bacterial strain	Study design	Main findings	Ref.
HA	Ceftriaxone–sulbactam (90 – 110 mg per 0,6 ± 1,0 g of the selected scaffold's formulation)	*S. aureus*	● 8 chronic osteomyelitis resistant patients in tibia, 1 in the temporal bone and 1 in the mandible; ● Age range: 18 to 54 years old.	● Average healing time of chronic osteomyelitis: . tibia: 9 months . mastoid bone: 14 months . mandible: 3 months ● Evidence of biocompatibility, biodegradability, and progressive bone repair in all cases; ● No evidence of either residual or new osteolysis. No adverse reaction to the local antibiotic implants.	(42)
Calcium sulphate HA	Gentamicin (17,5 mg/mL)	*S. aureus* (mainly)	● 100 patients; ● Mean age: 51.6 years old; Treatment according to a single-stage protocol for chronic osteomyelitis.	● 96% success rate; ● Adverse events were uncommon; ● Efficiency in chronic osteomyelitis treatment.	(35)
Calcium sulphate HA	Gentamicin (17,5 mg/mL)	*S. aureus* (mainly)	● 70 diabetic patients; Mean age: 68 years old.	● 90% infection eradication success rate; ● Mean time to ulcer healing: 12 weeks; Preventive reinfection management.	(36)
Calcium sulphate HA	Gentamicin (17,5 mg/mL)	Coagulase negative staphylococci (mainly)	● 13 patients with lower limb amputations for chronic medullary osteomyelitis; ● Local antibiotic eluting absorbable composite used as an endcap; Mean age: 59 years old.	● All wounds healed uneventfully without any surgical site infection; ● No instances of recurrent stump osteomyelitis or need for revision surgery; Satisfactory healing and successful prosthetic fitting.	(34)

Ref.: Reference; **HA:** hydroxyapatite*;* ***S.aureus*:** *Staphylococcus aureus*; The combination of calcium sulphate with hydroxyapatite and gentamicin-loading is designated as **cerement G.**

4.2 Osteoporosis

Osteoporosis is a common chronic systemic disease in which there is an imbalance between bone formation and its resorption carried out by osteoblasts and osteoclasts, respectively (43,44). Thus, bone tissue loss and bone microarchitecture degeneration take place (44). The decline in bone density and bone quality promotes bone fragility which in turn increases risk of fracture, even when a low force is applied (1,44). Usually, vertebrae, hip and distal radius are the main fracture regions and are associated with higher morbidity and mortality (1,45). Advanced age plus a decrease in oestrogen levels in menopause are related with osteoporosis occurrence (44). However, many other factors might be involved like nutrition deficiencies, inflammatory diseases or corticosteroids therapy (1,43,44). The main goal of osteoporosis treatment is the reduction in risk of fractures which can be difficult due to the poor bone healing ability (1,45). Scaffolds' use as local drug delivery systems may be advantageous because it can secure desirable amounts of drug at the bone level, enhance drug bioavailability and thereby enhance osteointegration and bone healing (43).

Tables 5 and 6 summarize the studies herein forward discussed. Distinct fabrication technologies as well as different materials and bisphosphonates have been assayed for a better understanding of the therapeutic interest of scaffolds as DDS in osteoporosis.

In vitro and in vivo studies

Alendronate is one of the first-line therapeutic agents for osteoporosis treatment (1,45). A fairly recent study reported that this drug's incorporation in collagen scaffolds resulted in high porosity and surface uniformity as well as an interconnected structure after crosslinking (between alendronate and collagen) had occurred (46). Plus, it did not interfere with the scaffolds' structure and could be consistently released during a minimum of 1 month. This fact was attributed not only to the blockage of direct drug exposure due to the spongiform constructs but also to the cross-linkage that contributed to the drug being more strictly locked in the scaffolds. The loaded scaffolds were shown to be biocompatible and capable of generating osteogenic differentiation. When inserted in an animal model, they could biodegrade in a 3-month time period, repair osteoporotic defects and resist bone loss. However, mineral content and mechanical strength evaluation were lacking in this experiment. The authors also referred that further validation for bone resistance loss could have been executed with a positive control. Nonetheless, they also mentioned that scarce studies on the application of biomaterials in osteoporosis therapeutic strategies conjointly with poor employment in clinical practice make

it quite hard to have a positive control (46). Van Houdt *et al.* also mentioned the influence of alendronate concentration in scaffolds as for drug release kinetics, having reported better outcomes with lower drug concentrations – 0,5% wt. – which were dependent on composite materials' degradation (47). The latter was observed at the end of 12 weeks. The constructs demonstrated acceptable clinical setting times (< 20 min), although alendronate slightly decreased the scaffolds' compressive strength. Interestingly, the *in vivo* assay showed that bone formation improved at the peri-defect region but not within the defect site itself. Still, it was concluded that the alendronate-loaded scaffolds could be valuable for clinical application (47).

Literature findings are not always consistent in describing positive or negative results related to the use of scaffolds in osteoporosis treatment. For example, Gómez-Cerezo *et al.* reported zoledronate incorporation into regular porous scaffolds was responsible not only for osteoblasts' apoptosis and lack of anti-osteoclastogenic activity, but also for fibrotic tissue infiltration accompanied by an inflammatory response (48). In fact, it was stressed out that the incorporation of zoledronate led to loss of regenerative properties of the scaffolds' mesoporous bioactive glass (MBG). A possible explanation given for this discovery was the development of osteoclasts' apoptosis resistance to the drug through inhibition and/or activation of enzymatic pathways. Notwithstanding, the *in vivo* experiment stated that zoledronate's concentration at the defect site was considerably elevated which could elucidate the inflammatory onset. The authors declared that this was most likely due to the great porosity of MBG, since the drug amount loaded into the scaffolds corresponded to the common dosage intravenously administered in humans: 4-5 mg/100 mL infusion once a year (48). On the other hand, Bai *et al.* obtained *in vitro* and *in vivo* results that significantly encourage zoledronate loading in scaffolds to enhance osteoporotic bone defect repair. Moreover, zoledronate release was found to be a great contributor for osteogenic differentiation, which allowed mineralization, and osteoclastic inhibition (49). Although these two studies show very contradictory outcomes, one must consider that the materials and the animal models used in each one were quite different.

So far, most of the studies here described used composite materials, polymers or ceramics. Regardless, metallic materials can also be useful. Through a rat model, Ray and co-workers showed that iron foam cells when coated with strontium or a bisphosphonate could be beneficial for new bone formation (50). An extremely recent report highlights strontium's pro-osteoblastic and anti-osteoclastic activity along with its profitable value in scaffolds' use as drug delivery systems (43). Despite this discovery, iron's degradation may lead to metal particles release and subsequently negatively affect cell viability. Thus, additional research is

necessary to a better understanding of the deterioration process and its impact on the bone tissue (50).

Bisphosphonates are first-line agents in osteoporosis treatment (1,45). Yet, there are therapeutic alternatives that also have antiresorptive action e.g., receptor activator of nuclear factor kappa-B ligand (RANKL) antibody – Denosumab – or selective oestrogen receptor modulators (SERM) – raloxifene (45). The latter was recently applied in 3D scaffolds and successfully improved osteoporotic bone regeneration (51). Other studies point out the importance of bone vascularization for bone osteoporotic defects regeneration. For example, Casarrubios *et al.* used vascular endothelial growth factor (VEGF) adsorbed silicon-scaffolds in two forms – nanocrystalline (NanoSiHA) and crystalline (SiHA) (52). No significant difference of VEGF adsorbed amount was observed between the scaffolds. The authors noticed that the growth-factor release was very low, thus indicating that it remained active while immobilized in the scaffolds. However, *in vitro* results showed that scaffolds' functionalization with VEGF led to endothelial cell proliferation similarly in both scaffolds whilst pre-osteoblasts cells could only differentiate in SiHA scaffolds. These results were strongly correlated through the translation to a post-menopause osteoporotic sheep model. SiHA scaffolds associated with VEGF evidenced a much higher bone growth along with a high presence of blood vessels and the inflammatory response was lower than in the sheep that were treated with NanoSiHA VEGF-functionalized implants (52).

Most of the above depicted studies (46–50) only incorporated alendronate or zoledronate as bisphosphonates. It would be interesting to figure out if similar findings could be obtained with ibandronate or risedronate. Likewise, the present works lack representative studies in which alternative therapeutic agents are employed. Another limitation is that all animal experiments consisted in mimicking a post-menopause osteoporosis scenario, but other causative factors and respective treatment response were not analysed. The literature shows evidence of drug-loaded scaffolds' potential clinical benefit in osteoporosis. Notwithstanding, up to date there are no completed or in-process clinical studies nor case reports regarding scaffolds loaded with synthetic drugs. However, it is of note that clinical trials involving more than 400 patients demonstrated good outcomes in open tibial fractures by employing recombinant bone morphogenetic protein 2 (rhBMP-2) impregnated in collagen sponge scaffolds (53). Further investigation should insight in a better understanding of the risk-benefit relation of scaffolds' use in animal models before scaling up to human models.

Table 5 - Examples of drug-loaded scaffolds for osteoporosis treatment (*in vitro* studies)

Scaffold Material(s)	Manufacturing technology	Therapeutic agent(s)	Cell line	Main findings	Ref.
Calcium Phosphate Cement PLGA	Ceramic/ Polymer paste extrusion (needle)	Alendronate (0,5% wt.; 5,0% wt.)	N.A.	• Appropriate setting time; • Appropriate compressive strength; • Controlled drug release dependent on composite degradation.	(47)
Silicon Substituted Hydroxyapatite in 2 different structures: crystalline (SiHA) and nanocrystalline (NanoSiHA)	Robocasting method	VEGF (adsorbed amount not specified)	EC2 MC3T3-E1 pre-osteoblasts	SiHA hinders pre-osteoblast proliferation, but when it is functionalized with VEGF it enhances the biological functions of pre-osteoblasts and of endothelial cells.	(52)
Mesoporous Bioactive Glass-polycaprolactone	Robocasting method	Zoledronic acid (4,15 mg – 1% in wt. of the scaffold)	Human Saos-2 osteoblasts	• Remarkably biocompatible, presenting osteoclast differentiation impediment time-dependent **only when the scaffolds are not drug-loaded**; • Zoledronic acid's incorporation leads to osteoblasts' apoptosis and lack of anti-osteoclastogenic activity.	(48)
Titanium Poloxamer 407 hydrogel	Robocasting method	Zoledronate (10 **µ**g/mL)	BMSCs	• Good biocompatibility; • Sustained drug release along with hydrogel degradation; • Cell proliferation and osteogenic differentiation; • Osteoclast differentiation inhibition.	(49)
Type I Collagen	Freeze-Drying	Alendronate (mixed with a collagen suspension in a 1:1 ratio)	BMSCs	Sustainable release of alendronate for osteoporotic bone defects.	(46)

Ref.: Reference; **PLGA:** Poly (DL-lactic-co-glycolic acid); **SiHA:** crystalline silicon substituted hydroxyapatite; **NanoSiHA:** nanocrystalline silicon substituted hydroxyapatite; **VEGF:** Vascular Endothelial Growth Factor; **EC2**: endothelial cell line; **BMSCs**: Bone Marrow Stem Cells.

Table 6 - Examples of drug-loaded scaffolds for osteoporosis treatment (*in vivo* studies)

Scaffold Material(s)	Manufacturing Technology	Therapeutic agent(s)	Animal model (Bone)	Main findings	Ref.
Iron (Fe)	Foam Replication Method	Strontium (Sr) (0,2 mg/cm^2) Zoledronate (35 **µ**g)	60 bilaterally ovariectomized Sprague-Dawley rats **(Femur)**	• Bone formation enhancement at the implant interface and mineralized tissue; • Increased osteoblast and decreased osteoclast activity; • Tissue in-growth into the scaffolds.	(50)
Calcium Phosphate Cement PLGA	Ceramic/ Polymer paste extrusion (needle)	Alendronate **(0,5 wt.)**	12 female, mature Wistar rats subjected to bilateral ovariectomy **(Femur)**	Stimulatory effects on bone formation.	(47)
Silicon Substituted Hydroxyapatite in 2 different structures: crystalline (SiHA) and nanocrystalline (NanoSiHA)	Robocasting method	VEGF (Adsorbed amount not specified)	6, 4-year-old female Merino sheep subjected to bilateral ovariectomy **(Different limb locations)**	SiHA scaffolds functionalized with VEGF resulted in enhanced bone vascularization, high ossification degree and higher presence of osteoblasts.	(52)
Mesoporous Bioactive Glass-polycaprolactone	Robocasting method	Zoledronic acid (4,15 mg – 1% in wt. of the scaffold)	6, 4-year-old female Merino sheep ovariectomized **(Different limb locations)**	• Excellent bone regeneration properties, at both the peripheral and the inner parts of scaffolds and high vascularization **when the scaffolds were not drug-loaded;** • Bone healing inhibition and intense inflammatory response when zoledronic acid was loaded in the scaffolds.	(48)
Titanium Poloxamer 407 hydrogel	Robocasting method	Zoledronate (10 **µ**g/mL)	5-month-old female New Zealand rabbits ovariectomized **(Femur)**	Significant osteointegration enhancement.	(49)

Table 6 - continue

Scaffold Material(s)	Manufacturing Technology	Therapeutic agent(s)	Animal model (Bone)	Main findings	Ref.
Type I Collagen	Freeze-Drying	Alendronate (mixed with a collagen suspension in a 1:1 ratio)	10-week-old female Sprague-Dawley rats subjected to bilateral ovariectomy **(Parietal bone of the skull; Femur)**	• Bone regeneration in defect area; • Promotion of trabecular bone formation.	(46)

Ref.: Reference; **PLGA:** Poly (DL-lactic-co-glycolic acid); **SiHA:** crystalline silicon substituted hydroxyapatite; **NanoSiHA:** nanocrystalline silicon substituted hydroxyapatite; **VEGF:** Vascular Endothelial Growth Factor.

4.3 Osteoarthritis

Osteoarthritis is a multifactorial, chronic, progressive joint disorder in which joint inflammation, cartilage tissue damage and loss of synovial fluid are present (1,54). Cartilage loss results from the imbalance of chondrocytes anabolic activity and the catabolic activity of enzymes that degrade the extracellular matrix (54,55). This process starts at the joint surface and as the disease progresses, spreads to the subchondral bone matrix, periarticular muscles and peripheral nerve (1). This disease can affect small joints (like those in the hand) and larger ones (suchlike knee and hip joints) (56). There are many risk factors which include ageing, female sex, obesity or joint injury. Main symptoms are pain, stiffness and functional impotence (56,57). Therefore, osteoarthritis is related to a higher morbidity and mortality (55,56). Numerous pharmacological therapies with the aim of pain management are available however, nonsteroidal anti-inflammatory drugs (NSAID) are the first-line therapeutic agents (54,56). Despite that, their oral administration is associated with cardiovascular and gastrointestinal toxicity (1,55,56). Local drug delivery through intra-articular injection can resolve this impasse, refine drug stability and its pharmacokinetics as well as targeting specific sites (55).

Several studies using diverse fabrication technologies, variant scaffold materials and drugs will be herein forward displayed for therapeutic potential analysis in osteoarthritis. They are summarized in Tables 7 and 8.

In vitro and in vivo studies

Diverse known natural polymers as collagen, chitosan or others can be used for scaffold construction. Even though cellulose acetate is not a common choice, Tsiapla and co-workers

successfully fabricated cytocompatible and biodegradable dexamethasone-loaded cellulose acetate scaffolds (58). It is also worthy of note that the selected drug has anti-inflammatory properties but is not part of the first-line treatment agents for osteoarthritis. Dexamethasone release profile showed to be biphasic: there was an initial burst release lately followed by a controlled one. It was also noted that the drug retarded the scaffolds' degradation rate due to its hydrophobicity and the scaffolds' diameter. Nonetheless, the authors considered it a potentially valuable option to avoid acute inflammation associated to implantation and its failure in bone replacement procedures (58).

According to a recent experiment, nanoparticles can act as cross-linkers and reinforce hydrogels structure (59). On the other hand, meloxicam can increase gelation time and hydrogel swelling due to its hydrophobicity with the capacity to weaken hydrophilic polymeric interactions. Nevertheless, the scaffolds obtained had a stable three-dimension that contributed to outstanding biocompatibility as well as cell viability and proliferation. A limitation of this study was the lack of clarity on meloxicam's amount previously loaded into the nanoparticles (59).

As previously showed, albeit NSAIDs are the main treatment choice, there are studies reporting the use of other anti-inflammatory drugs and growth factors in delivery systems. Mohammadi *et al*. combined prednisolone and **β**-Transforming Growth Factor (TGF-**β**) in a hyaluronic acid-collagen scaffold (60). The authors also referred to collagen's importance as a crosslinking agent for better mechanical properties maintaining an alluring porosity and pore network for tissue proliferation. A comparison between 2 dosage forms was made. Even though gel formulations exhibited a more controlled drug release profile, implantable disks were found to be more durable, having better mechanical properties and bigger cartilage similarity (60). A previous study using growth factor delivery alone through thermosensitive hydrogels determined these systems could present desirable viscosity, microstructure porosity and lead to a suitable release profile (owing to protection from the hydrogel). Additionally, great cartilage *in vivo* repair occurred at an 8-week time period (61). Moreover, small drug molecules such as gefitinib can also be delivered via acellular scaffolds while being administered by the intra-articular route. Once more, collagen scaffolds showed to be opportune by presenting good biocompatibility and *in situ* integration (62). When combined with gefitinib, they overhauled meniscal defect and osteoarthritis progression with no adverse effects. However, some limitations were present like *in vivo* models not being able to fully represent human physiological conditions and scaffolds' sustainability with quotidian tasks need of further

investigation (62). Hydroxyapatite-based scaffolds loaded with two distinct growth factors – Stromal Cell-Derived Factor-1**α** (SDF-1**α**) and TGF-**β**3 – were newly assessed in a porcine model (63). Even though SDF-1**α** portrayed effective bioactivity *in vitro*, it led to *in vivo* poor cartilage regeneration response. On the other hand, TGF-**β**3 provided good outcomes in both assessments. Nevertheless, the authors referred a major limitation to the study: lack of significance. Another negative point was the mechanical variability of scaffolds within and between joints. Thus, this study highlighted the importance of experimental evidence translation (63).

Overall, the prime limitations screened across the referred studies are need of further scaffold characterization, especially in terms of mechanical properties, and that most of *in vivo* assays relied on knee joints representation. It would be interesting to analyse if similar findings can be obtained in other commonly affected joints such as the hip. Plus, most of the reported experiments did not use the first-line therapeutic agents (NSAIDs) for drug delivery. Future studies should insight on this purpose. It is also of note that some clinical studies using scaffolds have already taken place, but not as local drug delivery systems (53,64). This hypothesis is yet to be further validated.

Table 7 - Examples of drug-loaded scaffolds for osteoarthritis treatment (*in vitro* studies)

Scaffold Material(s)	Manufacturing technology	Therapeutic agent(s)	Cell line	Main findings	Ref.
Cellulose acetate	Electrospinning	DEX (drug amount used not referred)	Fibroblasts L929	● Biocompatible; ● Biodegradable; ● Biphasic drug release profile.	(58)
CMC-MC-P	Physical crosslinking	Meloxicam (drug amount loaded unclear)	Chondrocytes	● Good porosity; ● Cell proliferation; ● Efficient drug encapsulation in nanoparticles.	(59)
Collagen	Customized transplantation (rabbit menisci)	Gefitinib (N.A)	Rat fibrochondrocytes	● Adequate mechanical strength; ● Biocompatible; ● Interconnected porosity.	(62)
PCEC	Freeze gelation	TGF-β1 (10 **μ**g/mL)	N.A.	● Porous microstructure; ● Sustained drug release.	(61)

Table 7 – continue

Scaffold Material(s)	Manufacturing Technology	Therapeutic agent(s)	Animal model (bone)	Main findings	Ref.
Hyaluronic acid Collagen	Multistep approach	Prednisolone (0,1%) TGF-β3 (20 pg)	N.A.	Implantable disks had longer durability and better mechanical properties.	(60)
Methacrylated Hyaluronic acid	Electrospinning	SDF-1**α** (≈ 21,25 ng) TGF-**β**3 (≈ 106,29 ng)	MSCs	Effective bioactivity (but TGF-**β**3's was superior).	(63)

Ref.: Reference; **DEX:** Dexamethasone; **CMC-MC-P:** Carboxymethyl Chitosan (CMC)-Methylcellulose (MC)-Pluronic (P); **PCEC:** Poly(ε-caprolactone)–poly (ethylene glycol)–poly(ε-caprolactone); **TGF-β1:** β1-Transforming Growth Factor; **TGF-β3: β**3-Transforming Growth Factor; **SDF-1α:** Stromal Cell-Derived Factor-1**α**; **N.A.:** Non applicable; **MSCs:** Mesenchymal stem cells.

Table 8 - Examples of drug-loaded scaffolds for osteoarthritis treatment (*in vivo* studies)

Scaffold Material(s)	Manufacturing Technology	Therapeutic agent(s)	Animal model (bone)	Main findings	Ref.
Collagen	Customized transplantation (rabbit menisci)	Gefitinib (5 mg/kg)	15, 5-month-old male New Zealand White rabbits (bw. 2.5-3.0kg) **(Menisci)**	Meniscal regeneration enhancement.	(62)
PCEC	Freeze gelation	TGF-β1 (10 **μ**g/mL)	80 healthy specific pathogen free Sprague-Dawley rats (12 weeks; bw. 280g) **(Knee joint)**	• Remarkable injectability; • Prime cartilage defect repair.	(61)
Hyaluronic acid Collagen	Multistep approach	Prednisolone (0,1%) TGF-β3 (20 pg)	90 male rats **(Knee joint)**	Implantable disks are a more effective formulation.	(60)
Methacrylated Hyaluronic acid	Electrospinning	SDF-1**α** (≈ 21,25 ng) TGF-**β**3 (≈ 106,29 ng)	6 Yucatan minipigs (6 months old; bw. 30-35kg) **(Knee joint)**	Only TGF-**β**3 enhances cartilage defect repair.	(63)

Ref.: Reference; **PCEC:** Poly(ε-caprolactone)–poly (ethylene glycol)–poly(ε-caprolactone); **TGF-β1:** β1-Transforming Growth Factor; **TGF-β3**: **β**3-Transforming Growth Factor; **SDF-1α:** Stromal Cell-Derived Factor-1**α**.

4.4 Osteosarcoma

Even though osteosarcoma's prevalence is rare, it is the most common type of bone cancer. It is more prominent in children and adolescents (1). It is known that the aggressive tumour originates in the mesenchymal tissue and is capable of compressing the adjoining muscular tissue as it grows. Thereupon, pain and swelling arise along with the possibility of joint dysfunction and fractures. Osteosarcoma can develop in any bone, but it generally appears in the proximal humerus, the distal femur or the proximal tibia (65,66). Therapeutic strategies consist in surgery, radiotherapy, gene therapy, immunotherapy and chemotherapy (1,65,66). The latter is essential to decrease tumour size and stop or delay metastasis development (65). Most common chemotherapeutic agents presently used are doxorubicin, cisplatin, ifosfamide (along with mesna), high-dose methotrexate (with leucovorin rescue) and adriamycin (1,65,66). Intra-arterial or intravenous administration can lead to ponderous toxic systemic effects. Thereby, scaffolds' application as local drug delivery systems could be a propitious tool in osteosarcoma treatment (1,65,66).

However, scaffolds employment as local drug delivery systems in osteosarcoma settings are still at an early experimental stage. There is still a lot of research needed before these can be transacted to clinical studies and later on in clinical practice. *In vitro* (Table 9) and *in vivo* (Table 10) studies will herein forward be described.

In vitro and in vivo studies

When it comes to oncologic treatments, it is important to assess if the different cytotoxic drugs can present synergism. With this thought in mind, Hess *et al* used calcium phosphate beads/hydroxyapatite matrix scaffolds loaded with doxorubicin and cisplatin (67) . The results underlined synergetic activity between chemotherapeutic agents. It was observed that for beads-based scaffolds considerably lower drug releases occurred in comparison to beads only and control, whilst tumour cell toxicity was higher. This indicated that beads-based scaffolds were more effective for osteosarcoma therapy. Additionally, by incorporating the drug-loaded beads, scaffolds impeded undesirable effects such as uncontrolled spreading and migration in the adjoining tissue (67).

Interestingly, another study decided to focus on resection defect repair using dexamethasone -loaded carbonate calcium microparticles incorporated in a PCL scaffold (68). Dexamethasone was more rapidly and greatly released in an acid environment (corresponding

to inflammation or cancer setting) compared to a physiological one. In addition, the loaded scaffolds increased cell adhesion, proliferation and differentiation (68).

Drugs don't have to be necessarily loaded directly into the inner structure of the scaffold in order to efficiently be released and induce their therapeutic effect. A recent study revealed that 5-fluorouracil (5-FU) adsorbed in CPC scaffolds could significantly reduce tumour growth and that total drug release occurred in 2 hours only (69).

A very recent experiment reported that polymer's, such as PLGA, inclusion in nanoparticles posteriorly incorporated into CPC scaffolds improved this material's injectability as its curing time. It also showed that the loaded nanoparticle-based scaffolds could offer a more controlled drug release (doxorubicin). Moreover, the authors stressed out the importance of tumour environment to successfully inhibit its progression (70).

Due to the high toxicity of chemotherapeutic agents, it is vital to evaluate the maximum tolerated dose (MTD). Yang *et al.* focused on this aspect using doxorubicin as the chemotherapeutic drug and demonstrated that its MTD, when integrated in a composite polymer hydrogel, was 15 mg/kg (71). *In vitro* assays revealed that doxorubicin-implanted hydrogels presented a sustainable drug release that preserved rather high local doxorubicin doses leading to an extended antitumoral activity. *In vivo* findings corroborated the substantial anti-tumour activity and showed an outstanding improvement in the survival rate with limited tissue damage (71). Another yet recent study led to the conclusion that calcium silicate microspheres and ferrite particles with magnetic properties integrated in chitosan scaffolds had a homogenous distribution (72). The microspheres contributed for bone regeneration and the ferrite particles ameliorated photothermal therapy conversion efficiency. Scaffolds alone were shown to potentially treat bone tumour defects and when combined with photothermal therapy exhibited synergistic activity against osteosarcoma (72).

Scaffold's shape can influence drug-loading efficiency which is an aspect worthy of consideration. Wang and co-workers underlined the importance of this point and in their case, spherical implants provided better outcomes (73). Those ones presented other desirable properties for cell adhesion and releasing rate, such as a rough and high porosity. Besides that, the scaffolds exhibited biocompatibility, biosafety and biodegradability. The latter increased when the environment turned acidic which favourably indicates that biodegradation can occur in an osteosarcoma setting. Drug concentration was markedly greater in the surrounding tissue of the implantation site than in the bloodstream, which confirms the hypothesis of scaffolds

acting as local DDS decreasing systemic toxic effects. Curiously, the authors did not make any comments about concentrations differences between the chemotherapeutic agents (73).

Table 9 - Examples of drug-loaded scaffolds for osteosarcoma treatment (*in vitro* studies)

Scaffold Material(s)	Manufacturing technology	Therapeutic agent(s)	Cell line	Main findings	Ref.
HA	Freeze gelation	Cisplatin (60 **µ**g/mL) Doxorubicin (60 **µ**g/mL)	MG63 osteoblast-cell like	• Porous structure; • Homogenous bead distribution through the matrix; • Synergetic drug toxicity.	(67)
PCL	Solvent casting and particulate leaching (SC/PL)	DEX (10 nM; 100 nM)	MG63 osteoblast-cell like	• Drug release time and pH dependent; • Osteosarcoma resection defect was successfully repaired.	(68)
Chitosan	Freeze-Drying	Doxorubicin (3,75 mg/L)	MG63 osteoblast-cell like	• Calcium silicate microspheres and magnetized SrFe12O19 uniformly distributed throughout the scaffold; • Rapid and efficient drug release.	(72)
PLGA-PEG-PLGA	Multistep approach (involving copolymerization)	Doxorubicin (1,0 mg/mL)	Saos-2 and K7 cells	• Controlled drug release; • Drug dose-dependent cell inhibition.	(71)
PLLA	3D-Printing	Cisplatin (120 mg/m^2/d) Ifosfamide (2 g/m^2/d) Methotrexate (10 g/m^2/d) Doxorubicin (45 mg/m^2/d)	U2OS	• Biodegradable; • Biocompatible; • Biosafe; • Spherical shape improves loading efficiency.	(73)
CPC	Multistep approach (involving crosslinking)	Doxorubicin (40 **µ**M; 100 **µ**M)	hMSC U2OS	Drug-loaded nanoparticles incorporated in scaffolds improved drug release profile.	(70)

Table 9 - continue

Scaffold Material(s)	Manufacturing Technology	Therapeutic agent(s)	Animal Model	Main findings	Ref.
CPC SOL PEG600	3D-Printing	5-Fluorouracil (0,5 g)	HeLa Hek293T	• Homogenous drug distribution throughout scaffolds' surface; • Entire drug release in 2 hours.	(69)

Ref.: Reference; **HA:** Hydroxyapatite; **PCL:** Polycaprolactone; **DEX:** Dexamethasone; **PLGA-PEG-PLGA:** Poly(D,L-lactide-co-glycolide)-poly(ethyleneglycol)-poly(D,L-lactide-co-glyco-lide); **N.A.:** Non applicable; **PLLA:** Poly L-lactic acid; **Saos-2:** Human osteosarcoma cells; **U2OS:** Human osteosarcoma cells; **K7:** mouse osteosarcoma cells; **CPC:** Calcium Phosphate Cement; **PLGA:** Poly (DL-lactic-co-glycolic acid); **hMSC:** Human Mesenchymal Stem Cells; **Hek293T:** Human kidney immortalized cell line; **SOL:** Hydrophilic Soluplus; **PEG600:** Polyethylene glycol 600. The drug-loading of the chemotherapeutic agents respectively to reference 73 was performed in accordance with the conventional clinical osteosarcoma postoperative chemotherapy regimen.

Table 10 - Examples of drug-loaded scaffolds for osteosarcoma treatment (*in vivo* studies)

Scaffold Material(s)	Manufacturing Technology	Therapeutic agent(s)	Animal Model	Main findings	Ref.
Chitosan	Freeze-Drying	Doxorubicin (3,75 mg/L)	20 Sprague-Dawley male rats (bw. 300–350 g)	• Synergetic effect of drug release with photothermal therapy; • Osteogenesis occurrence.	(72)
PLGA-PEG-PLGA	Multistep approach (involving copolymerization)	Doxorubicin (5 mg/kg 15 mg/kg 30 mg/kg)	Female BALB/c mice (18-20 g)	• Biocompatible; • **15 mg/kg dose exhibited great anticancer efficacy;** • Survival rate improvement.	(71)

Table 10 - continue

Scaffold Material(s)	Manufacturing Technology	Therapeutic agent(s)	Animal Model	Main findings	Ref.
PLLA	3D-Printing	Cisplatin (120 mg/m^2/d) Ifosfamide (2 g/m^2/d) Methotrexate (10 g/m^2/d) Doxorubicin (45 mg/m^2/d)	• Healthy ICR rats (bw. 275–300 g) (subacute toxicity testing); • New Zealand 6-month-old white rabbits (≈ 2.5 kg) (inflammatory response testing); • SD rats (efficacy test of PLLA drug-loaded scaffolds).	• Drug concentrations were higher at the lesion site than in the bloodstream; • Sustainable drug release; • No systemic toxic effects; Efficient tumour growth inhibition.	(73)

Ref.: Reference; **PLGA-PEG-PLGA:** Poly(D,L-lactide-co-glycolide)-poly(ethyleneglycol)-poly(D,L-lactide-co-glyco-lide); **N.A.:** Non applicable; **PLLA:** Poly L-lactic acid; **ICR:** Institute Cancer Research; **SD rats:** specific-pathogen-free (SPF) graded rats. The drug-loading of the chemotherapeutic agents respectively to reference 73 was performed in accordance with the conventional clinical osteosarcoma postoperative chemotherapy regimen.

4.5 Cancer bone metastasis

Bone metastasis is a major concern in patient morbidity and its main goal treatment consists in prolonging life expectancy. Bone is a recurrent metastasis site for breast and prostate cancers (65). In both cases, sharp bone pain and fractures are induced as a result of osteolytic or osteoblastic lesions (14). There are many therapeutic alternatives, but bisphosphonates are considered the most attractive ones. Bone-targeted drug delivery systems have been studied to surpass adverse effects and increase drug concentration at the specific sites (14,65).

Drug-loaded scaffolds' employment as local drug delivery systems in cancer bone metastasis in literature reports are exiguous and the few existing ones are yet at an extremely early experimental stage. Forwardly, three experiments summarized in Table 11 (*in vitro* studies) and Table 12 (*in vivo* study) will be discussed.

In vitro and in vivo studies

Using human breast tumour cells transplantation in a mouse model, Sun *et al*. discovered that composite scaffolds subcutaneously implanted had potential in decreasing multi-organ metastasis development (74). No cardiotoxicity or skin ulceration of doxorubicin were observed. Moreover, *in vitro* analysis showed that doxorubicin could be sustainably released for 4 weeks. This could not be completely corroborated by the *in vivo* experiment because the authors were only ethically allowed to keep the animals for a 2-week time observation. The authors did not evaluate local bone treatment and mentioned future studies with this aim should be performed. However, they mentioned this particular approach could be useful for patients with bone metastasis that would undergo tumour-debulking surgeries. The scaffold could be used as a void filler after bone tumour resection, consistently releasing small doxorubicin amounts that would eliminate remaining malignant cells and avoid systemic toxicity (74).

Other recent *in vitro* experiments using chemotherapeutic agents or bisphosphonates showed promising results. Ahangar *et al.* used two different cell lines and three types of scaffold materials to assess doxorubicin release in a bone metastatic environment (75). Drug release could be adjusted accordingly to the material type: greater porosity triggered the release of higher drug amounts, which was the case for Gel Lay. Doxorubicin was effectively released in both cell lines and was capable of decreasing metabolic activity in a dose-dependent manner, especially in patient cells. There were two main limitations pointed out to the study: lack of understanding the long-term possible cytotoxic effects of low-dose doxorubicin in healthy cells in the surrounding tissue and incomplete scaffolds' characterization namely in terms of mechanical properties (75). When combining the same materials to a completely different drug – zoledronate – and using identical cell types, similar conclusions were made (76). For instance, Gel Lay released bigger drug quantities, zoledronate-loaded scaffolds of all materials decreased both cell types proliferation and human cells were more susceptible to this effect. Once more, it was highlighted that these scaffolds could be modified in order to modulate drug release and that its materials conjugated with other biomaterials could ensure finer bone stability after bone tumour excision. Additionally, the authors stated that further validation was needed by applying this strategy in a *in vivo* model (76).

Table 11 - Examples of drug-loaded scaffolds for cancer bone metastasis treatment (*in vitro* studies)

Scaffold Material(s)	Manufacturing Technology	Therapeutic agent(s)	Cell Line	Main findings	Ref.
PCL	3D-Printing + Freeze Drying	Doxorubicin (2 mg/mL; 4 mg/mL)	MDA-MB-231-luc-D3H2LN Bioware	● Sustainable drug release profile; ● Inhibitory tumour growth.	(74)
Lay Fomm 60 Lay Fomm 40 Gel Lay	3D-Printing	Doxorubicin (500 ng)	LAPC4 Human resected metastatic spine tumours secondary to prostate cancer	● No defined mechanical properties, but Gel Lay scaffolds had better porosity which led to higher drug release amounts.	(75)
Lay Fomm 60 Lay Fomm 40 Gel Lay	3D-Printing	Zoledronate (500 ng)	LAPC4 Prostate-induced bone metastasis cells	● Gel Lay scaffolds had better porosity which led to the release of higher drug amounts; ● Bone metastasis proliferation reduced; ● Normal bone formation maintained.	(76)

Ref.: Reference; **PCL:** Polycaprolactone; **Lay Fomm 60, Lay Fomm 40 and Gel Lay:** materials composed of thermoplastic polyurethane and polyvinyl alcohol that differ in their shore hardness (Lay Fomm 60 and Lay Fomm 40) and/or in their appearance (Lay Fomm has a foamy appearance whilst Gel Lay has a gelatinous appearance). **MDA-MB-231-luc-D3H2LN Bioware:** luciferase- expressing cell line; **LAPC4:** prostate cancer cell line.

Table 12 - Examples of drug-loaded scaffolds for cancer bone metastasis treatment (*in vivo* study)

Scaffold Material(s)	Manufacturing technology	Therapeutic agent(s)	Animal Model	Main findings	Ref.
PCL	3D-Printing + Freeze Drying	Doxorubicin (2 mg/mL; 4 mg/mL)	10–14-week-old female BALB/ cATac-nude mice injected with human breast cancer cells	● Inhibitory tumour growth; ● No systemic toxicity; ● Multi-organ metastasis formation retardation; ● Potential use for bone metastasis as bone tumour resection fillers.	(74)

Ref.: Reference; **PCL:** Polycaprolactone.

5. Challenges and Future Perspectives

Despite the propitious progress of scaffolds as a BTE strategy for local drug delivery and subsequent bone repair enhancement, there are still challenges hampering the advancement towards its use in clinical practice.

The establishment of the adequate treatment is crucial to define the specific requirements for the selection of the biomaterial(s) but also of the fabrication process of the scaffold (12). It is very difficult to find in a biomaterial alone the desirable mechanical and biological properties mentioned in subsection 3.1. Composites (e.g., natural and synthetic polymers combination or ceramics assembled with polymers) offer a good solution, since they can provide enough mechanical strength and stiffness while supplying a bioactive component for new bone formation prompting (5). However, all biomaterials share the same vital limitation: deficient capacity to promote angiogenesis (7,15). Nevertheless, there are already studies suggesting that angiogenic agents' incorporation, such as growth factors (e.g., VEGF), can greatly contribute to scaffolds' revascularization. However, further research for angiogenic character improvement is required (7,77). The importance of the manufacturing process relies on the minimization of toxic residues and in the need for a cost-effective outcome enabling large scale production without negatively impacting the scaffold's macro and micro features (12). At the moment, there are not specific sterilization methods for scaffolds. Scaffolds are biomedical devices that are implanted in the body so they cannot be a source of infection (8). Therefore, future research should also focus on finding precise sterilization techniques for scaffolds to eradicate any possible source of microorganism contamination while maintaining their structural and biological properties.

Another crucial concern relies in drug-loading. Numerous studies still report an initial rapid burst release of the loaded drugs from the biomaterials (9). It has been implied that the addition of drugs during scaffolds' fabrication can prevent it however, other studies state the same occurrence even when drugs are priorly impregnated in nanoparticles or microparticles and are then added during the scaffold's fabrication (8,9). Further research should insight not only in improving drug-loading techniques to optimize drug release kinetics, but also in clarifying the appropriate loading dosages (9,12). Another interesting research field is dual drug delivery, which may prove to be advantageous through the synergistic effect that emerges when two distinct drugs are combined (20). There are other important limitations regarding the local drug delivery approach that need to be surpassed: difficult implantation in the bone, risk of

infection and inflammation - due to the foreign character of the scaffold to the body - in the surrounding tissue and problematic drug penetration into the targeted area (1).

Therefore, the future of scaffold design and manufacturing technology points out to a triggered drug delivery or 4D printing strategy using stimuli-responsive biomaterials. Examples of triggers are temperature, electrical and magnetic fields or pH change (8,20,23,78). This approach diminishes burst releases and optimizes drug specificity and quantity while lessening its toxicity (20).

Although *in vitro* models are required for cell activity and cell toxicity studies, they cannot portray the complexity of an organ or the interactions between the multiple ones existing in an organism (12). Hence, *in vivo* models become necessary. They rely on the use of small and large animals for up-scale purposes (12). Although small animals need shorter periods of observation because of their faster bone turnover rates, the osseous tissue of larger animals is much more similar to the human one (12). Most of the studies focus more on drug release testing and scaffolds' degradation but poor insight is given to the scaffolds' mechanical properties which are extremely important for a successful implantation. On this note, future research should further address mechanical testing. Drug-loaded bone scaffolds' employment in clinical practice priorly requires large, randomized and controlled trials that demand extensive monitoring, documentation and funding which in turn may discourage investigators (12,79).

Bone scaffolds' commercialization formerly entails a marketing authorization process which is expensive and time-consuming since it demands pre-clinical, clinical, long-term storage and packaging stability and safety studies before the final approval (12). There are already growth-factor-loaded scaffolds available on the United States' market such as Infuse™ or GEM 21S (80). Albeit there are already patented bone scaffolds loaded with antibiotics such as vancomycin, to the best of our knowledge, there are not commercialized synthetic-drug-loaded bone scaffolds (81). Thus, regulatory frameworks are not always consonant with the scientific progress. Moreover, the lack of worldwide regulatory harmonization of biomedical devices is a significant drawback so future work should also emphasize an easier access for the commercialization of these products (12).

6. Conclusion

Growth-factor-loaded and synthetic-drug-loaded bone scaffolds have proven to be worthy of attention as therapeutic strategies for bone repair in five distinct bone diseases – osteomyelitis, osteoporosis, osteoarthritis, osteosarcoma and cancer bone metastasis. Literature reports, especially *in vitro* and *in vivo* studies, are particularly vast for osteomyelitis when compared to the other four bone pathologies described. Additionally, synthetic-drug-loaded bone scaffolds have only been clinically assayed in osteomyelitis. For osteosarcoma and cancer bone metastasis, further research is still needed to better understand the therapeutic value of scaffolds for drug delivery.

There are critical steps to obtain a drug-loaded bone scaffold that can act as a proper drug delivery system and therefore be useful in clinical practice. These include the selection of adequate biomaterial(s) as well as manufacturing and drug-loading methods' eligibility, detailed pre-clinical and clinical studies in which the scaffold's mechanical, physical and biological properties are assessed alongside its safety.

Despite their great potential as therapeutic approaches in bone pathologies, there are a few hindrances that impede their translation into clinical practice such as the need for more pre-clinical and clinical research, drug release kinetics refinement but also the mismatch between the scientific evolution of drug-loaded bone scaffolds and their regulatory framework. We believe further research will lead to a better understanding of these BTE tools but also enable the development of other auspicious design technologies which in turn can conduct to a betterment of their bioactivity.

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