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https://doi.org/10.1016/j.jconrel.2021.09.004

Biphasic calcium phosphate microspheres in biomedical applications

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Abstract

Biphasic calcium phosphate (BCP) microspheres benefit from, on the one hand, a desired shape offering improved flowability and injectability to fill complex-shaped bone defects and on the other hand, a promising combination of bioresorbability, bioactivity, biocompatibility, osteogenesis and angiogenesis. The biofunctional characteristics of BCP microspheres are mainly controlled by varying the constitute phase ratio, porosity and surface roughness, which are all determined by the used production route and its parameters. In this paper, the manufacturing methods, properties and applications of BCP microspheres are reviewed and concluded in terms of future work directions to develop their uses in biomedicine, particularly in bone tissue regenerative and delivery applications.

Keywords: Bone void fillers; tissue engineering; drug delivery; hydroxyapatite (HA); tricalcium phosphate (TCP); non-stoichiometric calcium-deficient hydroxyapatite (CDHA)

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1. Introduction

Microspheres are three-dimensional spherical-shaped substances with diameters of 1-1000 μm. They were first introduced by Fox et al. [1] in 1959 during a research work on dissolution and aggregation of proteinoids. Afterwards, in the mid-1960s, microspheres were

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used as fillers in plastics [2] followed by other industrial areas, including aerospace, paints and coatings, transportation, abrasives, construction and so on [3, 4]. Applications of microspheres in the biomedical industry go back to the mid-1990s [5-7]. In biomedical engineering, they have two major applications; using as tissue engineering scaffolds and local delivery platforms [7-10]. In this regard, the suitable flowability and injectability of microspheres help them fill bone defects with complex shapes due to their desirable shape and size. The spherical shape of these substances also ensures the minimal invasiveness in comparison to irregular-shaped and sharp-edged alternatives [8, 11].

Microspheres have a promising ability to load different biological factors like drugs, DNAs and proteins, particularly in an openly porous state offering high surface areas. For this purpose, the surface morphology of the microspheres plays a critical role in the desired adsorption and release of the factors [12, 13]. Porosity inside each microsphere and among a batch of microspheres also provides biological fixation through mechanical interlocking with ingrown tissues, while bioactive fixation and osteointegration are offered in case of using bioactive materials to fabricate microspheres. In addition, the existence of these open pores facilities the interdiffusion of biological body fluids, proteins, nutrients and cells to the bulk of the filled bone void, helping cellular metabolism and tissue regeneration [14, 15].

Until now, a large number of materials have been used to produce biomedical microspheres, including polymers, ceramics and their composites. Among them, the most desirable combination of bioactivity, biocompatibility, and bioresorbability belongs to bioceramics. For this reason, bioinert ceramics (like zirconia [16], zinc oxide [17], carbon [18]), glasses (borates, silicates and phosphates) [19], glass-ceramics (like lithium ferrite [20]), calcium phosphates [7, 13, 15], calcium carbonates [21], calcium silicates [22] and calcium-magnesium silicates [23-26] have been frequently used to fabricate microspheres for

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biomedical applications. The suitable characteristics of these substances have made them great candidates for bone defect treatment, hard tissue engineering, local biological factors delivery and reinforcing of composites for soft tissue engineering and load-bearing applications. Despite these beneficial features, using a single-phase ceramic does not ideally satisfy needs for a medical therapy in some cases. For example, a desirable combination of bioactivity, bioresorbability, osteoconductivity and osteoinductivity is hardly integrated in a single-phase matter. To improve these features, there are two general approaches in terms of chemical modification, including using composites or multiphasic ceramics. In this regard, the most commonly used biphasic bioceramics are silicate and calcium phosphate materials. Calcium phosphates or apatites, which are the most applicable group of bioceramics in both research and technology, involve a variety of composition and thereby properties. Accordingly, the most studied multiphasic ceramic microspheres belong to the same bioactive/bioresorbable ceramic system.

In the literature, there are a good number of review papers published on both medical microspheres [6, 19, 27-33] and biphasic calcium phosphates (BCPs) [34-39] albeit in different compositions and shapes, respectively. Nevertheless, no dedicated review paper has been published on BCP microspheres which is the focus of the current paper in terms of composition, synthesis methods, characteristics and biomedical applications.

2. General features of biomedical calcium phosphates

Natural bone tissue is mainly made of collagen and non-stoichiometric calcium-deficient carbonate-containing hydroxyapatite. This reflects the potential of calcium phosphates as a promising material for bone grafting and reconstructive applications in the forms of scaffolds, powders, granulate, block, cements, microspheres and coatings [40].

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Apatites are a large group of materials which are classified based on their stoichiometry, particularly the ratio of Ca/P. The most commonly used calcium phosphates in biomedical applications are hydroxyapatite, tricalcium phosphates and BCPs [40].

Hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, with the Ca/P molar ratio of 1.67 is known to be the most stable form of calcium phosphates and to have the ability for osteointegration and osteoconduction [41]. Tricalcium phosphate ($Ca_3(PO_4)_2$) polymorphs, including alpha and beta tricalcium phosphates (TCPs) with the Ca/P ratio of 1.5, are more bioresorbable than HA [12], making them an excellent candidate for bone cements and tissue engineering scaffolds [13]. BCPs can be described as a mixture between two phases of apatites, for instance more stable HA and more soluble TCPs, allowing to control the bioactivity and bioresorbability of BCPs via controlling the HA/TCP ratio [40].

3. Fabrication and structure of biphasic calcium phosphate microspheres

The properties of microspheres strongly depend on their geometrical and phasic characteristics. On the one hand, the type, shape, size and percentage of internal pores and, on the other hand, the external size and surface roughness of microspheres play a determining role in their properties, which are entirely controlled by the production route used. Table 1 summarizes typical results published in the literature on the fabrication method, structure and properties of BCP microspheres.

Table 1. Overview of the fabrication method, structure and properties of BCP microspheres.

Author(s)	Fabrication route	Structural features	Biological properties	Reference
Chen et al.	Emulsion + Calcination, with the partial	HA/β-TCP microspheres with TCP promoted by increasing the substitution level	Enhanced bioresorbability with the Sr level, highest biocompatibility and	[42]

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	substitution of Sr for Ca		osteogenic ability at an optimal replacement (40%)	
Honda et al.	Ultrasonic spray pyrolysis	Hollow low-crystallinity HA/β-TCP microspheres of 0.5-2 μm in diameter	Ability to act a successful carrier in antibacterial drug-delivery systems	[43]
Kim et al.	Spray drying + calcination	Dependency of the phase structure on the calcination temperature, leading to HA/β-TCP, HA/β-TCP/α-TCP and HA/α-TCP microspheres	Not assessed	[44]
Lima et al.	Coacervation + sintering, incorporated into an injectable polymeric matrix	Porous HA/β-TCP microspheres	Considerable osteointegrability *in vivo* but with low biodegradability	[11]
Ohno and Izawa	Ultrasonic spray pyrolysis	Hollow microspheres of 0.5-4 μm in diameter, dependency of the phase structure on the solution concentration and pyrolysis temperature	Not assessed	[45]
Shen	Glass conversion + calcination	Different β-TCP/HA ratios, depending on the synthesis solution characteristics, with hollow cores and porous shells	Enhanced bioresorbability, osteoconductivity and angiogenic ability with increasing the β-TCP/HA ratio	[46]
Veljovic et al.	Hydrothermal synthesis + microwave-assisted sintering	Different multiphasic structures based on the Ca/P loading amount and sintering temperature	Enhanced hardness by increasing the Ca/P ratio and sintering temperature, enhanced fracture toughness only with increasing the sintering temperature	[14]
Victor and Kumar	Microwave-assisted hydrothermal method + emulsion, with gelatin porogen	Different HA/β-TCP and porosity amounts, based on initially loaded Ca/P and porogen levels, respectively	The need for optimizing the HA/β-TCP ratio and porosity level to meet the highest drug-delivery performance	[41]
Wu et al.	Emulsion + sintering, with camphene porogen	Microsphere diameter of 800-1500 μm and pore size to 250 μm, dependency of the phase structure on sintering temperature	Detected bioresorbability and apatite-formation ability for the biphasic structures, compressive strength slightly lower than trabecular bone	[13]

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Zarkesh at el.	Precipitation + Calcination	Different topographies including nanorods and nanosheets based on synthesis parameters	Suitable cytocompatibility and improved drug-delivery performance with long nanosheet topography	[12]

There are a lot of criteria to classify the manufacturing methods of microspheres, including the production atmosphere (wet and dry), reagents, dispersing media (gas, liquid or solid), starting material form (powder, solution, paste or slurry) and resulting diameter [19]. Particularly, two essential aims should be pursued to produce BCP microspheres; BCP formation and microspheroidization. On the criterion of the priority of achieving these two challenges, the fabrication methods of BCP microspheres can be categorized into two universal groups; direct and indirect approaches (Figure 1). Direct methods are involved with the simultaneous achievement of BCP formation and microspheroidization, whereas these structural features are separately obtained by using indirect approaches consisting of a sequence of processes. The latter groups, which are more common, can be also classified into microspheroidization-prior-to-BCP-formation and BCP-formation prior-to-microspheroidization groups.

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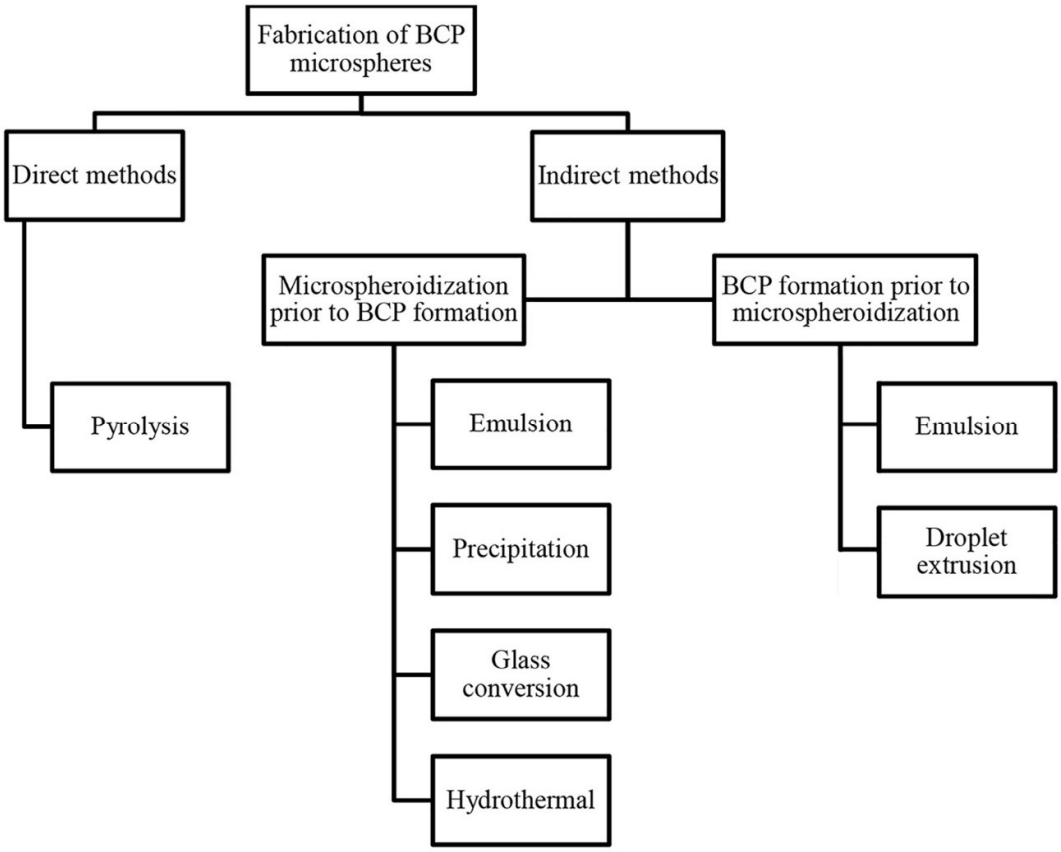

Figure 1. Manufacturing methods of BCP microspheres.

3.1. Indirect methods

3.1.1. Microspheroidization-prior-to-BCP-formation approaches

The most common approach to produce BCP microspheres is the pre-fabrication of non-stoichiometric apatite microspheres using ordinary methods, followed by calcination at appropriate temperatures at which desirable BCP is critically formed. For the former step, emulsion, glass conversion, precipitation and hydrothermal methods have been reported, although the other common processes of microspheroidization have the potential in this regard. It would be worth mentioning that pyrolysis [47], precipitation [12], hydrothermal [48], emulsion [13], sol-gel [49] and droplet extrusion [23] are commonly used methods to

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fabricate ceramic microspheres. The calcination schedule for the latter aim depends on the characteristics of the parent apatite and more importantly, desired BCP. The parent non-stoichiometric apatites used for this purpose include calcium-deficient hydroxyapatite (CDHA), ion-substituted apatites and non-stoichiometric HA. Typically, Equation 1 demonstrates the thermal decomposition reaction of CDHA into BCP, where 0<a<1 [50].

$$Ca_{10-a}(HPO_4)_a(PO_4)_{6-a}(OH)_{2-a} \rightarrow (1-a)\ Ca_{10}(PO_4)_6(OH)_2 + 3a\ \beta\text{-}Ca_3(PO_4)_2 + a\ H_2O \quad (1)$$

Wu et al. [13] used CDHA with Ca/P = 1.5 as the parent apatite and camphene as the porogen to produce porous apatite microspheres by a water-in-oil emulsion method. The fabricated microspheres were afterward sintered at 700-1400 ˚C. The sintered microspheres displayed the diameter range of 800-1500 µm with the pores size ranging from several microns to nearly 250 µm. The porosity and phase analyses of the prepared microspheres are listed in Table 2. In conclusion, the phasic structure exhibited a strong dependency on the sintering temperature from monophasic HA to biphasic and triphasic apatites, whereas a compromise of the sintering temperature and the loading ratio of the precursors determined the porosity amount and diameter of the microspheres.

Table 2. Characteristics of the CDHA microspheres produced by the emulsion method followed by sintering at different temperatures [13].

Sintering temperature (˚C)	**Phase amount (%)**			**Porosity level (%)**
	HA	**β-TCP**	**α-TCP**	
700	100	0	0	61.4±1.6
1100	16±1	84±1	0	54.9±1.3
1200	9±1	50±1	41±1	50.8±1.2
1400	7±1	0	93±1	49.6±1.2

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Chen et al. [42] fabricated strontium-containing BCP microspheres with the molar ratio of (Sr+Ca)/P = 1.67 by an oil-in-water emulsion method followed by sintering at 1100 ˚C. The result of this work indicated that by increasing the amount of Sr to 80% of partial substitution for Ca, the transformation of HA to β-TCP is promoted, as reflected in Figure 2. This is attributed to the reduction of the ideal Ca/P ratio for the HA stability (1.67) toward 1.5, thereby promoting the formation of TCP.

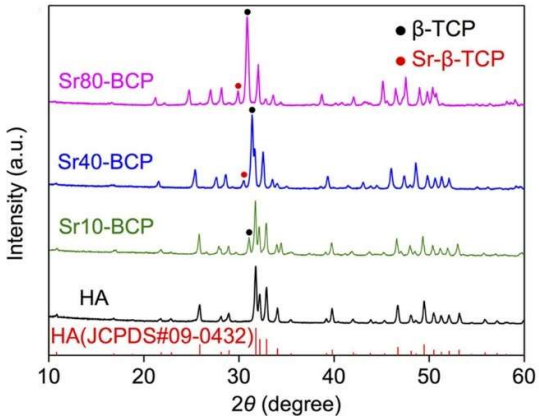

Figure 2. Dependency of the X-ray diffraction phase structure of the parent Sr-substituted HA microspheres on the concentration of Sr after sintering at 1100 ˚C [42].

Shen [46] produced hollow CDHA microspheres by the conversion of Li-Ca-B glass microspheres in phosphate solutions and then BCP microspheres by calcination at 800 ˚C. Results indicated that by increasing the pH and decreasing the phosphate concentration of the glass conversion solution, the molar ratio of Ca/P increases and β-TCP/HA decreases. The BCP microspheres fabricated by this method presented hollow cores and porous shells, so that a direct relation was concluded between the phosphate solution concentration and the size of the microspheres. However, the microspheres produced at the lower phosphate

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solution concentration (0.1 M) had a higher surface area as a result of the shell porosity configuration.

Zarkesh et al. [12] employed a precipitation method to develop CDHA microspheres using ethylenediaminetetra acetic acid disodium ($EDTANa_2$) as the template and urea as the reaction catalyst. The CDHA microspheres were afterward sintered at 900 ˚C to obtain BCP microspheres. The result of this research indicated that the concentration of EDTA, stirring speed and heating rate have a significant effect on the topography of the microspheres covered with nanorods and nanosheets (Figure 3). Noticeably, the increase in the concentration of the template encouraged the development of typical microspheres with short covering nanosheets, albeit at appropriate stirring speeds and heating rates.

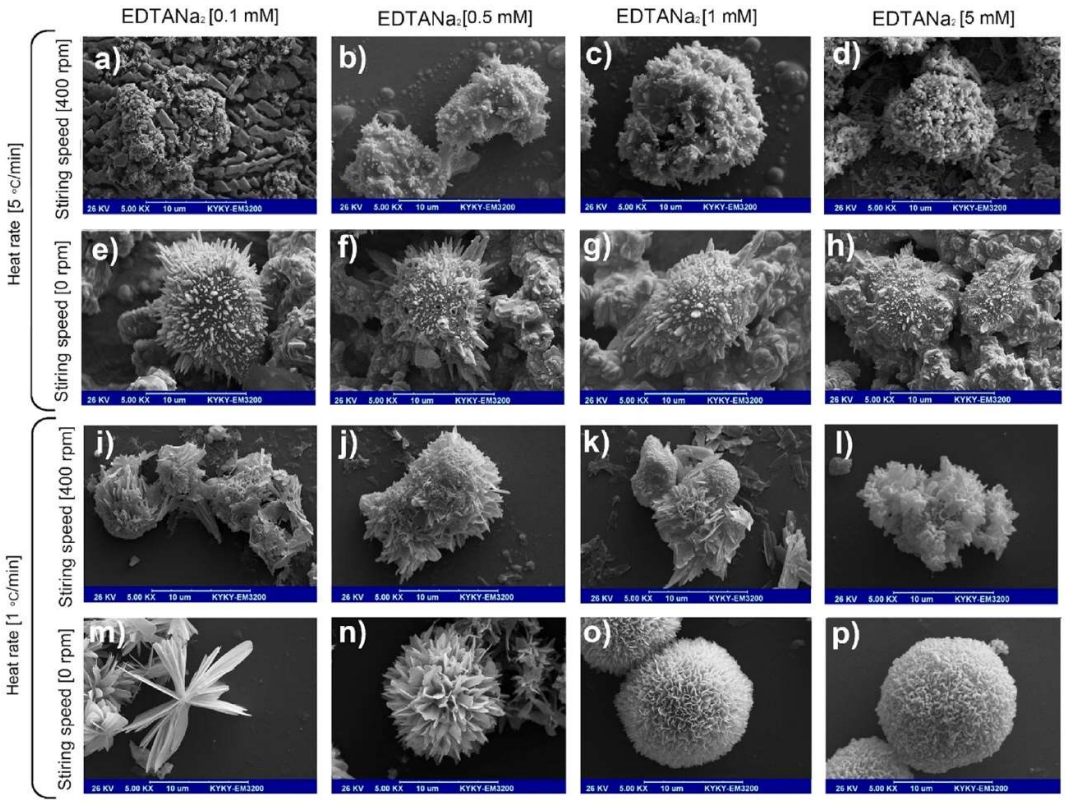

Figure 3. Effects of different precipitation parameters on the morphology of BCP microspheres [12].

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Veljovic et al. [14] also produced non-stoichiometric HA microspheres with the different ratios of Ca/P (1.42 and 0.96) by heating a solution containing calcium and phosphate sources at 160 ˚C and subsequent drying. Then, the hydrothermally-synthesized powders were processed by microwave sintering, which ensures a more uniform heating regime than conventional firing, at 900-1200 ˚C to produce BCP microspheres. Results of this work depicted that sintering at 900 and 1000 ˚C gives BCP microspheres composed of HA and β-TCP, but a third phase (α-TCP) appears due to processing at 1100 and 1200 ˚C. Also, by decreasing the Ca/P ratio which means a further deviation from stoichiometric HA, the amount of β-TCP in the sintered microspheres increases.

Kim et al. [44] fabricated CDHA microspheres by spray-drying of a solution with the Ca/P molar ratio of 1.602 and investigated the effect of sintering temperature on the characteristics of the microspheres. Sintering at 1000-1200 ˚C developed biphasic HA/β-TCP microspheres. But by increasing the sintering temperature to 1300 ˚C, the α-TCP phase was formed. The transformation of β-TCP to α-TCP was completed at 1400 ˚C, leading to a BCP structure of HA/α-TCP = 67.5/32.5. The mean size of the microspheres was decreased by increasing the sintering temperature from 1000 ˚C to 1200 ˚C due the sintering shrinkage, but by further increasing the sintering temperature, a slight increase in the size was detected as a result of volumetric expansion related to the TCP phase transformation.

3.1.2. BCP-formation-prior-to-microspheroidization approaches

Another indirect approach to fabricating BCP microspheres is the pre-synthesis of BCP powders or pre-mixing two types of apatite powders and then post-microspheroidization of the BCP feedstock. Despite that fact that this approach is less common than

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microspheroidization-prior-to-BCP-formation methods, it seems to have a higher potential for development, especially the approach that uses a mixture of pre-synthesized apatite powders. Advantageously, this approach allows an accurate quantitative control of the multiphasic composition. The methods used to spherodize a batch of BCP powders include emulsion and coacervation (droplet extrusion) methods.

Victor and Kumar [41] fabricated porous biphasic HA/β-TCP microspheres using an emulsion method from BCP powders synthesized by a microwave-assisted hydrothermal method along with gelatin as a porogen. Different ratios of the constituent phases were achieved by varying Ca/P in the used hydrothermal bath. It was also shown that a critical concentration of gelatin should be used to develop non-agglomerated and smooth-surface microspheres which are demanded for minimizing inflammatory reactions.

Lima et al. [11] also developed BCP microspheres by an alginate-based coacervation method using pre-synthesized HA and β-TCP powders, which exhibits a great potential for final porosity and phase composition controls. An aqueous slurry containing apatites (HA/β-TCP = 0.33), sodium alginate and a dispersant was atomized by a syringe into an aqueous calcium chloride solution for coagulation, forming microspheres. The microspheres were then sintered at 900 ˚C for condensation accompanied by removing alginate, giving a final structure composed of almost 23% HA and 77% β-TCP. The diameter of the microspheres could be controlled mainly by the flow rate of droplets at an inverse relation, where lower flow rates resulted in more spherical products (Figure 4). The removal of alginate left pores inside the microspheres, while a higher control of porosity is feasible by using porogens like carbon [23, 24].

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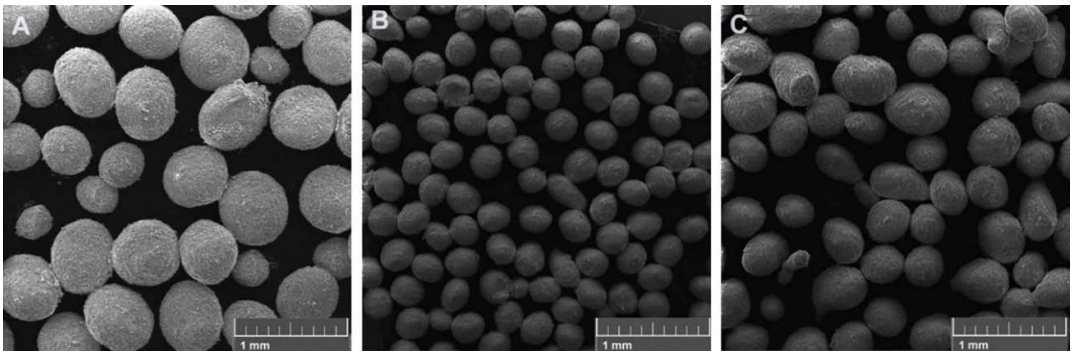

Figure 4. Scanning electron microcopy images of the BCP microspheres prepared by the coacervation method at different flow rates: 50 (A), 70 (B) and 90 (C) mL/h [11].

3.2. Direct methods

Despite the indirect methods that deal with two-step calcination and spherization, there are a few methods that use a direct chemical reaction, mainly based on pyrolysis, to produce BCP microspheres. Albeit the direct methods present a lower controllability in comparison to the indirect methods over the final structure in terms of size, phase composition and porosity.

Honda et al. [43] fabricated BCP microspheres by an ultrasonic spray pyrolysis (USSP) method consisting of atomizing a starting solution of nitric acid, tetrahydrate calcium nitrate and diammonium hydrogen phosphate (Ca/P = 1.5) at the reaction temperature of 850 ˚C. As revealed in Figure 5, the produced microspheres exhibited a hollow structure of HA/β-TCP with 0.5-2 µm diameter and low crystallinity.

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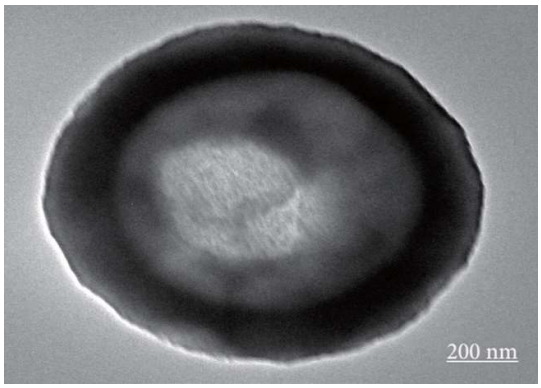

Figure 5. A hallow BCP microsphere produced directly by the USSP method [43].

Ohno and Aizawa [45] focused on the production of BCP microspheres by the USSP technique using an initial pyrolysis solution of Ca/P=1.5 as a function of the initial solution concentration and the upper furnace temperature. The fabricated microspheres represented a hollow and spherical shape with diameters of 0.5-4 μm, so that the mean diameter of the microspheres increased by increasing the solution concentration. Also, the specific surface area of the products indicated an inverse relation with the pyrolysis temperature. The phase composition of the microspheres strongly depended on the concentration of the solution and the temperature of the upper furnace.

4. Properties of biphasic calcium phosphate microspheres

In general, BCP microspheres exhibit properties similar to monophase apatites and more accurately between the constituent phases. The main parameters determining their properties are the ratio of the constituent phases and porosity. Considering the different solubility of apatite types in terms of α-TCP >> CDHA > β-TCP >> HA [15], the bioresorbability (ion release), cell interactions (viability, proliferation and differentiation),

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osteogenesis and drug release kinetics are critically affected by the ratio of the constituent phases. Also, it has been admitted that the existence of porosity can affect the biomaterials/body interactions in terms of proteins and cells adhesion, bone regeneration and drug loading. Micropores (diameter < 10 μm), which mainly correspond to the sintering progress, are hard controlled without changing other parameters, motivating more attention to macropores (diameters > 80-100 μm). Macropores can be introduced by the removal of volatile agents like organic substances or porogens at high temperatures, so that the size of macropores is more important than the amount of macropores [15, 51].

4.1. Phase stability

In order for an apatite arrangement to be considered as a BCP, it is obligatory for the candidate formulation to be stable. For instance, there are some self-setting calcium phosphates like monocalcium phosphate monohydrate + HA or dicalcium phosphate dihydrate + tetracalcium phosphate which can be theoretically considered as BCPs. However, they are rapidly transformed into monophase crystals such as CDHA in contact with moisture, unless kept under dry conditions [38].

4.2. Mechanical properties

Similar to other ceramic materials, BCP microspheres suffer from the drawback of brittleness and poor mechanical properties for load-bearing applications. The production method, especially in terms of the percentage and size of pores, highly affects the mechanical properties of BCP microspheres.

Wu et al. [13] investigated the compressive strength of BCP microspheres. It was concluded that by increasing the sintering temperature of the microspheres, the compressive

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strength is enhanced due to the sintering evolution and pore reduction (Figure 6). It seems that this contribution prevails over the phase evolution of HA to HA+TCP when the sintering temperature is increased from 700 ˚C to 1400 ˚C. The strength of the BCP and HA microspheres was slightly lower than natural trabecular bone (0.2-80 MPa). But the lower compressive strength of BCP microspheres is not a concern since their compressive strength increases upon implantation as a result of tissue ingrowth.

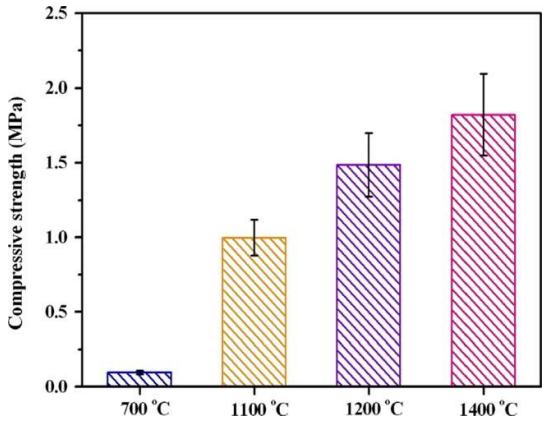

Figure 6. Compressive strength of BCP microspheres sintered at different temperatures. 1400 ˚C: α-TCP/HA, 1200 ˚C: α-TCP/β-TCP/HA, 1100 ˚C: β-TCP/HA and 700 ˚C: pure HA [13].

Veljovic et al. [14] investigated the effect of microwave sintering temperature on the mechanical behavior of BCP microspheres. It was realized that by increasing the sintering temperature and Ca/P ratio, the hardness of the microspheres increased. The hardness of the microspheres with the Ca/P ratio of 1.42 increased to 2.70 GPa, whereas that of the microspheres with the Ca/P ratio of 0.96 only increased to 1.80 GPa. This could be due to the introduction of residual stresses due to the higher expansion of the microspheres with the more transformation of HA to β-TCP (lower Ca/P ratio). Also, the Ca/P ratio did not have a substantial effect on the fracture toughness of the microspheres, but the increase of the

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sintering temperature, especially from 1000 ˚C to 1200 ˚C increased it from 0.5 MPa.m$^{1/2}$ to 1.25 MPa.m$^{1/2}$, which can be explained by the direct relation between fracture toughness and porosity.

4.3. Bioresorbability

Bioresorption is one of the most typical features of BCP substances, which makes them appropriate candidates for both bone tissue reconstruction and delivery applications. The resorbability of biphasic TCP/HA microspheres exhibits a strong dependency on the ratio of TCP/HA since TCP is very more bioresorbable than HA. Also, the porosity characteristics especially their interconnectivity level highly affect the bioresorbability of BCP microspheres; a property which strongly controls the biocompatibility and osteogenic ability of the substance based on ions release.

Shen [46] compared the bioresorbability of BCP microspheres including β-TCP/HA with the ratios of 70/30 and 44/56 with CDHA microspheres. It was found that the BCP microspheres are dissolved faster than the CDHA microspheres, although the CDHA microspheres had a more surface area. As expected, the higher percentage of β-TCP (β-TCP/HA =70/30) provided higher resorbability due to the lower physiochemical stability of TCP than HA.

Wu et al. [13] investigated the resorption of microspheres with different phase structures in distilled water. For HA microspheres, no noticeable degradation and weight loss were detected, but in case of basic β-TCP/HA microspheres, dissolution could be observed in the edges of pores. For BCP microspheres containing α-TCP, dissolution and weight loss were more obvious, accompanied by the precipitation of flake-like CDHA on their surfaces due to the fast dissolution of α-TCP providing the constitute ions of the precipitates.

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To improve the bioresorbability of biphasic HA/TCP microspheres, Chen et al. [42] investigated the impact of strontium doping. The results indicated that the addition of strontium to the microspheres can increase resorbability as a result of the increase in the formation of β-TCP.

4.4. Biocompatibility

Due to the high biocompatibility of calcium phosphates especially HA and TCP, the cytotoxicity of BCPs is not a concern. Typically, Zarkesh et al. [12] reported the high rabbit bone marrow mesenchymal stem cells cytocompatibility of BCP microspheres, equivalent to TCP, realized from fluorescent microscopy and MTT assay. In this research, the cells were exposed to the microspheres for 7 days and stained with a calcein AM/ethidium homodimer-1 solution for fluorescent microscopy characterized by green and red traces related to living and dead cells, respectively. The MTT cell viability was also conducted for the culture periods of 1, 3 and 7 days by measuring absorbance at 570 nm (Figure 7).

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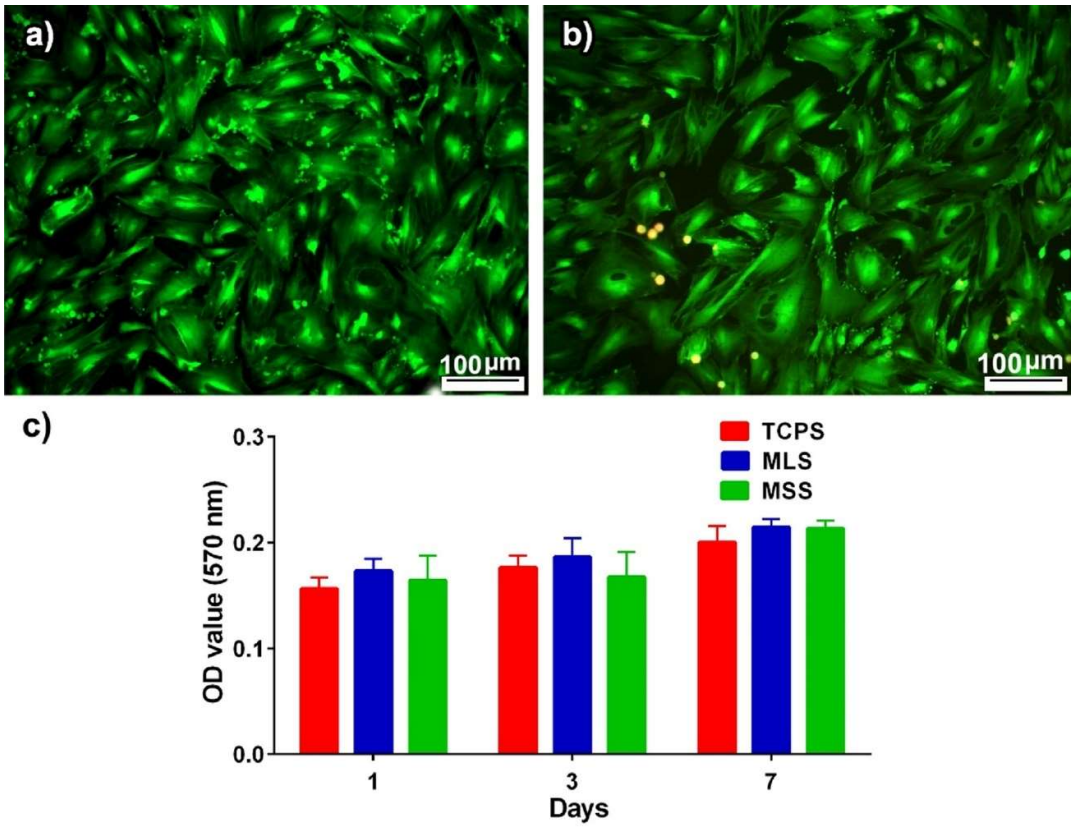

Figure 7. Significant biocompatibility of BCP microspheres: fluorescence microscopy images of fixed cells on BCP microspheres covered with short (MSS) (a) and long (MLS) (b) sheets. MTT cell viability of BCP microspheres along with tissue culture polystyrene (TCPS) positive control, where OD represents the optical density of viable cells (c) [12].

4.5. Osteogenic and angiogenic ability

Similar to other calcium phosphates, bone-like apatite can be precipitated *in vitro* or *in vivo* on the surface of BCP microspheres. Accordingly, BCPs are provided with the ability to adsorb proteins, cells and other bioorganic molecules, which helps to grow new bone from the edges of the bone filler and/or as a replacement of bioresorped regions [15, 38]. Comparatively, BCPs demonstrates a higher ability for osteogenesis and angiogenesis than

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the monophasic constituents. An interconnectedly porous structure also facilitates the vital transfer of nutrients and oxygen for cell adhesion and proliferation [52].

Shen [46] investigated the osteoconductivity of BCP microspheres by implanting them into the calvarial bone of rabbits and subcutaneously in rats. Results of the former implantation study indicated that after 8 weeks, new bones were formed as the replacement of the BCP microspheres, so that the BCP microspheres exhibited a significantly higher bone formation ability compared to the CDHA counterpart. The bone formation was also enhanced by increasing the β-TCP/HA ratio due to a more bioresorption rate and thereby the more release of Ca and P ions, triggering osteogenic differentiation. Nonetheless, for the latter, no newly formed bones could be observed in non-osseous areas, but the formation of blood vessels could be observed. In this regard, higher blood vessels were formed in case of the BCP microspheres compared to CDHA, due to the higher release of calcium ions that plays a key role in angiogenesis.

Similar to other calcium phosphates, BCPs are not substantially osteoinductive. It has been reported that the osteoinduction of BCPs is mostly limited to large animals and less in small animals like rats and mices [15]. However, it has been established that the osteoconductivity of BCPs is somewhat higher than the monophasic constituents [53], where the related *in vivo* mechanism has not been completely understood yet.

4.6. Drug delivery

Controlled drug delivery systems are being progressively developed to overcome the drawbacks of conventional systemic administration methods. In general, microspheres provide three main advantages for targeted drug delivery purposes; the protection of the drug against the environment, the ability to tailor the drug release kinetics in a desired dosage and

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the delivery of the drug to the targeted site. These features reduce the adverse effects of drugs on adjacent tissues and offer the maximum efficiency of drug delivery systems [54-56]. A promising drug delivery system is characterized by a high drug loading capacity and a prolonged and sustained drug release kinetics [57, 58]. The porosity level and hence specific surface area of microspheres are critical factors to determine the drug loading capacity. The application of porogens during the fabrication of microspheres is an effective strategy to improve this characteristic. Albeit the specific surface area of these substances, which is up to a few hundreds of m^2/g, is hardly comparable to that of mesoporous particles which can approach several thousands of m^2/g. However, microspheres are still promising carriers where there is a low level of blood circulations like infection sites, since they offer higher injectability and flowability than nanocarriers. Concerning the drug release kinetics, surface interactions between the carrier and drug and the biodegradability of the carrier are essential. In this regard, BCP microspheres are more ideal candidates than monophases as their surface charge and resorbability can be optimized by varying the constituent phases ratio. Encapsulation of these carriers into polymer coatings is an efficient approach to meet a sustained release profile.

Victor and Kumar [41] investigated the loading and release behavior of doxycycline from BCP porous microspheres to explore the role of the HA/β-TCP ratio and porosity introduced by using the gelatin porogen during microspherization. It was concluded that the surface area of the microspheres is the most critical parameter which determines the drug delivery behavior, which is its controlled by a compromise between the amount of the porogen used and the HA/β-TCP ratio. Typically, comparing the BCP microspheres with the different ratios of HA/β-TCP (80/20, 65/35 and 40/60) and various gelatin levels (4, 6 and 8

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% w/v), the highest loading and release characteristics were assigned to the ratio of 65/35 produced at 6% porogen due to benefiting from the optimal percentage and size of pores.

Zarkesh et al. [12] studied the loading and release behaviors of biphasic HA/TCP microspheres for dexamethasone (DEX) and bovine serum albumin (BSA). The hydrogen bonding of DEX and the BCP microspheres surface was responsible for the prolonged release. However, the loading and release behaviors of BSA strongly depended on the topography of the microspheres characterized by long (MLS) and short (MSS) nanosheets, according to Figure 8. The MLS microspheres also showed a higher capacity for the loading and sustained release of BSA. This was attributed to the more surface enrichment of this topography with calcium ions, which leads to further electrostatic interactions with negatively-charged BSA proteins and thereby a longer release duration.

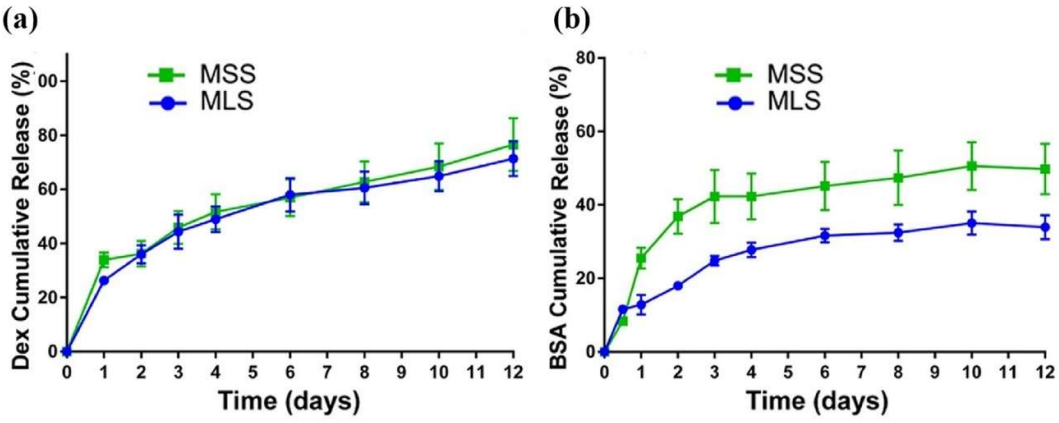

Figure **8**. *In vitro* cumulative release profile of DEX (a) and BSA (b) from the BCP microspheres with the different surface topographies [12].

Honda et al. [43] studied the immobilization of protamine on the surface of biphasic HA/TCP microspheres. Zeta potential measurements suggested the electrostatic adsorption of

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positively-charged protamine on the BCP microspheres via the negatively-charged sites of the constituent apatites. While the addition of protamine had no effect on the sphericality of the BCP carriers, needle-like precipitations could be observed on the surfaces due the transformation of TCP to HA promoted by a reaction with protamine. The results of Baclight LIVE/DEAD staining also revealed that the protamine-containing microspheres are successfully able to prevent bacterial proliferation.

5. Extrinsic modification approaches of biphasic calcium phosphate microspheres

BCP microspheres are indeed regarded as an effective modification to monophasic apatite microspheres as they exhibit a nearly ideal bioperformance. For this reason, apart from optimizing the intrinsic parameters of BCP microspheres including the constituent phases ratio and porosity, a few reports have been published on the further modification of BCP microspheres. This is in contrast to monophasic apatite microspheres which have been extensively altered by different extrinsic approaches, including dopants, polymer encapsulation and core-shell configuration.

Chen et al. [42] employed the partial substitution of strontium for calcium at different levels (10, 40 and 80 %) into BCP microspheres. The addition of strontium resulted in the increase of bioresorbability mainly due the promotion of β-TCP and Sr-β-TCP ($Ca_2Sr(PO_4)_2$). Comparatively, the microspheres with 40% substitution revealed the highest functionality in terms of cytocompatibility and osteogenesis, which could be owing to the optimal release concentrations of Ca, P and Sr ions. In this regard, the positive role of Sr in suppressing the osteoclast activity and encouraging the osteoblast metabolism is also noticeable. However, a negative influence is achieved at high release levels of Sr (more than 5 mmol/L), explaining the lower bioperformance of 80% replacement in comparison to 40%.

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Lima et al. [11] introduced a polymeric solution consisting of chitosan and polyethylene glycol along with BCP (HA/TCP = 1/3) microspheres for injectable bone substitute (IBS) applications. According to results, the used solution improved the injectability which depended on the liquid loading level and the microspheres size with no sign of cytotoxicity. Typically, a direct relation was found between the microspheres size and loading amount with the force needed to remove the composite substitutes. Due to the low bioresorption rate of HA, some of the injected bone substitute *in vivo* was retained even after 60 days (Figure 9). Typically, the key role of the ceramic constitute in osteointegration was also realized by the evidence of the new bone formation with no fibrous tissue interlayer.

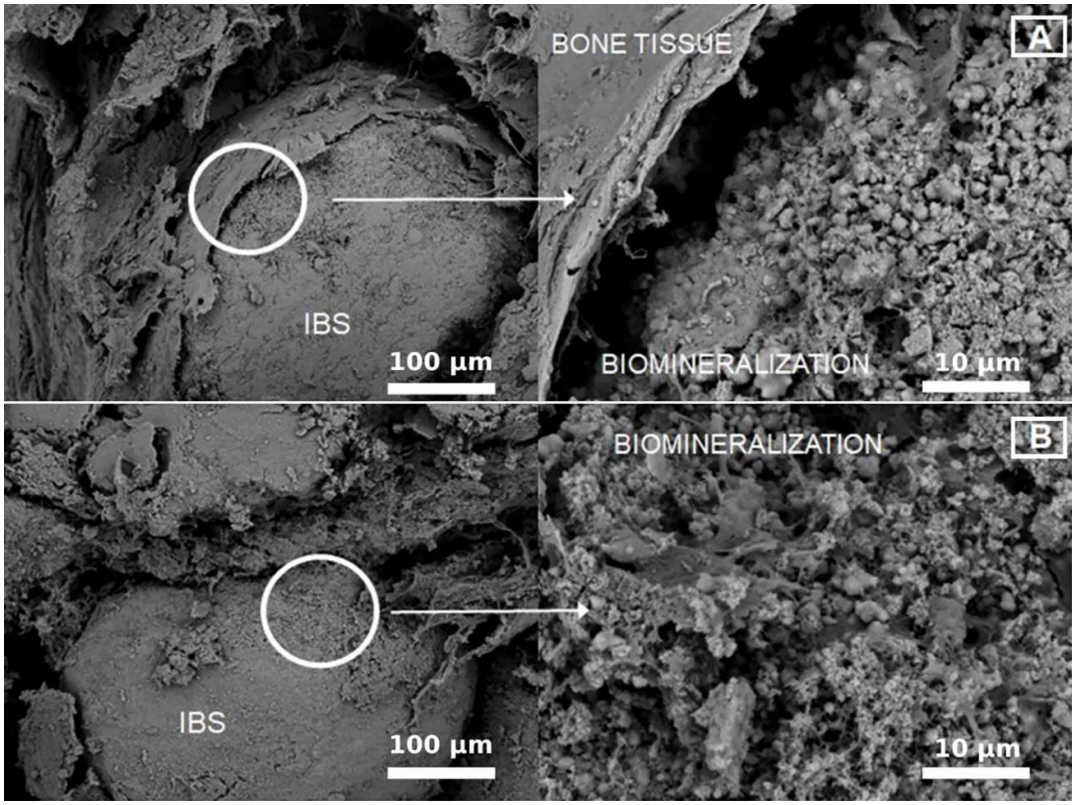

Figure 9. Retained injectable bone substitute of chitosan/polyethylene glycol/BCP microspheres and newly formed bone tissue after 30 (A) and 60 (B) days of implantation [11].

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6. Conclusions and future research directions

Calcium phosphates including HA, TCP and BCP have drawn a significant attention for medical applications owing to their composition similarity to natural bone. HA and TCP have some drawbacks related to bioresorbability and bioactivity, respectively. Nevertheless, BCPs exhibit a broad range of biodegradability, bioactivity and osteoconduction, converting them into promising biomaterials. Among the different forms of bone substitutes, microspheres with improved flowability and injectability are a suitable candidate to fill complex-shaped bone defects, especially in comparison to irregularly-shaped particles and nanofillers. Lower invasiveness to adjacent tissues and cells also ensures their higher biocompatibility than the aforementioned alternatives. In addition, these substances under porous conditions are promising for bone tissue regenerative and targeted delivery applications when compared to conventional scaffolds, allografts and systemic delivery systems. But it should be noted that their loading capacity is hardly comparable to that of mesoporous particles resulting from very high specific surface areas. Concerning BCP microspheres, there are a number of structural characteristics to control biofunction, including the HA/TCP ratio, size, roughness, pore size and amount, etc, which are determined by the production method of the substance. BCP microspheres show higher osteogenesis, angiogenesis and biocompatibility in comparison to the monophasic constitutes, where their bioresorbability could be optimized by varying the TCP/HA content. This variable also plays a critical role in the drug delivery kinetics under impregnated conditions. Despite the significant characteristics, BCP microspheres have not been still developed in clinical operations since researches on their structural optimizations should be pursued. Typically, the optimization of the constituent phases ratio involved with porosity and roughness is still complicated and unsolved. Also,

there are extrinsic ways to enhance the functionality of BCP microspheres that can be still investigated, such as applying polymer coatings and ionic substitution or doping into BCP composition. It is accordingly anticipated that a combination of intrinsic optimization and extrinsic modification approaches offers a hopeful prospect for future applications of BCP microspheres in clinical delivery systems and bone regenerative scaffolds. It seems that technical challenges related to the structural requirements of BCP microspheres, i.e. controlled phasic feature and spherization, demanding time-consuming and complicated steps still act as a barrier for their commercial development.

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