Review Article

Carrier systems for bone morphogenetic proteins: An overview of biomaterials used for dentoalveolar and maxillofacial bone regeneration

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ABSTRACT

Different types of biomaterials have been used to fabricate carriers to deliver bone morphogenetic proteins (BMPs) in both dentoalveolar and maxillofacial bone regeneration procedures. Despite that absorbable collagen sponge (ACS) is considered the gold standard for BMP delivery, there is still some concerns regarding its use mainly due to its poor mechanical properties. To overcome this, novel systems are being developed, however, due to the wide variety of biomaterial combination, the heterogeneous assessment of newly formed tissue, and the intended clinical applications, there is still no consensus regarding which is more efficient in a particular clinical scenario. The combination of two or more biomaterials in different topological configurations has allowed specific controlled-release patterns for BMPs, improving their biological and mechanical properties compared with classical single-material carriers. However, more basic research is needed. Since the BMPs can be used in multiple clinical scenarios having different biological and mechanical needs, novel carriers should be developed in a context-specific manner. Thus, the purpose of this review is to gather current knowledge about biomaterials used to fabricate delivery systems for BMPs in both dentoalveolar and maxillofacial contexts. Aspects related with the biological, physical and mechanical characteristics of each biomaterial are also presented and discussed.

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1. Introduction

The development of better strategies to achieve new bone formation is a major concern for orthopedic and maxillofacial specialists treating certain bone defects in which the self-healing capacity is insufficient. These defects may arise from trauma, congenital conditions, tumor excision and, in the case of dentoalveolar territory, infectious diseases such as periodontitis or peri-implantitis [1]. The destruction of periodontal tissues due to inflammatory conditions is a common cause of tooth loss, a main public health problem with a widely proven negative effect on quality of life among adults [2]. Patients with missing teeth usually experience accelerated bone resorption, which requires more complicated oral rehabilitation treatments such as the placement of prosthetic dentures or even dental implants [3]. In these cases, strategies for alveolar bone augmentation or sinus floor augmentation are needed in order to obtain sufficient bone volume to build an implant with adequate mechanical resistance [4].

Nowadays, regardless of the cause, the strategies used by dentist, implantologists or maxillofacial surgeons to promote periodontal and/or bone tissue formation involve a combination of guide bone-regeneration (GBR) techniques and bone grafting procedures [4,5]. GBR is a common method for the reconstruction of alveolar bone. It is based on the use of barrier membranes (resorbable and non-resorbable) to exclude non-osteogenic tissues (such as proliferating epithelium and connective tissue) from interfering with the natural bone healing process [5]. On the other hand, bone grafting procedures are also very common, mainly in orthopedic but also in maxillofacial surgery. They involve the use of filling materials to treat traumatic defects or lesions with loss of bone, to provide bone volume, and to stimulate the healing process [6,7]. Their use in combination with GBR techniques is highly recommended, for example, in socket preservation procedures following tooth extraction, implant placement in fresh extraction sockets and alveolar ridge width augmentations [8].

Currently, there are several options to treat dentoalveolar and maxillofacial bone defects based on the wide variety of grafting materials and barriers membranes available commercially. Autologous bone grafting remains the gold standard treatment for bone regeneration, due to its osteoinductive, osteoconductive and osteogenic capabilities [9]. Nevertheless, its undesirable secondary effects related with donor site morbidity and postoperative complications, have raised the question whether a new class of biomaterials with equal biological activity, and better physical properties, is needed. In recent decades, several allografts (tissues obtained from a donor of the same species but not genetically identical) and xenografts (from another species) have been formulated and successfully used in clinical practice [10]. However, the majority of these biomaterials have failed to achieve higher or at least the same levels of new bone formation as autografts [11]. For this reason, the use of bioactive molecules naturally involved in the bone healing process, such as bone morphogenetic proteins (BMPs), has been explored and included in new tissue engineering approaches [12].

BMPs have generally proved to be the most potent osteoinductive growth factors. Their use in combination with a proper delivery system or carrier for controlled release has been successfully demonstrated, serving as an initial framework to support cell growth and bone tissue regeneration [13–15]. However, despite their osteogenic potential, other properties derived from their molecular characteristics have limited their efficacy, preventing more widespread use in clinical practice. For example, their limited solubility at physiological pH [16], their high rate of clearance due to enzymatic activity [17] or their pleiotropic characteristics [18] can finally result in unwanted or insufficient biological actions or even in serious complications. These inherent pharmacological characteristics emphasize the importance of using BMPs and other growth factors in conjunction with delivery systems possessing adequate composition and a structure that permits a specific pattern of spatiotemporal release [12].

Since bone formation is a tightly controlled series of events in which several types of cytokines are delivered at precise locations and times, the ability of a carrier to retain growth factors and release them in a time- and dose-specific manner is crucial. For this reason, aspects such as chemical composition, mechanical strength, topological or architectural configuration and immobilization modalities must be considered in the selection of biomaterial to construct these carriers, as they may determine their ability to carry the bioactive molecules, and thus their suitability for clinical applications [19,20]. In addition, considering the treatment needs of the bone defect site, a good biomaterial should also act as a three-dimensional graft to restore lost bone volume; and as a scaffold whose surface is capable of promoting cellular migration, adhesion, proliferation, and differentiation [21]. Considering the above, the aim of this review was to give an overview of the different types of biomaterials used to fabricate delivery systems or carriers for BMPs in preclinical and clinical studies, for the treatment of dentoalveolar and maxillofacial bone defects. Moreover, aspects related with the composition and the biological and mechanical characteristics of each biomaterial are presented, highlighting their synergism with BMPs to induce new bone formation.

2. BMPs in bone tissue healing

BMPs are a subgroup of endogenous proteins, of low molecular weight, belonging to the transforming growth factor (TGB)-β superfamily of proteins [22]. They were first described in 1965 by Urist & McLean in experiments using animals where demineralized bone matrix (DBM) showed the ability to induce new bone formation in ectopic sites [23]. BMPs are dimeric molecules, with at least 120 amino acids in their composition, the presence of a cysteine knot with six highly conserved cysteine residues and a heparin binding site [24,25]. In general, it is well-known that BMPs have multiple biological effects, being involved in cell proliferation, cellular differentiation, hematopoiesis, production of extracellular matrix (ECM), embryogenesis and regulation of apoptosis [24,26]. To date, at least 20 different types of BMPs have been isolated and characterized, with evidence that some of them (such as BMP-2, 4, 5, 6, 7, 8 and 9) play important roles in cartilage and bone formation [25,27]. In Table 1 we summarize the main members of the BMP subgroup and their reported functions. Isolation and purification of BMPs from DBM is a well-known method, however it is a highly complex and inefficient process that requires a large amount of cortical bone. For this reason, the extraction of BMPs from bone tissue has now been replaced by genetic engineering-based methods involving the transfection of human cloned genes into organisms

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Table 1
Summary of BMPs, members of the TGF-β superfamily of proteins.

BMP	Other Names/ Homologs	Function	Ref.
BMP-2		Present during embryonic development and related with skeletogenesis. Necessary for bone fracture repair. Also involved in differentiation of osteoblasts from progenitor cells resident in the marrow (osteogenic differentiation)	[28,29]
BMP-3	Osteogenin	Most abundant BMP in demineralized bone. Osteogenin purified from bone has osteoinductive potential but rhBMP-3 has no osteogenic activity. BMP-3 inhibits BMP-2-mediated osteogenic differentiation in vitro and is a negative determinant of bone density	[30]
BMP-4		Important in early stages of embryogenesis. Present during fracture repair. Induces osteoblast differentiation (alkaline phosphatase activity) through the activation of Smads 1, 5 and 8.	[31,32]
BMP-5		Influences the generation of osteoclasts, increasing the RANKL/OPG ratio. Stimulates differentiation and proliferation of osteoblasts (increasing alkaline phosphatase activity). Suggested role in bone homeostasis	[33]
BMP-6	Vgr 1	Induces osteoblast differentiation (alkaline phosphatase activity) through the activation of Smads 1 and 5. Influences the generation of osteoclasts.	[31,33,34]
BMP-7	OP-1	Potent anti-inflammatory growth factor. Role in embryogenesis, hematopoiesis, neurogenesis and skeletogenesis. Induces osteoblast differentiation (alkaline phosphatase activity) through the activation of Smads 1 and 5. Important inducer of bone formation	[31,35,36]
BMP-8	OP-2	mRNA expression studies have suggested that OP-2 has a role in early stages of development. Also, its expression is higher during a restricted period in fractures healing when resorption of calcified cartilage and osteoblast recruitment are most active	[37–39]
BMP-9	GDF-2	Able to induce osteogenic differentiation of mesenchymal stem cells. Induces osteogenesis and chondrogenesis. Involved in differentiation of cholinergic neurons and synthesis of acetylcholine. Role as a regulator of glucose metabolism.	[40,41]
BMP-10		Its expression is restricted to the developing and postnatal heart. Essential role in regulation of cardiac growth and chamber maturation	[42,43]
BMP-11	GDF-11	Regulated axial skeletal patterning and skeletal formation of limbs	[44]
BMP-12	GDF-7; CDMP-3	Homolog GDF-7 induces connective tissue formation rich in type I collagen, resembling neonatal tendon and ligament. Acts as signaling molecule during embryonic formation of tendons, ligaments and joints	[45]
BMP-13	GDF-6; CDMP-2	Inhibits the osteogenic differentiation of human marrow multipotent mesenchymal stromal cells in vitro. Mutations or deficiencies may allow excess bone formation	[46]
BMP-14	GDF-5; CDMP-1	Affects chondrogenesis by increasing chondrocyte proliferation as well as cell adhesion in early chondrogenesis. Deficiency leads to a delay in fracture healing	[47]
BMP-15	GDF-9B	Present in oocytes throughout folliculogenesis. Physiological regulator of follicle cell proliferation and/or differentiation. Modulates the action of follicle-stimulating hormone	[48]

RANKL/OPG: Receptor Activator for Nuclear Factor κ B Ligand/Osteoprotegerin
Vgr: Vitellogenin related
OP: Osteogenic Protein
GDF: Growth Differentiation Factor
CDMP: Cartilage-derived Morphogenetic Protein.

such as bacteria, yeasts, baculovirus or mammalian cells to produce the mature protein [24,29,49].

Since the first reports by Urist, the use of BMPs to induce bone formation has become a major interest in the fields of orthopedics and maxillofacial surgery. In this context, in vitro and in vivo studies have already demonstrated the osteoinductive potential of BMPs, with results that are at least equivalent to those achieved using autologous bone [26,43,50], although success could depend on the particular clinical scenario [11]. During the first decade of 2000, the Food and Drug Administration (FDA) of the United States approved the use of recombinant human formulations of BMP-2 and BMP-7 (rhBMP-2 and rhBMP-7, respectively) coupled with collagen carriers in spinal fusion procedures, treatment of open fracture of the tibia and in cases where autologous graft has previously failed [27]. For application in the maxillofacial territory, in March 2007 the FDA approved the use of InFuse® device, a bone graft containing rhBMP-2 in an absorbable collagen sponge (ACS), as an alternative to autologous bone for sinus and alveolar ridge augmentations in defects associated with extraction sockets [51]. While InFuse® is still available for clinical use, formulations containing BMP-7 (OP-1) were removed from the market a couple of years ago [52]. Despite this, the use of BMPs in maxillofacial territory is not widespread, mainly because clinical trials have reported that its advantages are restricted to cases of lower morbidity, and that it does not necessarily induce a significant amount of newly formed bone when compared with autologous bone treatment [53–55]. Likewise, some adverse effects related with their use have been reported, including ectopic bone formation, osteolytic defects, and even graft failure and infection [56,57]. These complications are more frequently seen after

off-label uses of rhBMP-2 [57], including the use of higher doses [58] or utilization of inappropriate delivery systems [54].

Several requirements have been established to consider a carrier as appropriate to deliver BMPs, optimizing their therapeutic efficacy and safety [25]. First, an appropriate carrier should increase the retention of BMPs on the defect site, allowing the progressive migration of bone-forming cells [59]. The retention of these growth factors could also permit the use of lower doses of protein, prevents systemic diffusion and reduces the risk of adverse effects [60]. In addition, these carriers should be able to maintain an appropriate space inside the bone defect, a capacity closely related with physical and mechanical properties of biomaterials, allowing the gradual deposition of ECM to replace the concomitant reabsorption of the carrier [21]. Ideally, these carriers must be biocompatible and biodegradable to minimize inflammatory responses by the immune system [59,61]. A good carrier should have an adequate mechanical resistance and topological structure (including porosity, size and shape) according to the needs of the receiving tissue or defect site [61,62]. It has been seen that the lack of structural stability of the carrier could cause collapse of the soft tissue walls, promoting an initial increase in release of the BMPs and hindering their therapeutic effect [54]. This is especially relevant in dentoalveolar bone regeneration procedures in which masticatory movements and forces coupled with saliva contamination are present. Lastly, the biomaterial used to construct these scaffolds or carriers should be cost-effective and easy to fabricate, with chemical characteristics that allow adequate sterility, storage and stability over time, permitting large-scale production [63]. Table 2 summarizes the main requirements established for a carrier to be considered as an

Table 2
Characteristics of a good delivery system for BMPs (or other growth factors).

A carrier or delivery system should be/have:
- Biocompatible (non-immunogenic, non-toxic, and non-carcinogenic)
- Biodegradable (to permits the deposition of new bone)
- Controlled and sustained release of the bioactive molecules (allowing the use of lower doses)
- Proper mechanical strength (to resist compressive forces of the bone defect site)
- Proper physical configuration (including design, shape and porosity according to the size of bone defect)
- Easily handled by users
- Adequate sterility, storage and stability over time (closely related with its chemical composition)
- Inexpensive and easy to manufacture on a large scale (cost-effective)

Fig. 1. Schematic representation of scaffolds or carrier systems used to deliver growth factors (including BMPs) in bone regeneration procedures. The terms "scaffold" and "carrier" can be used to define any system that incorporates, transports and delivers molecules or cells for biomedical applications. However, a scaffold usually refers to a structure capable of supporting three-dimensional tissue formation and can include: a) a three-dimensional porous matrix in block or particles, b) a polymeric fiber mesh, c) hydrogels (injectable or not) and d) spheres or capsules (made of bioceramics or polymers), among others. A carrier, on the other hand, usually refers specifically to a system that incorporates and retains a precise amount of growth factor, enhancing its selectivity, bioavailability and efficiency. Different techniques have been used to immobilize growth factors, including: e) adsorption (by soaking a solid bulk), f) absorption (incorporation of the growth factor into the biomaterial), g) covalent conjunction (using intermediates molecules such as polydopamine) and h) encapsulation (in micro- or nanospheres, or capsules). Multiple possibilities for delivering growth factors can be generated by combining these modalities.

appropriate delivery system for BMPs. In addition to these factors, it is important to consider the possible cell-scaffold interactions in the designing of a new delivery system for BMPs. The scaffold properties not only could affect functions of surrounding tissue and cells; cells can also induce modifications in the scaffold (such as deformation or degradation) affecting its performance [64]. In Fig. 1 we schematize the main types of scaffolds or carriers used in preclinical and clinical studies, including topological architectures, types of growth factor immobilization/retention and possible combinations of these for carrier fabrications.

3. Delivery systems for BMPs in bone regeneration

As mentioned above, an appropriate carrier should allow the immobilization of the protein on the surface of or inside the biomaterial, and provide controlled release to induce cell migration, proliferation, differentiation, ECM deposition and mineralization. Although some biomaterials have been shown to be good options in allowing these events, so far there is no consensus regarding which biomaterial (or combination of biomaterials) is most effective and safest for the construction of delivery systems for the clinical setting. In the following sections we review some of the most important biomaterials used alone or in combination, highlighting their main outcomes in both preclinical and clinical studies.

3.1. Natural polymers

Inspired by the composition of ECM, collagen was one of the first natural polymers used to construct carriers for BMPs. Collagen is the most abundant protein in mammals; the major sources for scientific research are the skin, tendons, bones and cartilage of cows, pigs and sheep [65,66]. Collagen can also be obtained synthetically, and shorter sequences of the protein, including collagen mimetic peptides, collagen-like proteins and hydrolyzed collagen peptides, have been used as biomaterials for biomedical applications [67]. Collagen is considered the gold standard carrier for BMPs and ACS has been approved by the FDA to treat spinal fusion, long bone fractures and for periodontal regeneration procedures [68].

Early studies widely reported the ability of atelopeptide type I collagen, used as a carrier for BMPs, to induce ectopic bone formation [69,70]. ACS and slowly dissolving collagen membranes have been shown to induce bone formation when used with rhBMP-2 in periodontal defect models [71,72]. Recent systematic reviews have highlighted the superior bone formation achieved when rhBMP-2 delivered in ACS is used for both alveolar ridge preservation and alveolar ridge/maxillary sinus floor augmentation, compared with use of carrier alone [73,74]. Furthermore, these types of carriers have demonstrated the ability to induce cementum formation in animal models [75], and even to be effective in patients requiring local alveolar ridge augmentation for buccal wall defects [76].
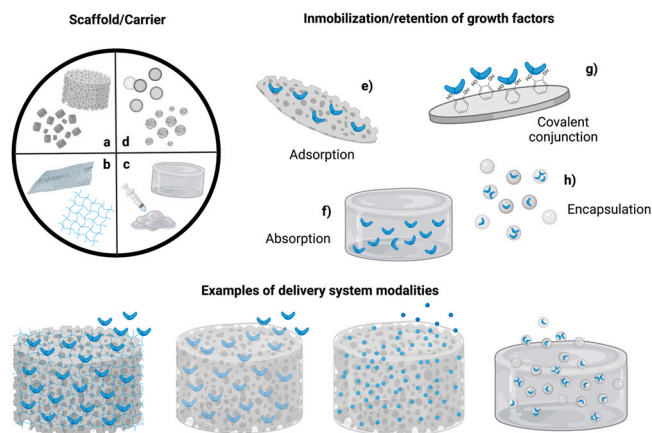
Similarly, the combination of ACS with rhBMP-9 formulations have also been shown to induce higher levels of newly formed bone after 8 weeks of treatment compared with the use of control or ACS alone in rat calvarial defect models [77,78]. Interestingly, when ACS loaded with formulations of rhBMP-9 and −2 were compared in vitro, it was reported that the osteoblast differentiation achieved by the first group was ten times higher than that achieved by the second [79]. The bi-layer collagen matrix, another interesting architectural conformation of collagen, has also been shown to induce new bone formation when used in conjunction with rhBMP-2 in the treatment of alveolar intrabony defects in dogs [80]. Meanwhile, the use of BMP-2 with collagen hydrogel scaffolds, which is the most stable architectural conformation for this biomaterial, have proved to enhance reconstruction of periodontal tissues in one-wall intrabony defects, including formation of cementum-like tissue, periodontal ligament and alveolar bone in animal models [81]. The lack of mechanical strength of collagen sponges and the rapid degradation of collagen fibers by collagenase enzymes have been pointed as crucial factors that could limit its use in clinical practice. For that reason, the incorporation of additional biomaterials into collagen hydrogels or ACS has been tested in both in vitro and in vivo studies [66]. In these trials, the addition of porous bioceramic to collagen resulted in more effective options to deliver growth factors and induce bone formation than controls [69,82–84]. More recent examples of these combinations are found in studies in which poly (lactic-co-glycolic acid) [85], alginate [86] or calcium phosphates salts (such as β-tri-calcium phosphate or biphasic calcium phosphate) were added, demonstrating a synergic effect with collagen and BMPs in bone formation [87–90].

Gelatin, a partially degraded type I collagen, has been also used to construct delivery systems for BMPs. The incorporation of BMP-2 into gelatin hydrogels have shown to induce bone regeneration in experimental alveolar clefts prepared in the maxillary bone of rabbits [91]. It has also been reported that fast-degrading gelatin carriers for BMP-2 are able to induce bone formation in rat periodontal

fenestration defects; however, slow-degrading gelatin allows more prominent new cementum formation [92]. As with other natural polymers, different gelatin-based carriers have been fabricated in combination with additional biomaterials to overcome gelatin's lack of mechanical strength. The most common is the combination of a gelatin sponge with a poly (lactic-co-glycolyc acid) copolymer, a formulation that retains the growth factor and can withstand the pressure exerted by soft tissues. This carrier showed favorable results in preclinical studies inducing new bone formation in periodontal defects [93], condylar defects [94] and alveolar ridge/vertical augmentations [95,96].

Another natural polymer used to construct carriers for BMPs is Hyaluronic acid (HA). HA is a highly hydrated glycosaminoglycan composed of repeating units of N-acetylglucosamine and glucuronic acid, distributed in the ECM of several tissues. HA is well-known for being biocompatible, non-toxic, non-immunogenic, non-inflammatory and biodegradable [97]. In preclinical studies, HA sponges appear to be suitable carriers for rhBMP-2 formulations in the treatment of alveolar ridge defects in animal models, however their superiority over ACS in forming new bone is still controversial [98]. For that reason, HA carriers are usually fabricated incorporating other polymers that confer a cross-linking conformation or a hydrogel structure. In preclinical studies, engineered HA hydrogel for the delivery of BMP-2 by adding fibronectin [99], polyvinyl alcohol [100], poly (ethylene glycol) [101] or heparin [102] have been tested, demonstrating positive results for bone regeneration.

Other less common natural polymers have been also used to deliver BMPs. One example is Chitosan, a polysaccharide that has a repeated structure of β-(1,4)-linked 2-amino-2-deoxy-D- glucose and produced commercially by the N-deacetylation of chitin [61]. Chitosan has been used to fabricate nanofiber membranes to immobilize rhBMP-2, a system able to induce osteoblast cell attachment, promote cell proliferation, and enhance both alkaline phosphatase (ALP) activity and calcium deposition in vitro [103]. In animal models the use of chitosan carriers for rhBMP-9 has not shown significant osteoinductive potential compared with either controls or ACS carriers [77]. Other example is alginate, a polysaccharide derived from algae, which in the form of microbeads proved to be an effective carrier for BMP-2 that enhanced ALP activity of mesenchymal stem cells (MSCs) in vitro after 14 days, and induced bone formation in both ectopic and calvarial defect sites of animal models [104]. Delivery of BMP-2 using oxidized alginate hydrogels with enhanced degradation rate to allow deposition of new tissue have achieved greater bone mineral density after 8 weeks of treatment in animal studies [105]. Another natural polymer used to fabricate carriers for BMPs is Fibronectin, a non-collagenous ECM glycoprotein also presents in plasma, that regulates several cellular functions including adhesion, migration, proliferation, differentiation and apoptosis [106]. A carrier system made of fibrin and fibronectin for rhBMP-4 delivery has been developed to treat critical-sized calvarial defects in rats, demonstrating greater new bone formation compared to controls at 2 and 8 weeks [107].

3.2. Synthetic polymers

During the development of new delivery systems for growth factors, synthetic polymers have received great attention in the last decades. Nowadays, the fabrication of copolymers allows the combination of various desirable properties of individual polymers into a single device enhancing stability, mechanical performance and biocompatibility [108]. In addition, the use of synthetic polymers instead of natural polymers (such as collagen) avoids the potential risks associated with the use of animal-derived biomaterials, including transfer of disease, unexpected inflammation or residual immunogenicity [109]. In the light of these considerations, polymers like poly-α-hydroxy acids (such as polylactic acid, polyglycolic acid

and copolymers), polyalkenoates, polyurethanes, polyorthoesters, polycarbonates, etc., have been studied intensely for biomedical and pharmaceutical applications [110]. Homopolymer of polylactic acid (PLA), a linear aliphatic thermoplastic polyester produced by ring-opening polymerization of lactides [111], in combination with BMP, has been shown to induce cartilage formation in one week, and induce bone at three weeks after implantation in muscle sites of mice [112]. PLA has also been used to fabricate 3D-printing scaffolds grafted with BMP-2 immobilized by polydopamine coatings demonstrating the ability to release growth factors in a sustained manner and promote in vitro ALP activity and osteocalcin in human MSCs [113]. PLA scaffold containing 30% weight of nano-sized β-tricalcium phosphate (a calcium phosphate salt described below) and loaded with rhBMP-2 also showed the ability to induce new bone formation in ectopic sites of rabbits, with outstanding mechanical properties [114]. A copolymer combining PLA with polyglycolic acid (poly (lactic-co-glycolic acid) or PLGA), applied as a coating on a compressed gelatin sponge, has been described as one of the most promising biodegradable carriers for rhBMP-2 due to its porous structure permitting cellular infiltration, its biocompatibility, and its sufficient mechanical strength to maintain space [95]. This carrier has been reported to induce bone formation in segmental bone defects in tibiae of dogs [115], mandibular bone defects in rats [116], periodontitis in the dog models [117] and in ectopic sites in rats [118]. Furthermore, the use of rhBMP-2 with PLGA copolymer sponge has also been shown to induce greater bone formation after dental implant in maxillary sinus floor augmentation in sheep compared with the use of autologous pelvic cancellous bone [119]. Copolymers of PLA with polyethylene glycol (PEG), a thermoplastic polyester polymer, in a molar ratio of 3:2 approximately, has shown superior ectopic bone formation when is used to deliver rhBMP-2 compared to others PLA/PEG copolymers in different ratios [120]. In a later study, since remains of this copolymer were seen in the cores of the ossification sites its biodegradability was improved by adding random linkage of p-dioxanone [121].

3.3. Bioceramics

Hydroxyapatite with the formula $Ca_{10}(PO_4)_6(OH)_2$ is a type of biological apatite and the main inorganic mineral component (70%) of bones and teeth [14]. As a calcium phosphate salt, it impregnates the organic collagen matrix of bones giving them hardness and rigidity [122]. Due to its well-known similarity, in both physico-chemical and biological properties, to that found in living organisms, synthetic hydroxyapatite has been widely applied as a biomaterial for bone scaffold and fillers, implant coatings, and drug delivery systems [123]. Its osteoconductive capacity allows the migration of host bone-forming cells into porous scaffolds, thus slowly promoting new bone formation [124]. Due to its lack of osteoinductive properties, it has been combined with other biomaterials such as bioglass [125], or growth factors like BMP-2 [126]. Since the 1980 s, several preclinical studies have investigated the feasibility of combining hydroxyapatite with soluble BMPs. The combination of BMPs with calcium-phosphate-based materials was inspired by the natural delivery system present in bone. Bone cells produce BMPs in an extracellular matrix impregnated in calcium phosphate salts [127]. In these studies, it was demonstrated that this combination allows faster and more pronounced bone formation (before 8 weeks) in comparison with hydroxyapatite alone [128,129]. In addition, clinical studies have also reported the effectiveness of this combination, achieving major new bone formation in maxillary sinus augmentation [130], alveolar ridge preservation [131] and alveolar ridge augmentation [132]. Despite these findings, it was reported that the porous structure of hydroxyapatite scaffolds coupled with their non-absorbable characteristics might facilitate the rapid diffusion and loss of soluble proteins, limiting the capacity of BMPs to promote

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osteogenesis [82]. According to some studies, a pore size of 90–200 μm could be suitable for osteoconduction [128]; however, a pore size of 300–400 μm could also be considered as optimal for cell attachment, differentiation and growth [133]. In more recent studies, the use of novel micro- or nanostructures has improved the performance of hydroxyapatite carriers for BMPs. For example, it has been demonstrated in vitro that the nanotopography of four experimental hydroxyapatite bioceramics was a critical factor that improved the bioactivity and osteoinductivity of BMPs, enhancing the response of bone marrow stromal cells [134]. Similarly, the use of hollow hydroxyapatite microspheres (100 μm) as an osteoconductive matrix and carrier for controlled local delivery of rhBMP-2 has shown potential as a bone graft substitute compared with control or hollow microspheres without the protein [135]. Nanostructured microspheres of hydroxyapatite loaded with rhBMP-2 have been shown to improve osteogenesis compared to conventional microspheres also loaded with rhBMP-2 in the treatment of rat femoral bone defect [136]. A recent study has shown the advantage of using mesoporous hydroxyapatite nanoparticles as a carrier for binding BMP-2 to a scaffold of silk fibroin/chitosan, achieving osteogenic differentiation of bone marrow stem cells in vitro and inducing more pronounced bone formation in vivo [15]. In a study of complex hydroxyapatite-based carriers, a 3D-printed scaffold composed of gelatin, chitosan and hydroxyapatite nanoparticles was reported as producing sustained co-delivery of BMP-2 and Vascular Endothelial Growth Factor (VEGF), promoting osteogenesis and angiogenesis, and accelerating new bone formation in both in vitro and in vivo [137]. Similar results were reported using a collagen/hydroxyapatite scaffold for the dual delivery of growth factors (BMP-2 and VEGF), achieving complete bridging of a critical-sized rodent calvarial defect and facilitating the use of low doses of growth factors [138]. The combination of micro- or nanohydroxyapatite particles with cellulose scaffolds has also shown promising results, demonstrating the ability to promote greater cell adhesion and spreading, increasing metabolic activity and osteoblast gene expression in vitro, and inducing a significantly higher amount of newly formed mineralized tissue in vivo [139].

β-Tri-Calcium Phosphate (β-TCP), with the abbreviated formula $(Ca_3(PO_4)_2)$, is another type of calcium phosphate salt used in bone regeneration. It derives from apatitic tricalcium phosphate $(Ca_9(HPO_4)(PO_4))$ and its calcium/phosphate ratio can vary widely according to the pH value and temperature used during production [140]. Its biocompatibility has been widely verified, making it feasible to use it as bone graft biomaterial alone [141] or in combination with BMPs in spinal fusion procedures and for bone augmentation in implant dentistry [142–144]. The use of β-TCP in combination with BMPs has demonstrated an increase in trabecular bone formation and a higher mechanical stiffness [145]. This combination has also been shown to have a more potent osteoinductive effect compared with the use of BMP alone [146] or β-TCP alone [147–149] in animal models. Most importantly, the combination of β-TCP with BMPs has shown similar results to those achieved using autologous bone graft in the regeneration of bone in rat calvarial defects [150]. Like other bioceramics, β-TCP has been combined with different biomaterials to fabricate complex carriers with improved performance in the delivery of BMPs. For example, a thermosensitive alginate/β-TCP hydrogel combined with BMP-2 has been shown to induce a significantly higher percentage of mineralized tissue in critical-sized calvarial defects in rats [151]. Combining β-TCP with polycaprolactone as a carrier for rhBMP-2 has also been shown to induce new bone in mandibular bone defect models of animals [152]. The combination of β-TCP and hydroxyapatite granules in a synthetic matrix of PEG to deliver rhBMP-2 has been shown to significantly enhance bone regeneration in calvarial bone defect in rabbits [153]. However, it has been also reported that the addition of PEG to a construct of β-

TCP and hydroxyapatite could compromise the osteogenic effect of BMP-2, possibly due to the lower degree of cell attachment described for the polymer [154]. Finally, while polymers are typically used to contribute to overall physical and chemical properties, it is important to clarify that to date there is no evidence of them contributing directly to more mineralized tissue formation, unlike the addition of BMP-2 [155].

Another type of bioceramic used to fabricate carriers for BMPs is Biphasic Calcium Phosphate (BCP), which is a mixture of a more stable hydroxyapatite and a more soluble β-TCP in different ratios [156]. The term BCP was first used by Nery and colleagues and originally described as a "two-phased calcium phosphate" [157]. The use of BCP to deliver rhBMP-2 has been shown to increase osteopromotive differentiation in vitro [158], and enhance bone regeneration in critical-sized cranial defects in mice [159] compared to controls. Similarly, the combination of BCP and rhBMP-2 has been shown to induce higher percentages of bone formation in animal models when compared with the use of hydroxyapatite carriers [160] or collagen scaffolds [161,162]. Adding collagen to the combination of BCP and BMP-2 has proved to increase the bone formation capabilities even further [163]; however, although bone formation appears to be higher in the early stages of regeneration, a study concluded that at 8 weeks the substantial difference in bone growth between the use of BCP with rhBMP-2 versus ACS with rhBMP-2 in calvarial defects diminished [164]. Despite this evidence, some clinical studies have shown disparities in their results. In a human maxillary sinus floor augmentation study, the combination of BCP and rhBMP-2 was found to be inferior at regenerating bone than DBM at 24 weeks after surgery [165]. A 12-week clinical trial compared a test group receiving ACS soaked in rhBMP-2 with a control group receiving BCP immersed in rhBMP-2; the two treatments showed similar efficacy and healing in alveolar ridge preservation [166]. Since the amounts of hydroxyapatite and β-TCP in the BCP composites may differ, the question of which proportions are best for bone regeneration has been addressed repeatedly. Various studies comparing different ratios of hydroxyapatite/β-TCP (20/80, 30/70, 40/60 and 50/50) have demonstrated that higher hydroxyapatite ratios (over 30%) could be considered more appropriate for the construction of carriers for rhBMP-2 [167,168]. Regarding the dosage of BMPs, the combination of BCP with a high concentration of rhBMP-2 (1.5 mg/ml) has proved to inhibit bone regeneration from pristine bone and increase the inflammatory response in the early stages in animal models [169]. Meanwhile, a low dosage of rhBMP-2 (0.05 mg/ml) in a BCP carrier has been shown to promote an osteoinductive effect with accelerated mineralization in animal models [170]. Moreover, it has been reported that a combination of BCP with 0.5 mg/ml of rhBMP-2 formed a greater volume of bone in a rabbit model than BCP combined with 1.0 mg/ml of rhBMP-2 [171]. The use of collagenated BCP-based carriers have allowed a more controlled and sustained release of BMP-2 which showed a significant new bone formation in maxillary sinus floor augmentation procedures in rabbits [172].

Finally, an alternative to the calcium phosphates salts discussed above is to construct carriers using biomimetic calcium phosphate particles with co-precipitation of the osteoinductive protein. This type of bioceramic is made using amorphous calcium phosphate microparticles coated with crystalline calcium phosphate layers in a supersaturated calcium phosphate solution. After several coating cycles, the amorphous calcium phosphate and crystalline calcium phosphate are assembled layer-by-layer until the addition of the final crystalline calcium phosphate layer, in which the soluble BMPs are introduced into the solution and precipitated [173]. This biomimetic calcium phosphate allows slow and continuous release of the protein, which was shown to induce bone formation in both ectopic and orthotopic sites in animal models [174].

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3.4. Complex combinations

Novel delivery systems for BMPs have been fabricated by combining two or more classes of biomaterials. These complex combinations have improved several characteristics of classic carriers such as mechanical performance and controlled release profile of growth factors. For example, the use of a carrier composed of collagen and BCP (hydroxyapatite/β-TCP ratio 60:40) with low doses of rhBMP-2 showed strong osteogenic potential and faster new bone formation compared with the same carrier but using higher doses of the protein [175]. A different carrier fabricated with polycaprolactone containing osteoblasts encapsulated in a HA hydrogel and incorporating BMP-7, showed the ability to produce mineralized collagenous matrix after 6 weeks in vitro, and vascularized-bone-like tissue after 4 weeks in vivo [176]. Another example is the combination of chitosan-alginate gel with MSCs and BMP-2, which has been shown to stimulate new bone formation with trabecular pattern after injection into the subcutaneous space on the dorsum of nude mice [177]. More recent, an "injectable bone" loaded with BMPs has been developed through the combination of a 3D-printed polylactic-co-glycolic acid/nano-hydroxyapatite scaffold with rhBMP-2 encapsulated in chitosan nanoparticles embedded in a chitosan hydrogel. This injectable bone complex demonstrated good biocompatibility, appropriate growth factor release profile and a potent osteogenic effect in animal models [178]. Another innovative approach is the sequential delivery of BMPs using PLGA nanocapsules loaded with BMP-2, and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) nanocapsules loaded with BMP-7, both incorporated on a 3D fiber mesh prepared from chitosan and poly(ethyleneoxide) [179]. This scaffold allowed early release of BMP-2 and longer-term release of BMP-7, enhancing cell proliferation of rat bone marrow mesenchymal stem cells and increasing ALP activity showing a synergistic effect between both growth factors [179,180]. The feasibility of using dual growth factor release has also been tested for rhBMP-2 and rhVEGF, using a polymer carrier composed of poly-DL-lactic acid and calcium carbonate ($CaCO_3$) particles. This carrier was able to induce bone formation in mandibular bone defects of minipigs after 4 and 13 weeks. The combination of angiogenic and osteogenic growth factors allowed the dose required previously to be reduced, while still inducing bone formation [181].

Focusing on the mechanical characteristics of the carriers, highly porous complex combination of biomaterials with the desired compressive strength and degradation rate have also been investigated. A three-dimensional β-TCP scaffold with internal canals, coated with gelatin layers and filled with BMP-2-loaded chitosan nanoparticles dispersed into collagen hydrogel, has been shown sustained growth factor release, inducing osteoblast-like differentiation of human buccal fat pad-derived stem cells in vitro [182]. Another example of a compression-resistant scaffold for BMPs was fabricated using resorbable lysine-derived poly (ester urethane) and BCP particles of 15% hydroxyapatite and 85% β-TCP with size ranging from 100 to 500 μm [183]. In combination with rhBMP-2, this scaffold has shown the ability to promote significant new bone formation in alveolar ridge defects of non-human primates [184].

Other delivery systems made of complex combination of biomaterials have been formulated and tested in both in vitro and in vivo studies; however, due to disparities in the types and number of biomaterials combined, and differences in the topological architectures and modalities of construction/fabrication, their classification and comparison in terms of biological and mechanical performance is harsh. Considering the heterogeneity of these studies, there is an urgent need for a rational systematization of which biomaterials should or should not be combined to construct carriers, in order to fulfill all the requirements for growth factor delivery to repair bone defect sites.

3.5. Gene therapy for BMPs

Although the topical delivery of BMPs using carriers has shown promising results for bone regeneration in vitro, in vivo and even in some clinical studies, there are still some important limitations and concerns regarding their use. Surprisingly, despite the large number of carriers developed and the possibilities for biomaterial combinations, there is as yet no consensus on which exhibit the best performance in enhancing the efficiency of BMPs. Furthermore, due to the disparities in study design, biomaterials used and assessment methods, it seems that this consensus is far from being established. A new approach based on gene therapy has therefore been explored to bypass the limitations associated with the use of biomaterials and the local delivery of soluble proteins. Somatic gene therapy consists in the insertion of genes into single cells and tissues to treat genetically based disease or, in the context of bone formation, to induce the expression of key growth factors [185,186]. Either the desired gene can be transfected directly into the target site (in vivo approach), or target cells can be harvested, expanded and genetically manipulated before being re-implanted in the defect site (*ex vivo* approach) [187]. Viral and non-viral vectors have been used for both approaches, achieving bone formation in ectopic and orthotopic sites [188]. In the field of dentistry, gene therapy has been applied in preclinical periodontal regeneration models using *ex vivo* BMP-7 transfection of syngeneic dermal fibroblast of rats [189]. This was the first evidence for chrondrogenesis, osteogenesis and cementogenesis in large mandibular bone defects using this technique. The *ex vivo* approach has been also used to induce bone formation in rat calvarial defects through transfection of human gingival fibroblast with BMP-2 gene [190]. Meanwhile, in vivo gene therapy has demonstrated efficacy in bone formation for the treatment of large maxillary osteotomy defects in rats using recombinant adenoviral vectors encoding BMP-7 [191]. Despite this evidence, gene therapy studies applied to bone regeneration in the maxillofacial territory are still in early stages, and more research is needed to overcome problems associated with the use of viral vectors, the current limited timing of effectiveness, the current use of a single gene in complex diseases or contexts, and the possibilities of rejection caused by immune response [186].

4. Concluding remarks

The effective use of bioactive molecules such as BMPs represents the new frontier in bone tissue regeneration. Today, great efforts are being made to determine which BMP or combination of molecules is most appropriate in a given scenario, and which is the most effective kinetic release profile and dose. To achieve this goal, several delivery systems fabricated with different combinations of biomaterials have been tested; however, due to the heterogeneity of the studies and intended clinical applications, there is still no consensus on which exhibits the best performance. To the best of our knowledge, in the maxillofacial territory only BMP-2, BMP-4, BMP-7 and BMP-9 have been used for bone regeneration in human or animal models; they have been applied using a wide variety of carrier systems and doses.

Various requirements have been established for a carrier to be considered a good delivery system for growth factors. A good carrier should allow the use of minimum doses of these molecules, with long-term activity and few side effects, while fulfilling the mechanical requirements of the bone defect site to be treated. Thanks to the advances achieved in biomaterial science, today there is a longer list of carriers that have been shown to be weakly immunogenic, with great biodegradability, appropriate resistance to mechanical stress and the modulatory capacity to immobilize, retain and release BMPs during the healing process. The capacity of different carrier systems to provide controlled and sustained release of growth factors has been achieved thanks to modifications in particle

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size, percentage of porosity, three-dimensional configuration and stiffness that polymer chemistry has allowed efficiently. In this sense, the development of more complex scaffolds and carriers that combine, for example, the rigidity and biocompatibility of calcium phosphate salts with the malleability and different architectural conformations of natural or synthetic cross-linked polymers has allowed high percentages of bone formation in many preclinical models. Despite the above, there is still a gap between the development of new carrier systems and the fulfillment of clinical needs for bone formation. In general, all these carriers are tested under experimental conditions that do not consider the mechanical forces usually involved in a functional stomatognathic system. Given these needs, parameters such as biodegradability, stiffness, mechanical strength and distribution of forces should probably be the main concerns of researchers in developing new carriers.

Finally, the development of new carrier systems requires deep knowledge of the highly regulated control of cell organization, cytokine interactions and cell behavior in physiological conditions, combined with knowledge of the physical and mechanical requirements of each bone defect site, in order to fabricate a functional three-dimensional construct that does not interfere with the natural bone healing process but facilitates the actions of bioactive agents and guides cellular activities.

Conflict of interest

None.

Acknowledgments

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