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Review article

Biphasic calcium phosphate ceramics for bone reconstruction: A review of biological response

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ABSTRACT

Autologous bone graft is considered as the gold standard in bone reconstructive surgery. However, the quantity of bone available is limited and the harvesting procedure requires a second surgical site resulting in severe complications. Due to these limits, scientists and clinicians have considered alternatives to autologous bone graft. Calcium phosphates (CaPs) biomaterials including biphasic calcium phosphate (BCP) ceramics have proven efficacy in numerous clinical indications. Their specific physico-chemical properties (HA/TCP ratio, dual porosity and subsequent interconnected architecture) control (regulate/condition) the progressive resorption and the bone substitution process.

By describing the most significant biological responses reported in the last 30 years, we review the main events that made their clinical success. We also discuss about their exciting future applications as osteoconductive scaffold for delivering various bioactive molecules or bone cells in bone tissue engineering and regenerative medicine.

Statement of Significance

Nowadays, BCPs are definitely considered as the gold standard of bone substitutes in bone reconstructive surgery. Among the numerous clinical studies in literature demonstrating the performance of BCP, Passuti et al. and Randsford et al. studies largely contributed to the emergence of the BCPs. It could be interesting to come back to the main events that made their success and could explain their large adhesion from scientists to clinicians. This paper aims to review the most significant biological responses reported in the last 30 years, of these BCP-based materials. We also discuss about their exciting future applications as osteoconductive scaffold for delivering various bioactive molecules or bone cells in bone tissue engineering and regenerative medicine.

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1. Introduction

1.1. Clinical context

Despite the benefits that minimally invasive osteosynthesis and surgery have brought to fracture and bone healing, there are still many circumstances where achieving bone healing may prove challenging. Autologous bone grafts are still considered the gold standard in bone repair and regeneration because of their osteogenicity, osteoinductivity and osteoconductivity [1]. But in human medicine, some instances clearly demonstrated the clinical equivalence of synthetic bone substitutes over autografts which then are no longer recommended as they are time consuming in the OR expansive in terms of hospitalization and available in limited quantity. They induce morbidity and chronic pain and can be associated with unpredictable outcomes [2–7]. The harvesting procedure requires a second surgical site, with which complications have been reported, and the quantity of bone graft is limited. In addition, autologous bone grafts may be too rapidly resorbable as they can be degraded before bone healing has been completed [8].

Allogenic and xenogenic bone substitutes have also been proposed and are still used in some clinical applications [9]. But viral transmission and a lack availability of native bone have led to the development of synthetic bone substitution biomaterials whose use dramatically increased in the last 15 years, because of their reliable manufacturing process and the possibility of combining them with bioactive molecules, therapeutic agents and cells for tissue engineering, cell-therapy and gene-therapy applications.

1.2. Biomaterials as bone graft substitutes

Synthetic bone graft materials available as alternatives to autogenous bone for repair, substitution or augmentation include: metals; resorbable and non-resorbable polymers; inert ceramics (e.g., alumina, zirconia); special glass ceramics described as bioactive glasses; calcium sulfates, calcium carbonates and calcium phosphates (CaP). These inorganic materials differ in composition and physical properties from each other and from bone [10–12].

Since bone mineral is made of non-stoichiometric and polysubstituted CaP apatite, CaP materials were rapidly preferred as they can be part of the bone remodeling process. Based on composition, synthetic calcium phosphates presently used as biomaterials are classified as calcium hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$; alpha- or beta-tricalcium phosphate (α- or β-TCP), $Ca_3(PO4)_2$; biphasic calcium phosphates (BCPs) for mixtures of HA and β-TCP; and unsintered apatites or calcium-deficient apatites (CDA). HA and β-TCP ceramics can be prepared by grounding CaO and P_2O_5 powders with Ca/P equals to 1.67 and 1.5 respectively. These mixtures have to be subsequently sintered over than 1100 °C and generally submitted to further grounding/sintering processes until the final powder presents a homogeneous final Ca/P. CDAs can be prepared either by aqueous precipitation from calcium and phosphate salts or alkaline hydrolysis of acidic calcium phosphates [13–15]. BCPs, with varying β-TCP/ HA ratios can be prepared by sintering precipitated CDAs of varying Ca/P ratio [16–18]. Calcium phosphate biomaterials differ in their solubility or extent of dissolution in acidic buffer which may reflect the comparative dissolution or degradation *in vivo* [14]. The comparative extent of dissolution is α-TCP ≫ CDAs > β-TCP ≫ HA. For BCPs, extent of dissolution depends on the β-TCP/HA ratio, the higher the ratio, the higher the extent of dissolution [14,19]. BCP have been described for the first time in 1985 at the 11th Annual Meeting of the Society for Biomaterials [20,21]. They were used by Nery et al. in 1975 [22] but the preparation was wrongly described as 'tricalcium phosphate' which was corrected by these authors in 1986 [23] and confirmed by LeGeros in 1988 [18].

- Nowadays, BCPs are definitely considered as the gold standard of bone substitutes in bone reconstructive surgery. Among the numerous clinical studies in literature demonstrating the performance of BCPs [4,6,7,24,25], Passuti et al. [26] and Randsford et al. [27] studies largely contributed to the emergence of the BCPs. It could be interesting to come back to the main events that made their success and could explain their large adhesion from scientists to clinicians. This paper aims to review the most significant biological responses reported in the last 30 years, of these BCP-based materials.

2. The biological responses of BCP ceramics

2.1. The role of HA/β-TCP ratios

Chemical properties of ceramics may influence the resorption activity by osteoclasts. Among the chemical properties, solubility of ceramic is probably one of the most important to control. It is irrelevant to affirm that by increasing the solubility of ceramic, the resorption activity would be optimal. By contrast, synthesis of a ceramic too soluble might create an important gradient of calcium ions extremely deleterious for the activity of osteoclasts. Given that solubility of ceramic is mainly dependant on the ratio HA/β-TCP, some studies were interested in determine which is the best selected ratio [28–30].

In this attempt, Yamada et al. have tested CaP ceramics with various degrees of solubility according to HA/β-TCP ratios [30]. Resorption activity was observed on pure β-TCP and BCP 25/75 (25% HA/75% β-TCP). Osteoclasts did not resorb BCP 75/25 (75% HA/25% β-TCP) or pure HA. Interestingly, they observed that solubility influences the pattern of osteoclastic resorption in terms of shape and distribution of resorption lacunae. For example, on pure β-TCP, lacunae appear discontinuous like a chain of small islands whereas they are large and continuous on BCP 25/75 (25% HA/75% β-TCP). resembling those on bone. In addition, the shift in functional phases from resorption to migration seems to occur earlier on β-TCP than on BCP 25/75 (25% HA/75% β-TCP). Data in literature are often contradictory considering the various ceramics tested. Their properties tend to vary depending on the mode and sintering processes which induce different phases in ceramics and various amounts of lattice defects crystals and even when the

ceramic is the same material. That could explain why Badran et al. showed that osteoclasts can resorb easier β-TCP than BCP [31].

In response to the degradation of ceramics (ie by solubility and by resorption activity) and depending on the HA/b-TCP ratio, some free ions released may subsist in the vicinity of bone progenitor cells, which could thus trigger the osteogenic differentiation, thereby participating in new bone formation. For example, osteoblasts respond directly to changes in Ca^{2+} concentration in bone microenvironment [32], and osteoblastic differentiation of mesenchymal stem cells is accompanied by the expression of Ca^{2+} binding-proteins and Ca^{2+} incorporation into the extracellular matrix [33]. Moreover the increase in inorganic phosphate (Pi) has also been shown to act as a specific signal, affecting the expression of various genes implicated in the proliferation, differentiation, mineralization and apoptosis of skeletal cells [34]. More precisely, Pi regulates the expression of several mineralization-associated genes such as osteopontin (OPN) and matrix gla protein (MGP) [35]. In addition, by hydrolyzing inorganic pyrophosphate ions (PPi) into Pi, ALP promotes type I collagen mineralization by preventing the inhibitory action of PPi on mineralization. A sustained release of Pi was shown to upregulate the mineralization process of collagen by overriding the inhibitory effect of PPi.

To sum up, by controlling the ratio of HA-betaTCP of BCPs, it should be possible to control not only the resorption rate of BCPs but also the release of ions in the vicinity of bone cells, and consequently modulate the biological properties of BCPs.

Considering the importance of the HA/beta-TCP ratio of the BCPs on their bioactivity, attention must be paid to the possible thermal decomposition of HA to beta-TCP during the sintering process of BCP synthesis. For example, with pre-sinter HA/beta-TCP = 40/60 wt%, approximately 80% of the HA decomposed to beta-TCP during sintering at 1000 °C. As mentioned by Nilen et al., HA content appeared to influence the reverse transformation of alpha-TCP to beta-TCP expected upon gradual cooling from sintering temperatures >1125 °C [36].

2.2. Biological interactions with ceramic surfaces: The role of microporosity

As pertinently evidenced by Bohner et al., it remains complex to define what is the optimum scaffold architecture despite the numerous studies in literature [37]. In fact, the scaffold architecture evolves constantly with the degradation process and consequently modifying the ions profile release and the degradation by-products that make difficult to predict the biological response of biomaterials.

However, it has been largely admitted that structure of ceramics including macro/microstructure and interconnectivity determines interactions with biological fluids and influences behaviour of bone cells [38–40]. Major cellular events such as growth, colonization and differentiation are dependent of early cell adhesion mechanisms including protein adsorption and cellular attachment. Understanding the molecular mechanism of cellular adhesion on biomaterials is necessary for the development of the future biomaterials. Various molecules are involved in adhesion such as cytoskeleton proteins, cell membrane proteins and extracellular matrix proteins. Following the aggregation of integrins, tensin and FAK (focal adhesion kinase) accumulate and bind the integrins. Interaction between integrins and extracellular matrix results in accumulation of vinculin, talin, actin and activation of FAK in the focal adhesion site of material. Both microporosity (pore size < 10 μm) and surface rugosity affects the expression of these proteins in a time-dependent manner. For example, expression of vinculin gradually decreases with time once cells become stable on HA [41].

Rouahi et al. investigated the *in vitro* influence of the microstructure of a microporous HA as compared to non-microporous HA on serum protein adsorption and bone cells attachment and their proliferation [42]. The structural characteristics of microporous HA were roughness amplitude estimated at 4.35 μm (vs 0.065 μm) and open microporosity around 12% whereas non-microporous HA displayed only closed pores (2.5%). Microporous HA adsorbed 10-fold more proteins, essentially fibronectin and albumin, than non-microporous HA. The higher levels of c-fos and c-jun gene expression observed could explain a better presentation of extracellular matrix molecules on microporous HA [43]. By contrast, a weak expression of integrin genes has been observed in non-microporous HA that could result in its lowest adhesion. As also observed by Yuan et al., microporosity of ceramic increases considerably its protein adsorption [44].

Presence of these proteins on ceramics promotes bone cells adhesion that directly impacts on morphology of cells. In fact, cells appear like "adsorbed" by the HA surface and exhibit the particularity of the cytoplasmic edge undistinguishable from the surface, with only the extremity of the cells and lamellipodia visible. In consequence of this higher attachment capacity, cellular proliferation is decreased. Isaac et al. have studied the effects of ceramic (β-TCP) microporosity (0, 25 or 45%) on the behaviour of osteoprogenitor cells [45]. Interestingly, they observed that a high microporosity decreased the viability of human bone marrow stromal cells (BMSC) in a time and rate-dependent manner. They also showed that increased microporosity inhibited osteoblastic differentiation as compared with non-microporous ceramics, as revealed by decreased alkaline phosphatase activity and osteocalcin secretion. These results are in agreement with reports from the literature [46,47]. For example, Rosa et al. demonstrated rat BMSC proliferation and osteoblastic differentiation are greater on ceramics with microporosity rates of 5% and 15%, as compared with 30%. By affecting bone cells spreading, microporosity may inhibit their viability and their differentiation potential. It is also possible that high microporosity rates may modulate ceramic solubility and affect levels of calcium and phosphate ions which in turn affect the osteoblast viability [48], commitment [49] and maturation [50].

To sum up this duality effect observed *in vitro*, on one hand microporosity displayed an important role to protein adsorption and on the other hand microporosity inhibited osteoblastic differentiation. These *in vitro* studies did not take into consideration the essential role of the microvascularisation into the microporores as recently shown *in vivo* by Rustom et al. [51]. This result is in accordance with few studies proving the beneficial role of microporosity on osteointegration and neoformation of bone [52–56]. Despite these interesting data, little attention has been paid to elucidate the ideal microporosity. In fact, interest continues to focus on macroporous ceramics with pores >100 μm in diameter. Lack of relevant studies is probably due to the difficulty to vary micropore size without changing other parameters such as porosity. Moreover assessment of the sample microporosity depends on the methods used (i.e. microCT, materialography or mercure porosimetry) which making comparison difficult between *in vivo* studies in literature. For example, Klein et al. addressed the impact of the microporosity on the BCPs behaviour [57]. They observed that resorption rate increased with microporosity whereas Lapczyna et al. conclude on absence of significant differences of bone formation between four types of BCPs scaffolds with various microporosities from 10 to 30% [58]. As pertinently suggested by Lapczyna et al., further experiments have to be conducted to decipher the mechanisms of resorption and bone formation in microporous BCPs.

2.3. Biological interactions with ceramic volume: the role of macroporosity and macrointerconnection

Macroporosity (pore size >80–100 μm) is defined by its capacity to be colonized by cells. It can be induced in the material by the addition of organic substances (e.g., naphthalene or sucrose particles) that are sublimated or calcinated before sintering at higher temperatures [17,18]. Both macroporosity (pore size >80–100 μm) and interconnectivity support the cellular invasion on ceramics. According to Bignon et al., interconnection size of 15 μm appeared to be effective to promote cellular colonization without bringing down mechanical strength [59]. It appeared from histological observations that osteoblasts were able to cross interconnections measuring less than 5 μm. By consequent, it can be assume that larger connections would optimize cellular penetration. Cell growth occurred from the surface to the depth of ceramics. In fact, cells bridged macropores with their long cytoplasmic sprouts that linked to the walls and on the micropores. Cells formed a lining covering the surface by creating anastomoses with each other. Chouteau et al. have observed this flourishing cell growth into the macropores and a very dense network of cytoplasmic extensions [60]. Increasing the size of macroporosity would reduce the number of interconnections to cross and therefore would accelerate the cellular colonization. However, that would extremely compromise the mechanical resistance of ceramics [61,62]. Toquet et al. studied the *in vitro* osteogenic potential of human bone marrow cells cultured on macroporous BCP pellets [63]. They showed that macroporosity of biomaterials play a key role in both cellular phenotype expression and osteogenesis process. They considered that macropores provide an appropriate environment for the differentiation and growth of bone progenitor cells and subsequent new bone formation (Fig 1) [63].

To document the impact of total porosity of BCP on cell microenvironment, different BCP scaffolds were prepared with 25%, 50%, 65%, and 75% of total porosities and mean pore size of 300 μm [64]. The extracts of these scaffolds were assessed with regard to viability, proliferation and differentiation of human dental pulp cells. In presence of scaffolds with 65% and 75% of total porosities, viability and proliferation of human dental pulp cells were reduced probably due to both high alkalinity and calcium and phosphate ions release. Despite this reduction, BCP scaffold with 65% of total porosity displays a great odontoblastic differentiation strongly suggesting it can support human dental pulp cells differentiation for dentin tissue regeneration. Similarly Tang et al. evaluated the effect of macroporosity on the biocompatibility of BCP bioceramic scaffolds with different macropore sizes (100 to 600 μm) by using human dental pulp stem cells (hDPSCs) [65]. The hDPSCs exhibit favorable cellular adhering capacity on the pore surface of scaffolds, especially on the scaffolds with 100–200 mm pore diameter.

Gauthier et al. implanted macroporous biphasic calcium phosphate ceramics into distal femoral defects in rabbits [66]. Results indicated that the influence of macropore size is greater than that of macroporosity percentage. For similar macropore size, no significant difference in newly formed bone was noted for implants of 40 and 50% macroporosity. Osteoconduction was more efficient for BCP implants with a 565 μm than a 300 μm macropore diameter. A 565 μm pore diameter and a 50% macroporosity percentage should provide mechanical improvements and preserve optimal bone ingrowth in porous BCP ceramics (Fig 2). In 1999, elaboration procedures of BCP implants were found to have significant influence on their *in vivo* degradation [67] and emphasized the possibility of a thermally controlled microporosity. Low sintering temperatures, eg calcination of CDA powder, increase their reactivity and biodegradation properties when compared with high sintered BCP with similar HA/β-TCP ratio. Thus BCP ceramics present an intermediate *in vivo* degradation behaviour that provides a good compromise between sustainability of the CaP matrix for bone colonization and progressive resorbability. Such a progressive resorption-substitution process for ultimate replacement by a viable new bone proved optimal for biomaterial-supported bone regeneration.

2.4. Biocompatible behaviour of BCP ceramics: the role of the microparticles

Microparticles released from CaP ceramics may be phagocytosed by cells of the monocytic-macrophage lineage, triggering an inflammatory response characterized by the secretion of inflammatory cytokines. Among them, tumor necrosis factor (TNF-α) plays a crucial role in promoting bone repair through the induction of osteoprogenitor cell recruitment [68]. Interestingly Lu et al. postulated that this release could be related to the sintering process [69]. It is well known that BCP ceramic have to be sintered at 1160 °C. Consequently, HA micro-particles of BCP ceramic are incompletely sintered and easily released after immersion or

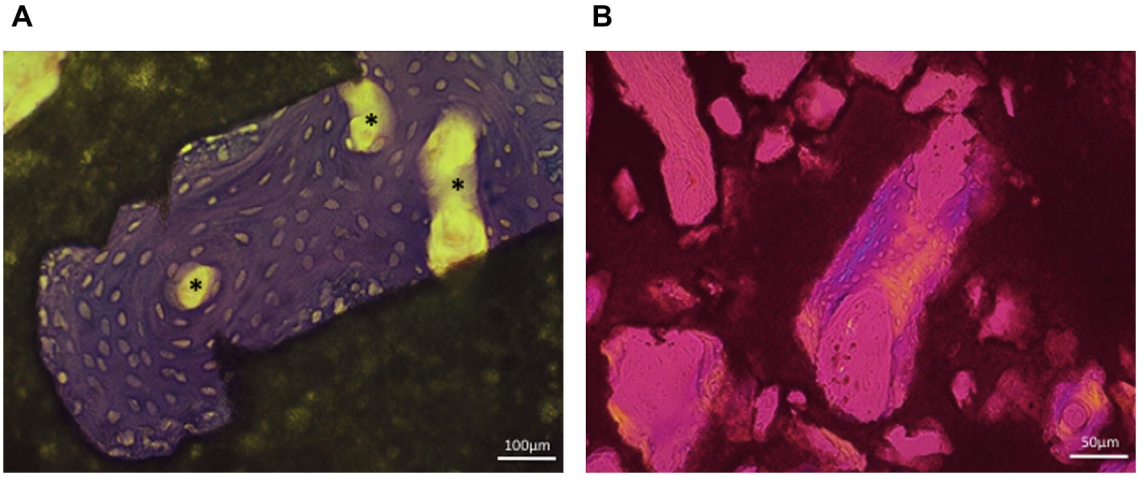

Fig. 1. Histological features of macroporous BCP new bone colonization. A. New-bone formation inside a macropore of a macroporous BCP ceramic implant 8 weeks after implantation in rabbit femoral defects. Lamellar and mineralized newly-formed bone grew onto the surface of the ceramic and restored an haversian system (*) that completely colonized a 200 μm macropore (solochrome cyanine staining; BCP ceramic appears in black, newly-formed bone in purple). B. New bone colonization of a macroporous BCP ceramic implant as early as 3 weeks after implantation in rabbit femoral defects (polarized light).

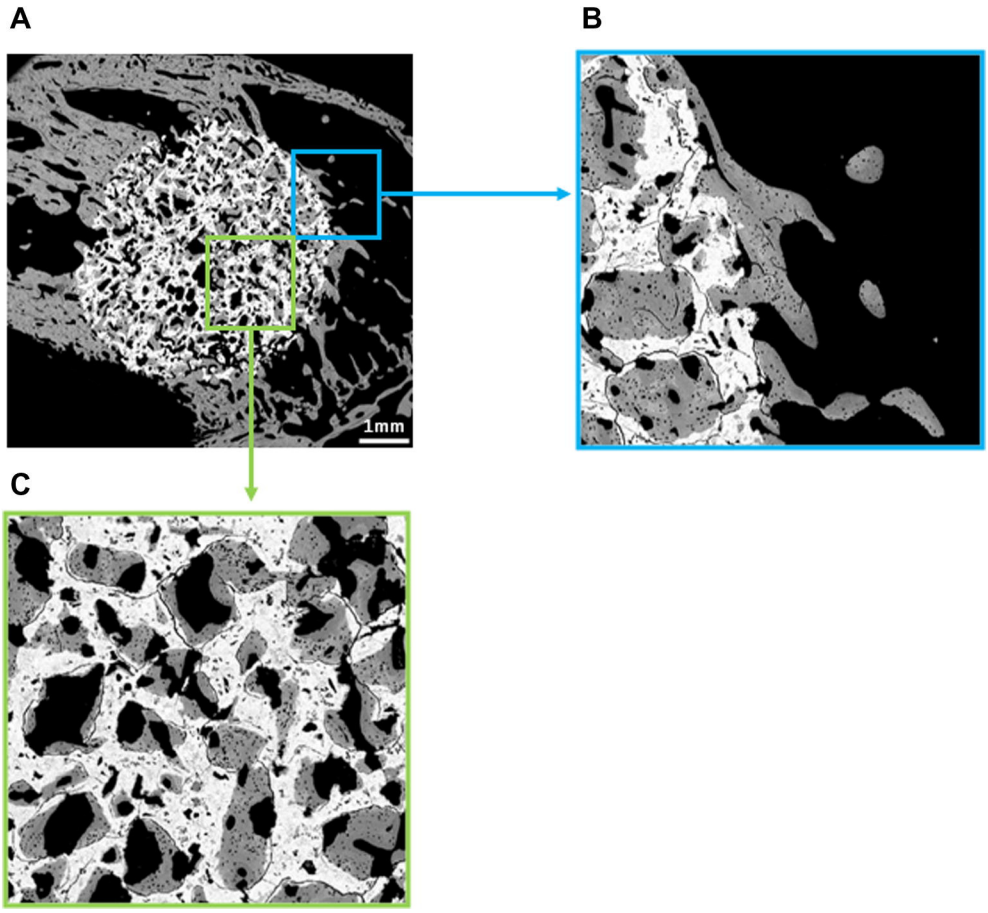

Fig. 2. A. SEM microradiographs of a macroporous BCP implant (mean macropore diameter 565 μm, macroporosity percentage 50%) in a 6-mm in diameter critical-size defect in rabbit femur. 12 weeks after implantation, bone ingrowth is extensive in both the peripheric macropores (B) and in the central part of the implant (C). In SEM BCP appears white, native host bone and newly-formed bone grey, non-mineralized tissues black.

implantation. They demonstrated that these microparticles released from BCP could induce local inflammation and cell damage resulting in affecting osteogenesis. This deleterious inflammatory response due to microparticles on osteogenesis was also confirmed by Fellah et al. [70].

Furthermore, Silva et al. analysed BCP microparticles (37 μm in size) impact on human macrophages locomotion and secretion [71]. They observed that cells and BCP granules attached to each other. Interestingly, cells attached to BCP presented a higher intracellular free Ca^{2+} concentration compared with nonattached neighbors and secreted CaP particles into the medium. By using Energy dispersive X-ray analysis, they also showed that the secreted particles presented a Ca/P ratio of 1.64 (±0.05) which is similar to hydroxyapatite. These secreted particles could create a transition zone favorable for further macrophage adhesion.

It has been also observed that fibroblasts and osteoblasts efficiently internalize particles that compromise their cellular functions. Finally the response of human mesenchymal stem cells as precursors of osteoblasts to particles released from bioceramics may be critical for successful bone regeneration [63,72].

A few reports in the literature document the impact of larger size of BCP particles on the cell behaviour. By seeding human mesenchymal stem cells (hMSCs) on BCP microparticles (140–200 μm), Cordonnier et al. observed that cells adhered and proliferated more rapidly in the first days of culture as compared to culture on plastic [73]. Analyses of hMSCs cultured without osteogenic factors on BCP particles revealed an abundant extracellular matrix production forming 3-dimensional hMSCs/BCP particles constructs after few days.

2.5. Bioactivity of BCP ceramics: the role of the granulometry

To complete these *in vitro* results, granulometry influence on both BCP resorption and osteoconduction were also studied *in vivo* by Malard et al. [74]. Three particle sizes were compared: 10–20, 80–100, and 200–400 μm. The 10–20 μm powders provided the best bone ingrowth, with a higher resorption/degradation rate in conjunction with stronger early inflammatory reactions. The 200–400 μm powders showed higher bone ingrowth than 80–100 μm ones, indicating that properties of cell recruitment for osseous apposition and mechanical support for bone bonding may both play a role in both ingrowth mechanisms.

Gauthier et al. investigated the *in vivo* performance of two suspensions of BCP particles presenting the same composition and two different granulometric profiles [40–80] and [200–500] μm [75]. These biomaterials were injected for 2, 3, 8, or 12 weeks into bone defects at the distal end of rabbit femurs. Bone colonization occurred more extensively during early implantation times for [40–80] μm suspension than for the [200–500] μm one (Fig 3). For the latter, BCP degradation occurred regularly throughout the implantation period, whereas it was very intensive during the first 2 weeks for the [40–80] μm BCP suspension. In *vivo* response of this [200–500] μm injectable suspension was also compared with the implantation of massive porous BCP cylinders in rabbit critical size and non load-bearing defects [76]. Histological studies and scanning electron microscopy (SEM) were used to compare their respective biological efficiency 3 and 8 weeks after implantation. The presence of intergranular spaces in the suspension was found to be particularly favorable to early neoangiogenesis and cellular

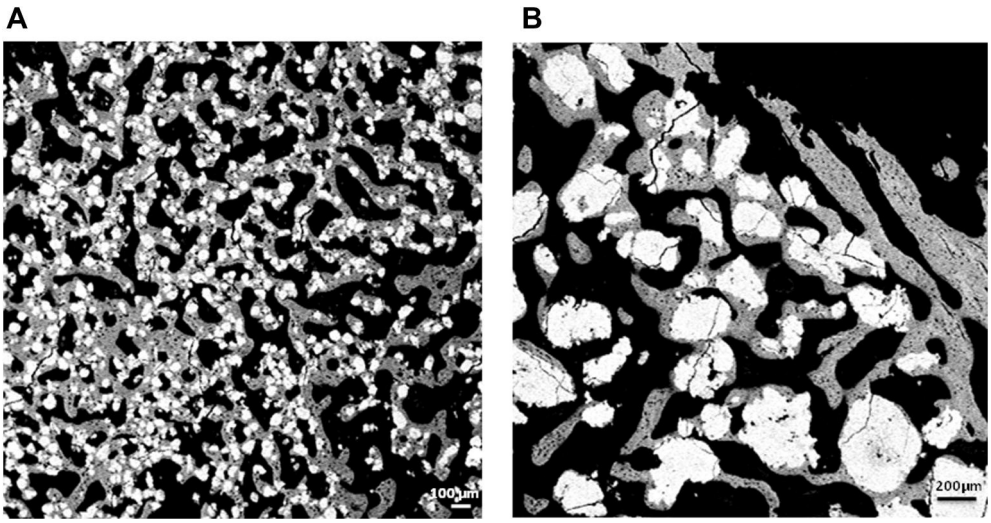

Fig. 3. SEM microradiographs of BCP particles in suspension in a cellulosic polymer and injected in rabbit femoral critical-size defects. Three weeks after implantation, a newly-formed bone network joined the granules the ones to the others, regardless the granules size (A: BCP particles 40 to 80 µm in diameter; B: BCP particles 200 to 500 µm in diameter), restoring the trabecular bone architecture.

colonization by osteoblastic cells from bone marrow as depicted in Fig. 4 [77]. However the respective rates of newly-formed bone after eight weeks of implantation did not differ significantly from one group to the other one. BCP resorption occurred regularly throughout the implantation period, though to a greater extent with the suspension than the massive ceramic cylinders [76].

A [200–500] µm fraction of BCP particles suspended in a viscous cellulosic-ether solution was studied for alveolar ridge preservation after tooth extraction in a canine model [78]. A SEM histomorphometric comparison was performed on fresh extraction sockets. Results always showed an alveolar bone resorption in unfilled sockets. Interestingly, an alveolar ridge augmentation was measured in mandibular filled sockets including 30% of newly-formed bone. In extraction sites with standardized vestibular bone defects, both intraoral retroalveolar radiographs and computed tomographic imaging showed that extraction site filling with an injectable ceramic with small BCP particles could preserve alveolar bone volume and alveolar width, as confirmed by subsequent histological analysis. Such properties could be of the highest interest to support dental implant placement [79]. In a canine model where dental implants were placed after mandibular premolars extraction a peri-implant mesial bone defect were surgically created and immediately filled with an injectable BCP ceramic. Such peri-implant bone defect filling with BCP particles enhanced significantly high bone-to-implant contact, and high peri-implant bone density in filled sites as illustrated in Fig. 5 [80]. A sinus lift model was developed in sheep by Saffarzadeh et al. in order to evaluate functionality of this BCP suspension for maxillary oral implantology purposes [81]. The biomaterial had been injected for 3 months and explanted for histologic and SEM quantitative studies. After 3 months, the mean rate of newly formed bone was significantly higher in sinuses filled with BCP particles than in control ones filled with morcellized autograft. Thus, newly formed bone represented 18.9% ± 5.4 of the surface of tested sinuses whereas in control ones, 12.9% ± 7.9 of the surface was occupied by bone tissue. In the same model, BCP granules were associated with fibrin glue to provide sinus floor augmentation and implanted for 6 months before dental implants were placed into the previously grafted area. No difference in implant stability was then observed 3 months after implant placement between autografts and BCP granules + fibrin glue, using a Resonance Frequency Analysis evaluation method [81]. In order

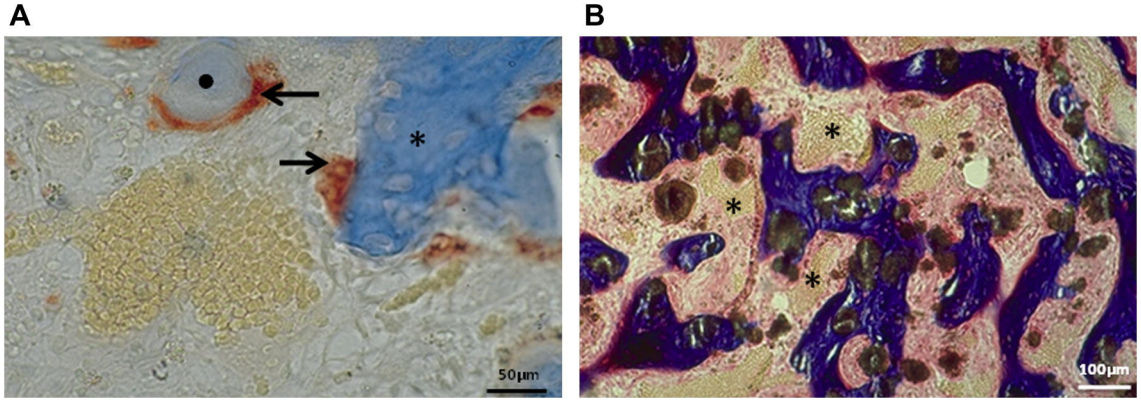

Fig. 4. Injectable BCP ceramic particles associated with a cellulosic polymer. A. Positive TRAP-stained cells (arrows) in contact with newly formed bone (*) and BCP particles (•) 2 weeks after implantation 40–80-µm BCP particles (original magnification X400). Note the very close presence of blood vessels. B. 40–80-µm BCP particles (black) are joined the ones to the others by newly-formed bone trabeculae (dark blue), in close relationship with new vascular structures (*).

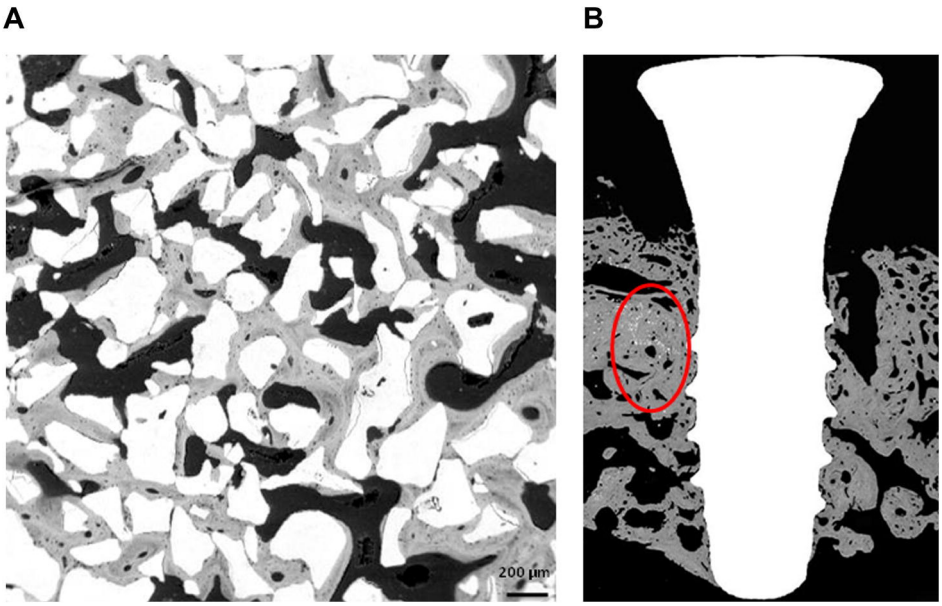

Fig. 5. Bone augmentation with BCP particles after dental extraction. A. SEM microradiograph of BCP particles in a cellulosic polymer injected into dental extraction sites in dogs. Twelve weeks after implantation a abundant new bone network developed into the intergranular spaces exhiting the osteoconductive properties of large BCP granules (200–500 µm). B. SEM microradiograph of BCP particles in a cellulosic polymer injected into dental extraction sites in dogs for immediate dental implant placement. 3 months after implantation, the whole implant surface is in close contact with newly-formed in an extraction socket filled with 40–80 µm BCP particles that appear almost completely resorbed (white points within the red circle).

to improve BCP particles handling, BCP granules were associated to a self-reticulating Si-HPMC hydrogel and proved to support bone filling in fenestration, furcation and peri-implants periodontal standardized osseous defects in a canine model. This suggests that such a self-reticulating hydrogel may not act only as a vehicle for BCP particles whose osteoconductive properties were preserved, but may also enhance intergranular cohesion and act as an exclusion barrier to prevent epithelial colonization of the defects [82].

3. From BCP ceramics to bone tissue engineering: Some extrinsic ways to increase osteoformation

3.1. Osteoconductive properties

Osteoconduction refers to bone ingrowth from bone defect edges towards the surface or down into the pores of a biomaterial, which serves as a scaffold or template to guide the formation of the new bone tissue [83]. This phenomenon is regularly seen upon the implantation of BCP and is considered dependent both on biological factors for bone repair, but also on the intrinsic properties (geometry, porosity) of the implanted biomaterial (fig 6).

Osteoconductive properties of ceramics have been extensively documented from pre-clinical experiments. For example, *in vivo* studies reported the use of BCP as bone substitute to fill defects generated in rabbit [69,84–87], and these experiments highlighted the osteoconductive properties of BCP particles with a size ranging from 40 µm up to 1500 µm. Reconstructed images obtained from SEM 2D and microtomographic 3D analysis reveal good osteointegration and excellent osteoconductive properties of 80–200 µm BCP particles after 8 weeks of implantation in a rabbit femoral defect (fig 7). BCP was also implanted *in vivo* in mouse [88], in rat [89] and in dog [90]. Depending on the study and the animal model, BCP microparticles (less than 20 µm in diameter, or 40–500 µm particles) or larger granules (> 1000 µm) were used. At last, several recent reports in the field of maxillofacial surgery for sinus augmentation documented the use of BCP for human patients [91–95].

3.2. Osteoinductive properties

In contrast to osteoconduction, osteoinductive properties of ceramics are still controversial in literature. In the past decade, osteoinduction has been observed by diverse calcium phosphate biomaterials in various forms such as (i) sintered ceramics including HA [96–99], β-tricalcium phosphate (β-TCP) [100,101], biphasic calcium phosphate (BCP), (ii) cements [102,103], (iii) coatings [104,105], as well as (iv) coral-derived ceramics [97,106] in various animal models. Also composites consisting of a polymer and HA have shown to be able to induce bone formation heterotopically [107,108].

Despite all these studies flourishing in literature, the exact mechanism of osteoinduction by biomaterials is still incompletely understood (Fig. 6). This is complicated by the fact that properties of the end material mostly depend on the processing parameters, which often differ among research groups. According to the synthesis modes of ceramics, macroporosity, grain size and surface roughness can differ resulting in different osteoinductive potential. Indeed, as strongly suggested by Habibovic et al., microstructural surface properties, including grain size, microporosity, surface roughness and specific surface area have been suggested as critical factors in osteoinduction [83,109,110]. Since these parameters determine the ability for BCP ceramics to link with proteins or peptides, that may account for their reported intrinsic osteoinductive properties. Micro/macroporous BCP ceramics particles proved to induce ectopic bone formation in muscular sites or osteoformation in bone sites without any osteoconduction contribution, especially in large animal models. It has been hypothetized that endogenous BMPs or other proteins can be adsorbed and can concentrate on the ceramic surface and contribute to attract osteogenic cells. In addition, surface topography and inorganic ion release from ceramics may also be a direct trigger of the process of osteogenic differentiation and bone formation.

It seems that osteoinductive properties might be animal model dependent. In contrast to large animal models, bone induction by biomaterials is weakly observed in smaller animals such as rodents. Besides interspecies variation, intra species variations

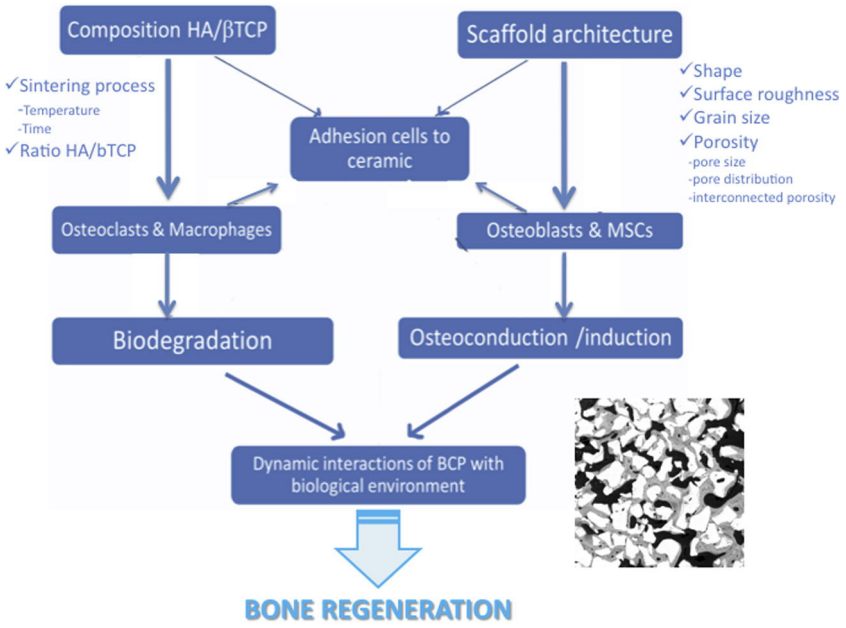

Fig. 6. This scheme recapitulates the dynamic and complex interaction between BCP ceramics and the biological environment. Chemical composition and scaffold architecture of BCP ceramics are essential parameters for controlling bone cells adhesion including bone-formation cells and osteoclasts. The concomitant action of these adherent cells mediates the biodegradation process and the osteoconduction/ induction properties of BCPs.

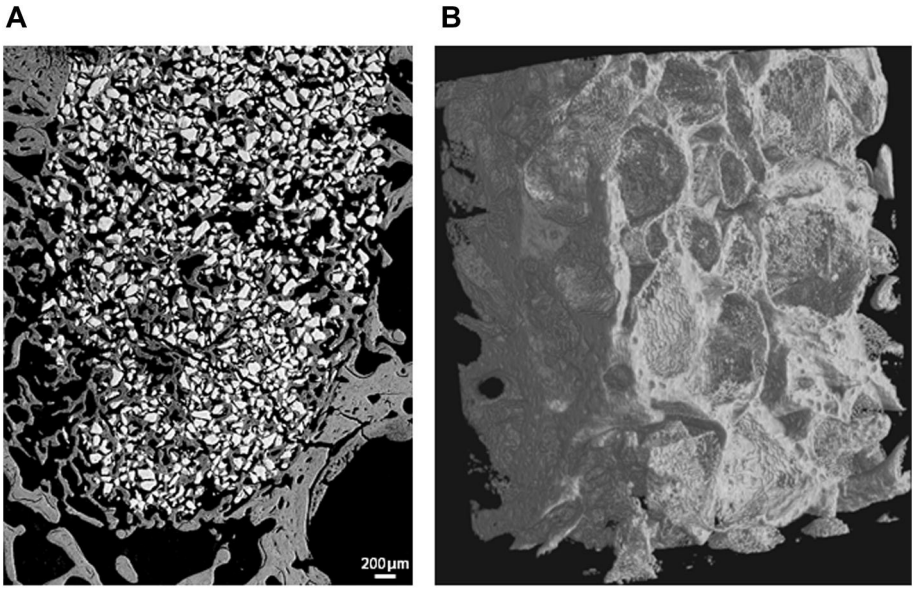

Fig. 7. BCP ceramic 80–200 µm particles in a cellulosic polymer 8 weeks after implantation in a rabbit femoral defect. A. SEM 2D microradiograph: new bone trabeculae developed into the intergranular spaces, filling the whole defect. B. Microtomographic 3D Synchrotron image: BCP ceramic particles have been retrieved by the image analysis system to show the new bone trabecular network that developed onto the BCP particles and into the intergranular spaces, providing an homogenous bone colonization throughout the whole defect volume [136].

have been found [111]. Moreover, the osteoinductive capacity of ceramics depends on the implantation site and duration of implantation.

From all of that, it is a real challenge to ascertain and to compare the osteoinductive potential of biomaterials after implantation. Osteoinductive properties of BCP ceramics still remain under investigation but to date, their clinical relevance does not seem yet established [112,113]. By the way, one could question about the real pertinence to document the relevance of osteoinduction in clinical practice since the injury in itself is sufficient to recruit a great number of osteoprogenitor cells and factors.

3.3. Tissue engineering

3.3.1. Cellular therapy

Bone marrow is a source of osteoprogenitor cells that can be stimulated to proliferate under appropriate conditions to form bone [114]. Quite recent studies have proposed to combine BCP materials with whole bone marrow (BM) aspirates or isolated mesenchymal stem cells coming from BM bone marrow aspirates in order to stimulate new bone formation in large critical-size defects or in bony environments where cellular activity is poor [115,116]. In 2010, Jégoux et al. performed 15-mm-long segmental defect in

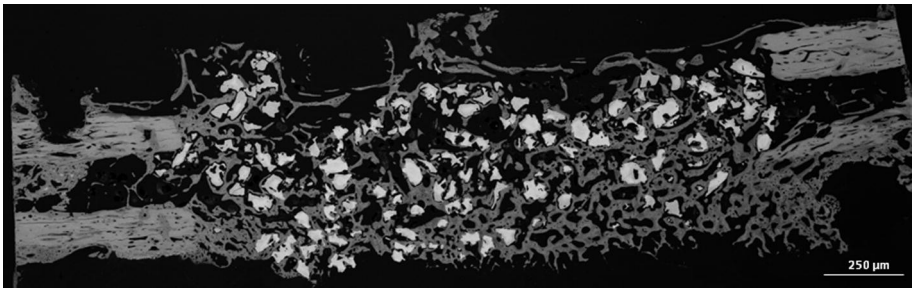

Fig. 8. SEM microradiograph of bone regeneration in a segmental critical-size defect model of non-union in dogs using BCP ceramic granules in an hydrogel delivering BMP-2. Local release of BMP-2 allowed complete regeneration of the defect, similarly to the one obtained with autologous cancellous bone graft, as BCP granules without any BMP-2 only showed very limited osteoconduction at the junctions with the host bone.

dogs mandibula that were filled with BCP granules previously wrapped in a collagen membrane [117]. Two months later, a bone marrow graft was injected into the center of the implants and after 16 week, an osseous colonization was observed in implanted sites bridging the whole length of the defects. In control conditions, nonunions were observed but the precise role of BM remained unclear as these control conditions were empty defects only.

In the perspective to perform bone substitutions after treatment of head and neck squamous cell carcinomas, the bioactivity of BCP associated with bone marrow cells was studied in irradiated bone. In 2005 a dog model replicating human oncologic treatments was developed and hollowed BCP blocks were implanted in tibial and femoral bone defects, then irradiated and injected with autologous bone marrow aspirations [118]. Implants were removed after 18 weeks and were analyzed, using scanning electron microscopy linked to quantitative image analysis. Autologous cells were found able to increase significantly new bone formation inside the BCP implant. The possibilities for bone reconstruction of (i) bone marrow, (ii) BCP granules [40–80 µm fraction] and (iii) association of the 2 former grafts were compared at 3 weeks in a rat model of bone irradiation [119]. Although this study did not provide information on the mechanisms that were involved in the bone repair (i.e. osteoprogenitor recruitment, neoangiogenesis), it evidenced that bone marrow graft associated with BCP granules significantly increased ceramic degradation and bone ingrowth in irradiated bone defects.

Similarly, Bléry et al. demonstrated the efficacy of BCP and total fresh bone marrow (TBM) in regenerating irradiated bone defect [120]. Interestingly, they observed that adding a high concentration of MSC didn't improve the bone regeneration. The association BCP + TBM remains the most efficient material for bone substitution in irradiated areas.

Osteogenic properties of combinations of a blood clot with BCP particles [5–500 µm fraction] were studied in syngenic mice ectopic and in rats long bone critical-sized defects [121]. Implantation in bony and ectopic sites revealed that this composite biomaterial is able to repair a 6-mm critical femoral defect in rat and induced woven bone formation after subcutaneous implantation. Parameters such as particle size and loading into the clot were found critical for its osteogenic properties.

Espitalier et al. compared bone reconstruction in irradiated areas using either mesenchymal stem cells (MSCs) or total bone marrow (TBM) in association with biphasic calcium phosphate (BCP) granules in a rat model [122]. Due to the presence of all components in TBM, the BCP-TBM mixture provides the best results in terms of vascularization and bone substitution.

3.3.2. Drug combined-devices

In a view to optimize their osteogenic properties, BCPs, in massive or granular forms, have been associated with various active molecules or growths factors [123,124]. Early studies investigated the association of the macroporous BCP blocks with human Growth Factor (hGF) and the consequences of the molecule local release on the resorption-substitution process of the BCP ceramic. Implantations in rabbit bone showed that hGH local release significantly increased both bone ingrowth and ceramic resorption compared to BCP ceramics without the associated growth factor, emphasizing that BCP ceramics could be a suitable matrix for the local delivery of bioactive molecules [125,126].

Considering the great interest in the last decade for the bone morphogenetic protein family (BMP) including BMP-2, some studies were interested in developing composites BCPs [127–131]. For example, BCP (87% HA, 13% β-TCP) loaded with BMP-2 was implanted into porcine mandibular defects for 24 weeks [129]. BMP-2 loading significantly increased the bone-specific surface area by 17%, as compared to BCP alone. In addition, BMP-2 accelerated the healing process 4-fold as compared to BCP alone; in fact, healing in the BCP-BMP groups and in the BCP group required 6 weeks and at least 24 weeks, respectively. In addition, the efficiency of a composite material (β-TCP plus PLA-DX-PEG copolymers) associated with BMP-2 (15–30 µg) on L4-L5 vertebral fusion has been reported in rabbits [130]. Full vertebral fusion was observed after 6 weeks of implantation. Moreover, efficiency of local BMP-2 release using BCP ceramic granules in an hydrogel was also demonstrated by Minier et al. in a segmental critical-size defect model of non-union in dogs (Fig 8) [131].

BCP bone substitutes appear a suitable matrix to vehicle osteoinductive molecules and optimize bone regeneration, provided that both dosage and release profile of the bioactive molecule are adequately adjusted.

4. Conclusion & perspectives

The ideal bone graft substitute must combine several biological properties in order to favorably compare with an autologous graft: biocompatibility, osteoconductivity, bioactivity (interactions with the implantation site environment that can result in biodegradability, ionic release and exchange). Ideally, bone substitutes should be able to repair large defects, provide mechanical resistance and be resorbed to allow osteogenesis as the bone tissue is remodeled. Biphasic calcium phosphate (BCP) ceramics are now successfully used for bone substitution in many different clinical situations such as repair of bone defects, bone augmentation in spinal arthrodesis, periodontal treatment, or as coatings for metallic implants [2–7,25,132,133]. In perspectives, innovative strategies of bone tissue engineering with BCP-based composites, by including cells or active agents, may eliminate the need for autologous bone grafting procedure. Among them, three-dimensional (3D) printing may offer great opportunity in the field of bone reconstructive medicine. Three-D printing, which was firstly described by Charles Hull, can be defined as a digital fabrication process in which geometrical data are used to produce 3D solids by

incremental addition of material layers. This smart approach is becoming extremely popular in the fields of biomedical research and tissue engineering, due to the ability to replicate the architecture as well as the cellular and matrix components of tissue. Particularly for bone tissue, this 3D-method raises great potential as it allows producing patient-specific geometries that are derived from medical images, such as CT scans and therefore fitting perfectly to the bone defect [134,135]. In this attempt, Detsch et al. designed 3D printed BCP scaffold by gluing granules together by a binder liquid following with a sintering step [135]. After seeding monocytic RAW 264.7 cells on the surface of these 3D BCPs, they observed a great cell differentiation and resorption activity demonstrating the ability of these 3D surfaces to serve as bone substitute scaffolds.

Despite several positive biological results, technological challenges have to be solved particularly to improve the mechanical properties of these printed BCP scaffolds at biologically-relevant temperatures.

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