Elaboration conditions influence physicochemical properties and *in vivo* bioactivity of macroporous biphasic calcium phosphate ceramics

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Two different preparations of biphasic calcium phosphate (BCP) were characterized *in vitro*: BCP_1 from a mechanical mixture of hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) powders, and BCP_2 from calcination of a calcium-deficient apatite (CDA). The structural, physicochemical and mechanical parameters of these two preparations were investigated, and two different macroporous BCP_1 ($MBCP_1$) and BCP_2 ($MBCP_2$) implants were manufactured and implanted in rabbit bone for *in vivo* bioactivity studies. Scanning electron microscopy observations showed that $MBCP_1$ implants had a significantly higher degradation rate ($P < 0.0001$) than $MBCP_2$ implants. This was probably caused by the presence of calcium oxide impurities in BCP_1 and the more intimate mixture and stable ultrastructure of BCP_2. No significant difference about the newly formed bone rate in these two BCP preparations was observed. Very slight variations in sintering conditions appeared to influence the biodegradation behavior of the two MBCP implants despite their identical HA/β-TCP ratios and similar porosity. Precise and complete *in vitro* characterization enabled us to understand and predict *in vivo* degradation behavior.

1. Introduction

Calcium phosphate ceramics such as hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) possess a mineral composition very close to that of normal bone [1] and a total biocompatibility, which make them successful bone substitutes [2–5]. Various procedures have been developed for the preparation of these ceramics, based on parameters such as sintering temperature, purity of starting products, and morphological characteristics (specific surface area, microporosity and macroporosity), resulting in differences in composition and physical forms. These factors can affect the stability of calcium phosphate ceramics and their *in vivo* degradation properties [6–11]. The development of biphasic calcium phosphate (BCP) ceramics has provided materials whose activity in macroporous forms is controlled by an association of HA and β-TCP in ratios that promote material resorption/bone substitution events [12–16]. Recent improvements in the biological evaluation of biomaterials, involving scanning electron microscopy (SEM) with backscattered electrons and image analysis, have provided quantitative measurements for ceramic evaluation which are easier to perform and more precise than with classic histomorphometric methods [17–19]. SEM and image analysis were used in the present work to compare the *in vivo* bioactive properties of two preparations of macroporous BCP (MBCP) ceramics. The main purpose was to provide adequate physicochemical characterization of these two BCP preparations to evaluate the influence of elaboration procedures on their *in vivo* biodegradation and correlate degradation events with their composition and structure.

2. Materials and methods

2.1. BCP powder preparation

Two different batches of 60/40 HA/ β-TCP powders were prepared:

- BCP_1 consisted of a mechanical mixture of pure commercial HA and β-TCP powders (Merck, Darmstadt, Germany);

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- BCP_2 was obtained by hydrolysis of commercial dicalcium phosphate dihydrate and subsequent sintering of the formed calcium-deficient apatite (CDA) [20, 21].

Macroporous biphasic calcium phosphate ceramics (MBCP) were manufactured from these two BCP powders according to a previously described method [22, 23]. In addition to physicochemical characterization, *in vivo* studies were performed on BCP_1 and BCP_2 implants. Accordingly, two kinds of cylindrical implants (6×6 mm $MBCP_1$ and $MBCP_2$ implants) with identical macroporosity percentage ($50 \pm 2.02\%$) and macropore diameter ($565 \pm 21.7\,\mu m$) were prepared.

2.2. Physicochemical characterization

Crystallographic phase purity and chemical composition were checked by the following methods:

1. X-ray diffraction (XRD), according to a standard French process [AFNOR 1994]. BCP_1 and BCP_2 powders were examined ($20° < 2\theta < 40°$) using a Siemens D5000 Kristalloflex diffractometer with $0.02°$ resolution, allowing HA/β-TCP ratios to be checked.
2. Infrared spectroscopy (i.r.): pellets of a 300 mg KBr/1 mg sample were prepared and examined ($400\,cm^{-1} < 1 < 4000\,cm^{-1}$) by a Nicolet Magnat II 550 Fourier transform infrared (FTIR) spectrometer with $4\,cm^{-1}$ resolution.
3. A chemical test using phenolphthalein [AFNOR 1994] was performed to detect any calcium oxide (CaO) content.

The dissolution properties of $MBCP_1$ and $MBCP_2$ (after crushing and ignition) were investigated. They were expressed as $mg\,l^{-1}$ Ca released with time in acetate buffer.

Specific surface area measurements were performed on these two BCP powders and on their starting products, using a Quantasorb Jr instrument and a 70/30 He/N_2 gas mixture. Macroporosity was checked with SEM and image analysis on $MBCP_1$ and $MBCP_2$ implants.

2.3. Mechanical compression tests

Six-by-six mm MBCP cylinders of each kind of BCP preparation were tested for compressive strength on an MTS machine with a displacement control adjusted to $0.1\,mm\,min^{-1}$. Ten measurements were performed for each kind of MBCP implants. Compressive strength (σ_{comp}) was deduced from the load–deformation curve: $\sigma_{comp}(Pa) = F_{comp}(N)/S(m^2)$ where F_{comp} is the applied force and S the cylinder surface.

2.4. Surgical procedures

Statistical evaluation in *in vivo* studies required the manufacture of 20 MBCP cylinders (10 for each type of implant). Comparison of these two types of implants with different BCP preparations and similar macroporosity parameters ($MBCP_1$ and $MBCP_2$ implants) allowed the influence of elaboration conditions on both biodegradation and bone ingrowth to be evaluated. Bilateral implantations were performed in aseptic conditions under general anaesthesia in 10 New Zealand White rabbits. After skin incision and lateral arthrotomy of the knee joint, a cylindrical defect was created at the distal end of the femur. The defect was then filled with an MBCP cylinder after saline irrigation of the cavity. Subcutaneous tissues and skin were closed in different layers. The distribution of the implants was randomized to prevent the surgeon from knowing which kind of implant was used.

No antibiotics were given after surgery, but oxytetracycline was injected intramuscularly for double labeling (Terramycine®, Pfizer, France) 13 and 12, and then 3 and 2 days before the animals were killed.

When the MBCP materials were retrieved after 8 weeks, the femoral ends were excised, fixed in glutaraldehyde solution, dehydrated in graded ethanol and embedded in glycolmethylmethacrylate.

2.5. Histological evaluation

Femur sections from each group were analyzed by undecalcified histological examination in light microscopy and SEM. For each implant, two serial sections were cut perpendicular to the long axis of the implant using a low-speed diamond saw (Leitz, Germany): a 20 μm thick section for light microscopy (stained with solochrome-ocyanine), and a 30 μm thick section for fluorescence and polarized light. Qualitative observations were performed by light microscopy on the stained sections and by polarized light for the unstained sections. The latter were also examined by fluorescence microscopy to detect tetracycline labeling. To obtain data from the entire length of the sample, all sections were repeated twice in two areas separated by at least 2 mm. The two retained implant surface areas were then sputtered with gold–palladium for SEM studies.

The quantity of resorbed ceramic and newly formed bone were determined using a semi-automatic image analyzer (Leica Quantimeter 500, Cambridge, UK) from SEM observations of implant surfaces obtained with backscattered electrons (Jeol JSM 600, Japan) at 20 kV. The total implant surface area was divided into 12 adjacent fields and recorded on SEM (magnification × 50). The threshold was determined by the operator on the image analyzer, and the three surface tissue components (ceramic, soft tissues and newly formed bone) were identified using artificial colors. Their respective areas were automatically calculated and expressed as a percentage of the total surface area.

2.6. Statistical analysis

The differences in compressive strength, biodegradation and new bone formation between the two kinds of MBCP implants were studied for statistical purposes by one way analysis of variance followed by an *a posteriori* Duncan test. P values < 0.05 were considered statistically significant.

3. Results

3.1. Physicochemical characterization

The infrared spectrum of BCP_1 showed an extra peak due to carbonate groups not detected with BCP_2 (Fig. 1). X-ray diffraction (XRD) confirmed the 60/40 HA/β-TCP weight ratio, and no extra phases were detected in either BCP_1 or BCP_2 powders. The phenolphthalein test was slightly positive for MBCP samples obtained from BCP_1 (mechanical mixture), revealing the presence of CaO impurities in these implants. After the starting materials were checked, it was determined that the HA phase alone presented such a positive reaction.

Dissolution rate was much higher for BCP_1 (obtained by a powder mixture) than BCP_2 (Fig. 2). Specific surface area measurements are shown in Table I. Values are the means $\pm$ SD of 10 measurements each. Specific surface area was not significantly different in BCP_1 and BCP_2, but the CDA starting product from BCP_2 had a much higher SSA than HA and β-TCP powders. The resulting SEM images of the microporous structure of BCP_1 and BCP_2 are presented in Fig. 3.

3.2. Compression tests

Results from mechanical compressive strength of $MBCP_1$ and $MBCP_2$ cylinders are presented in Table II. Values are the means $\pm$ SD of 10 measurements each. Implants from BCP_2 preparation expressed significantly higher compressive strength than implants from BCP_1 preparation ($P < 0.01$).

3.3. Clinical data

Concerning the *in vivo* study, all sites healed uneventfully, with no clinical evidence of inflammatory response to the ceramic implant and no toxic signs during the experimental period. Histological examination proved impossible for one implant because of a problem during the embedding period. Thus, 19 implants were available for qualitative and quantitative histological analysis.

Fluorescence microscopy showed intense bone remodeling activity in all sections, including bone apposition and ceramic degradation.

Analysis of implant surfaces (Fig. 4) after processing of back-scattered electron (BSE) images (two sputtered sections per implant with a total of 38 sections) indicated

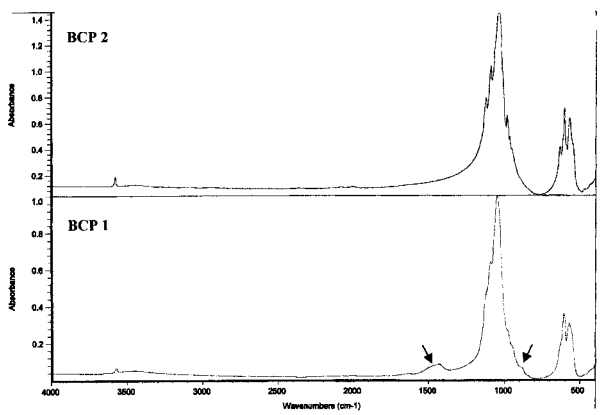


Figure 1 Infrared spectra of the two BCP preparations. Note the extra peaks (arrows) due to carbonate groups in BCP_1.

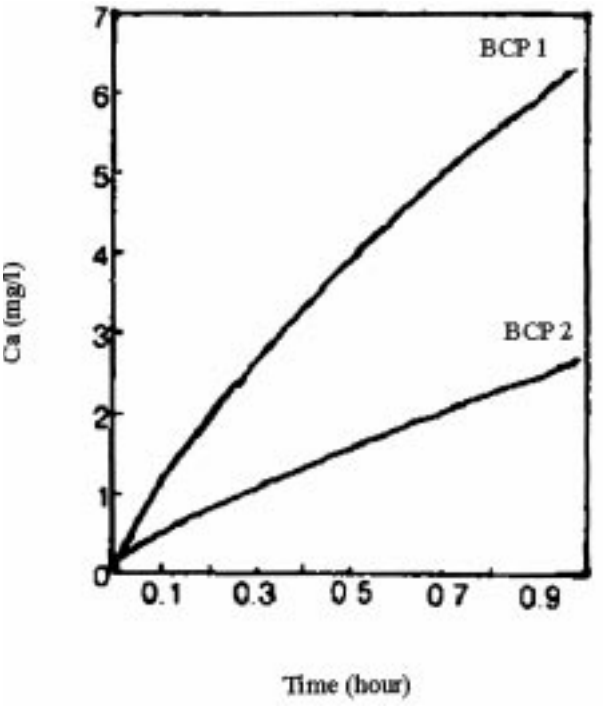


Figure 2 Diagram of the dissolution of BCP_1 and BCP_2.

that all implants were partially degraded, i.e. their BCP percentage 8 weeks after implantation was lower than before implantation. The degradation rate was calculated as the difference between the BCP percentage before and after implantation.

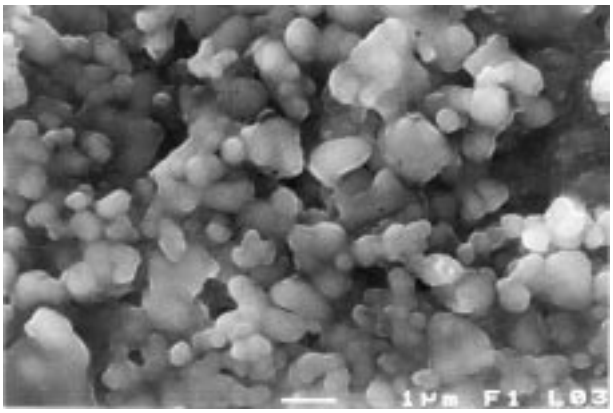
The degradation rate for $MBCP_1$ implants was much higher than for $MBCP_2$ implants. Statistical study showed that the degradation rate was significantly greater for nine $MBCP_1$ implants than for ten $MBCP_2$ implants ($P < 0.0001$). Newly formed bone rate was very similar in the two kinds of implants which presented the same macroporosity parameters. Characteristics of the two kinds of MBCP implants are summarized in Table II.

4. Discussion

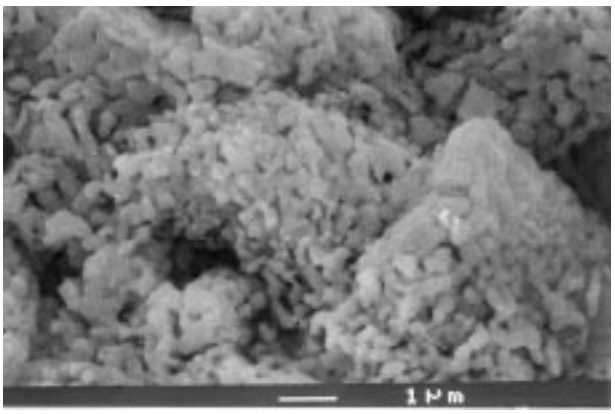
The degradation of bone substitutes is necessary as they are ultimately to be replaced by newly formed bone [24]. In our study, qualitative observations in light microscopy provided very little information about implant resorption. Therefore, a very precise and previously described measurement method was needed to calculate the percentage of ceramic on the implant surface and determine the differences in degradation rates of these MBCP materials [19].

TABLE I Specific surface area measurements of BCP_1 and BCP_2 and their respective starting products. Values are means $\pm$ SD of 10 measurements each

	SSA ($m^2 g^{-1}$)
BCP_1	3.53 ± 0.02
HA_{powder}	6.02 ± 0.05
$\beta\text{-}TCP_{powder}$	5.80 ± 0.05
BCP_2	3.88 ± 0.01
CDA	63.80 ± 0.30

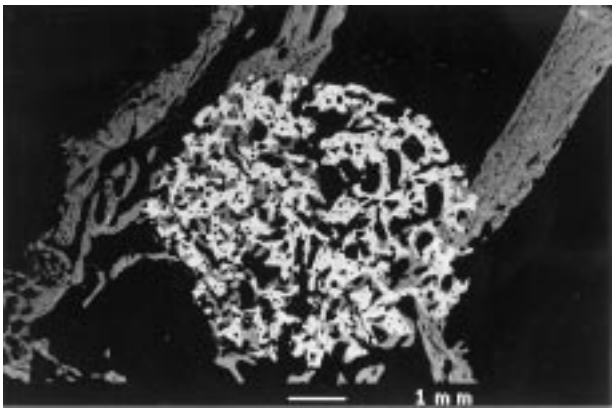


(a)

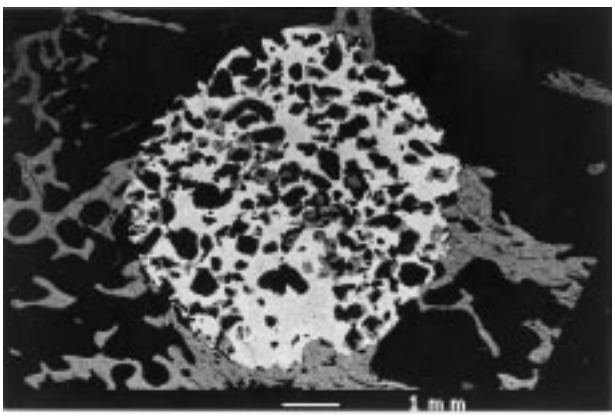


(b)

Figure 3 SEM images of microporous structure (magnification × 10 000) in: (a) $MBCP_1$, (b) $MBCP_2$. The resulting microporosity after sintering was different for the two BCP preparations: the grains were smaller for $MBCP_2$ obtained by precipitation, with more boundaries than in $MBCP_1$. $MBCP_1$ implants expressed higher dissolution rate, lower compressive strength and higher *in vivo* degradation rate than $MBCP_2$ implants.



(a)



(b)

Figure 4 Backscattered electron image of the surface of $MBCP_1$ implant (a) and $MBCP_2$ implant (b). BCP ceramic appears in lighter gray, and newly formed bone in darker gray. Soft tissues and bone marrow appear black. The implants, though identical in starting macroporosity, differed in their preparation conditions. The general appearance of the image indicates that $MBCP_1$ implant (degradation rate = 14.8%) is more degraded than $MBCP_2$ implant (degradation rate = 3.6%).

MBCP degradation was confirmed for all implants through the disappearance of the ceramic observed in quantitative analysis. $MBCP_1$ and $MBCP_2$ implants had similar macroporous features, but their degradation rates were very different. Although macroporosity is essential in promoting cell activity (especially bone growth) in calcium phosphate ceramics [14, 15, 19], biodegradation did not seem to depend on macroporous properties, contrary to the findings of other authors [25].

Elaboration conditions, including pressure and temperature parameters during sintering and the presence of impurities, can influence the degradation rate of calcium phosphate ceramics [8, 11, 26]. Microporosity has a major effect on this dissolution rate because of the presence of biological fluids: the more microporous implants are, the more degradable they become.

High sintering temperature increases particle size by crystal fusion, reduces microporosity and slows down the degradation process of calcium phosphate ceramics, which become denser after sintering [6, 7, 11].

The biphasic structure of BCP ceramics, characterized by different degradation rates for HA and β-TCP, accounts for their controlled bioactivity. BCP ceramics present an intermediate degradation behavior, so that their progressive resorption and ultimate replacement are adapted to bone ingrowth [12, 16, 27]. HA and β-TCP are both degradable, but β-TCP is much more soluble than HA as shown by *in vitro* and *in vivo* studies

TABLE II Characteristics of the two different kinds of MBCP implants

	$MBCP_1$ Macroporosity = 50% Macropore diameter = 565 µm	**$MBCP_2$** Macroporosity = 50% Macropore diameter = 565 µm
Elaboration method	Mixture of HA and β-TCP	Precipitation of CDA
Ceramic % before implantation	50 ± 2	50 ± 2
Compressive strength (MPa)	13.9 ± 1.65 ($n = 10$)	16.2 ± 0.81 ($n = 10$)
Ceramic % after implantation	35.1 ± 3.50 ($n = 9$)	46.3 ± 3.09 ($n = 10$)
Biodegradation rate (%)	14.8 ± 3.38 ($n = 9$)	3.6 ± 2.96 ($n = 10$)
Newly formed bone rate (%)	22.0 ± 6.93 ($n = 9$)	22.0 ± 5.30 ($n = 10$)

[7, 8, 26, 28, 29], and their association in a 60/40 ratio appears well adapted to bone substitution. Resorption of calcium phosphate ceramics occurs as a result of two different mechanisms: chemical dissolution due to the circulation of biological fluids, and cellular degradation by both macrophagic cells and osteoclasts or osteoclast-like cells [6, 11, 30–33]. Some authors have recently shown that ceramic solubility can influence osteoclast resorption activity *in vitro* and that too high a solubility, as with pure β-TCP, could inhibit this activity because too much calcium is released in the cell resorption microenvironment [34]. BCP ceramics, because of their intermediate composition, apparently undergo cellular resorption activity involving osteoclasts, osteoclast-like cells and other types of cells which have been implicated in their *in vitro* and *in vivo* biodegradation [31, 35, 36]. The present study did not allow us to determine whether these cellular events were different in $MBCP_1$ and $MBCP_2$ or to assess the possible effects of high Ca release from $MBCP_1$. Bone ingrowth was very similar in $MBCP_1$ and $MBCP_2$ implants and, as bone ingrowth and bone resorption are very closely related, it is unlikely that cellular resorption events differed for these two BCP preparations.

Therefore, the difference in degradation rates appeared to be due to the structure and ultrastructure of the BCP implants.

Classic physicochemical characterization, such as XRD, i.r. spectroscopy and chemical analysis are efficient to describe the composition of BCP ceramics. In this study very similar MBCP ceramics with an identical 60/40 HA/β-TCP ratio showed significantly different *in vivo* degradation properties. The dissolution *in vitro* process seemed to be predictive for the *in vivo* biodegradation properties of MBCP implants. $MBCP_1$ implants were found to be more biodegradable than $MBCP_2$ implants and the extent of dissolution of $MBCP_1$ was also much higher than for $MBCP_2$.

Specific surface area was not significantly different in BCP_1 and BCP_2. All BCP samples were sintered at 1150 °C, but $MBCP_1$ implants were sintered after a mechanical mixture of HA and β-TCP powders, whereas $MBCP_2$ implants were sintered after precipitation of a calcium-deficient apatite. Moreover, it may be considered that BCP_1 implants were ignited twice because the starting HA and β-TCP commercial powders had already been heated above 800 °C before their mixture and a second sintering in BCP. The results observed were quite unexpected and seemed paradoxical as BCP_1 was more resorbable than BCP_2 in spite of its double sintering.

Several explanations could account for these differences. The sintering conditions for BCP_1 created CaO impurities in $MBCP_1$ implants that were not detected with XRD but revealed by a positive phenolphthalein test. The CaO in BCP_1 was probably related to the presence of carbonate groups observed in the infrared spectrum (Fig. 1). CaO is a very hydrosoluble component often found, like other calcium-rich phases, during non-pure HA preparation above 1000 °C [37, 38]. It represented about 0.2% of our final BCP_1 product, which could explain the higher degradation rate observed in $MBCP_1$ implants. CaO impurities reduce the mechanical strength of calcium phosphate ceramics, especially since the compound is present homogeneously in the material and very hydrosoluble [39]. Dissolution by biological fluids weakens ceramic very quickly and contributes to its more rapid degradation [10]. The *in vivo* results confirmed the difference in the extent of dissolution between the two preparation methods.

As observed *in vitro*, calcium release from calcium phosphate ceramics has recently been measured *in vivo*, confirming the biodegradability of synthetic HA implants [40]. In our study, the BCP_2 preparation appeared to provide a more stable ceramic, probably because of the purity of the raw powders.

The BCP_2 preparation, as a result of precipitation of calcium-deficient apatite and sintering, also promoted the formation of a more intimate mixture that could have reduced the degradation rate. Even though the specific surface areas (SSAs) of final $MBCP_1$ and $MBCP_2$ were similar, the study of the starting products showed that CDA had a much higher SSA than HA and β-TCP powders. Thus, CDA could have been more reactive, i.e. a higher SSA would be likely to produce small grains but with many more boundaries than a lower SSA [41, 42]. This particular feature could have given the BCP_2 preparation a more stable and resistant composition and provided qualitative differences in the resulting microporosity, as shown on Fig. 3.

Finally, favorable mechanical effects can be expected from the BCP_2 preparation. $MBCP_2$ implants showed higher compressive strength than $MBCP_1$ implants, a quality related to both the number and size of grain boundaries achieved in BCP_2 [43]. Given the optimal macroporosity proposed in a recent work [19], this BCP preparation could also contribute to enlarging the clinical applications of this type of bone substitute.

Thus, the *in vivo* biodegradation behavior of these two BCP preparations could be predicted with a precise physicochemical characterization with standard techniques that can relate their ultrastructure to their elaboration process.

5. Conclusion

These results confirmed the *in vivo* biodegradation of MBCP ceramics, regardless of elaboration conditions of the implants. They also indicated that the elaboration process could have an influence on *in vivo* degradation properties because of the presence of impurities or the intimate mixture of the biphasic structure and showed how two preparations with the same HA/β-TCP ratio and porosity, both qualified as MBCP ceramics, could display different *in vivo* behavior. The elaboration of BCP ceramics involving the calcination of a calcium-deficient apatite obtained by an aqueous precipitation method would appear to be an effective process. This preparation conserved bioactivity and biodegradability, increased the purity of the ceramic and displayed interesting mechanical properties.

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