# Synthesis and biocompatibility of porous nano-hydroxyapatite/collagen/alginate composite

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Porous nano-hydroxyapatite/collagen/alginate (nHAC/Alginate) composite containing nHAC and Ca-crosslinked alginate is synthesized biomimetically. This composite shows a significant improvement in mechanical properties over nHAC material. Mechanical test results show that the compressive modulus and yield strength of this composite are in direct proportion to the percentage of Ca-crosslinked alginate in the composite. Primary biocompatibility experiments *in vitro* including fibroblasts and osteoblasts co-culture with nHAC/alginate composite indicated the high biocompatibility of this composite. Therefore the composite can be a promising candidate of scaffold material for bone tissue engineering.


## 1. Introduction

Nano-hydroxyapatite/collagen (nHAC) composites have been developed for bone tissue engineering in recent years [1]. Such composite is synthesized by simultaneous titration coprecipitation of hydroxyapatite (HA) and collagen. Experiment results show that it not only mimics the composition of natural bone, but also has high osteoconductive activity and is able to induce bone-remodeling units [2–9]. However the composite is still limited in use because of its poor mechanical properties. Much effort has been made to improve the mechanical properties of nHAC composites. Cold isostatic pressed (CIP) HA/collagen nanocomposite with a quarter of the mechanical strength of bone was developed [2–4]. Porous nHAC composite using glutaraldehyde as cross-linkage agent, nHAC composite developed by mineralizing the type I collagen sheets, were also reported [5, 7]. In this paper we report a new method to improve the mechanical properties of nHAC material.

Our method is based on the following considerations. Since there is some polysaccharide in natural bone, the addition of some structural polysaccharide may be helpful to improve mechanical properties of nHAC composite while maintaining its biological properties. Polysaccharide alginate fulfills the requirements, it possesses good biocompatibility and it can provide satisfying mechanical support. In our method, nHAC composite was integrated by a small fraction of Ca-crosslinked alginate into a porous structure. Mechanical test results indicate that even the highly porous composite shows a significant improvement in mechanical properties over nHAC composite. Both the compressive modulus and yield strength of the composite are in direct proportion to the content of Ca-crosslinked alginate. Primary biocompatibility experiments *in vitro* including fibroblasts and osteoblasts co-culture with nHAC/alginate composite indicated the high biocompatibility of this composite. Therefore the composite can be a promising candidate of scaffold material for bone tissue engineering.

## 2. Materials and methods

### 2.1. Materials

0.3% purified bovine dermal collagen solution from Cellon S.A. Strassen, Luxembourg, was used as obtained. Sodium alginate [$(C_6H_7O_6Na)_n$] was purchased from Acros Oganics, Belgium. Analysis grade $H_3PO_4$, $CaCl_2 \cdot 6H_2O$ and NaOH were acquired from Chemical Agents Co. Ltd., Beijing, China.

### 2.2. Preparation of nHAC/alginate porous composites

Type I collagen solution was adjusted to a concentration of 0.67 g collagen/L. Solution of $CaCl_2$ and $H_3PO_4$ (Ca/P = 1.66) were then added in drops separately. For every liter of collagen solution 13.4 g $CaCl_2 \cdot 6H_2O$ was added. Gently stirred and titrated the solution at room temperature with sodium hydroxide solution to pH 7.4. After 48 h, the deposition was harvested by centrifugation. Mix the grounded deposition powder with sodium alginate at a weight ratio of 9 : 1–2 : 1. Distilled water was added to make it into a paste. The paste was shaped and soaked in solution of 5% $CaCl_2$ for 24 h, later soaked in distilled water for another 12 h. Samples were then frozen and lyophilized. Such samples were ready for experiments.

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## 2.3. Characterization

### 2.3.1. Porosity and density measurement

The density and porosity of the porous composite were measured by water displacement. A sample with a known weight $w_1$ was immersed in a beaker holding a known volume of water $v_1$. A series of brief evacuation–represurization cycles were performed to force the water into the pores of the composite. Then total volume of the water plus the water-impregnated scaffold $(v_2)$ and the residual water volume after the water-impregnated composite was removed $(v_3)$ were recorded. The density of the porous composite $(d)$ and the porosity of the composite $(\varepsilon)$ are expressed as follows:

$$d = w_1/(v_2 - v_3)$$
$$\varepsilon = (v_1 - v_3)/(v_2 - v_3) \times 100\%$$

### 2.3.2. Mechanical testing

The compressive mechanical properties of the porous composite were tested with an Instron 1122 mechanical tester (Instron Co., Canton, MA). The specimens were cut into column (diameter = 8 mm, height = 12 mm). Crosshead speed of 0.5 mm/min was used. The compressive modulus was determined from the linear part of the stress–strain curve and the yield strength was determined from the highest point. Five specimens were tested for each sample and their average values were plotted.

### 2.3.3. Morphology observation

The porous morphology of the porous composite was studied with scanning electron microscopy (SEM, Hitachi, S-450, 20 kV). The specimens were cut with a razor blade after being frozen in liquid nitrogen for 5 min, and were coated with gold.

### 2.3.4. Biological test

*2.3.4.1. Fibroblasts culture test.* Primary biocompatibility of the composite biomaterial was evaluated by fibroblasts proliferation *in vitro*. Fibroblasts were co-incubated with exchange 50% 0.9% NaCl extraction of nHAC (defining the negative control group) and nHAC/alginate. A positive control group of fibroblasts in exchange 50% 64 g/L phenol was used [10]. The results in 2 h, 1 day, 2 days, 4 days and 7 days of the above co-incubated groups were recorded. The decline of fibroblasts was evaluated by counting the dead cells on a hemacytometer after trypsinization stained with trypan blue.

*2.3.4.2. Osteoblasts co-culture with material.* Osteoblasts were isolated via sequential digestions of neonatal rat calvaria according to established procedures [11, 12], characterized by alkaline phophatase activity, and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under strandard cell culture conditions (that is, 37 °C, humidified, 5% $CO_2$). Osteoblasts were seeded on the surface of the materials and cultured for 7 days in 3.5-cm dishes at a concentration of $5 \times 10^4$ cells/cm$^2$. *In vitro* cell test samples for SEM were fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2), followed by 1% osmium tetroxide in acetone. The specimens were dehydrated through a graded series of ethanol and acetonitrile, vacuum dried and gold coated for SEM observation.

*2.3.4.3. MTT assay.* After the osteoblasts (4000 cells/cm$^2$) were cultured in the 24-well plates containing different medium under the standard cell condition for 7 days, the cell viability was evaluated using MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrasodium bromide) (Merck). At the end of the prescribed time period, 100 μL of MTT solution (5 mg/mL MTT powder in phosphate buffer saline, sterilized through a 0.2-μm filter) were added into each well and incubated at 37 °C for 4 h. The medium in the wells was aspirated carefully. Then 0.5 mL DMSO was added to each well, vibrating the plates for 15 min to make formanzan dissolved sufficiently. Light absorbance of these samples was measured at 490 nm on an Ultrospec 3100 pro UV/visible Spectrophotometer (Biochrom Ltd., England).

*2.3.4.4. Alkaline phosphatase activity.* This procedure is performed according to Lowry [13]. Aliquots (100 μL of the distilled water with 500 μL of reaction solution, Diagnostic Kit) were added to the 24-well plates and incubated at 37 °C for 30 min. The reaction of *p*-nitrophenol conversion to *p*-nitrophenylate was stopped by adding 1.5 mL of 0.25 N NaOH [14]. Light absorbance of these samples was measured at 405 nm on an Ultrospec 3100 pro UV/visible Spectrophotometer (Biochrom Ltd., England). The alkaline phosphatase (ALP) activity was expressed as nano-moles of converted *p*-nitrophenol/min/mg protein. Alkaline phosphatase activity of osteoblasts cultured in 64 g/mL sterilized phenol solution, and DMEM (supplemented with 10% FBS) served as controls.

## 2.4. Statistical analysis

Experiments were run in the triplicate and reapeated at three different times per sample. Numerical data were analyzed using standard analysis of variance (ANOVA) techniques; statistical significance was considered at $P < 0.05$. All the statistical analysis was undertaken using statistical software program (SPSS 10.0, USA).

# 3. Results

Porous nHAC/alginate composites have been prepared by the method described above. Their composition, densities and porosities are listed in Table I. Vary the

TABLE I Densities and porosities of nHAC/alginate porous composites

| Alginate content (wt %) | Density (g/cm$^3$) | Porosity |
|---|---|---|
| 33.3 | 0.62 | 65 |
| 25 | 0.66 | 66 |
| 20 | 0.65 | 70 |
| 15 | 0.53 | 70 |
| 10 | 0.31 | 80 |

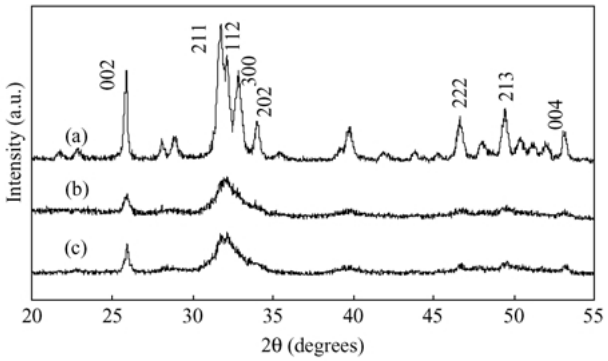


*Figure 1* XRD patterns of (a) commercially purchased HA; (b) nHAC/alginate composites; (c) nHAC composite.

alginate component from 15% to 33.3%, the average porosity is around 60–70%. There is not distinct difference in density and porosity among samples above 10% alginate in the composite. The weight ratio of HA and collagen in the composite stay at the constant which equal to 3 : 1, according to the result of Thermogravimetric Analysis (TGA). The weight fractions of HA in various composite vary from 67.5% to 50% (67.5%, 63.5%, 60%, 56.3% and 50%, respectively).

Fig. 1 shows the X-ray diffraction (XRD) spectra of nHAC, nHAC/alginate composite and commercially purchased HA powder with good crystallinity. The inorganic phase in nHAC and nHAC/alginate composite was both determined as HA. The extensive broadening and overlap of the peaks indicate that the crystal grains of HA in nHAC and nHAC/alginate composites are extremely fine to nanometer level that is similar to natural bone [1]. Because powder XRD analysis revealed no significant difference between nHAC/alginate and nHAC sample, it is demonstrated that the nano-sized HA crystals were not changed by the Ca-crosslinking procedure.

SEM micrograph of the nHAC/alginate composites shows a highly porous structure. Fig. 2 indicates that the irregular pores range from tens of microns up to about 300 μm with a mean value around 100 μm, which this pore size distribution is favored by cell [15, 16]. There is no distinct difference for the pore size distribution among samples of different alginate content. The high porosity is expected to satisfy better the cell penetration and mass transport requirements for nutrient, metabolites, and soluble signals.

Improved mechanical properties of the composite scaffold over the nHAC composite alone were observed obviously. Without alginate, nHAC composite is very weak with its compressive yield strength is only around 62 kPa. Fig. 3 shows the mechanical properties of nHAC/alginate as a function of alginate addition. Both the compressive modulus and the compressive yield strength of the composite increase linearly as alginate addition increase (the thick black line in the Fig. 3). While with 20–33% alginate addition, nHAC/alginate composite can stand a compressive strength up to 120–330 MPa. These data demonstrate the positive effects of the $Ca^{2+}$ crosslinked alginate in enhancing the mechanical performance of the scaffolds above 10% alginate.

In the cell culture experiments, the decline rate of the fibroblasts co-incubated with sample extraction showed the same magnitude as those of the blank control group and the negative control group in the whole week, which was about 1% or less. While positive control groups showed 20% decline rate in the 1-h culture, and the 50% decline rate in the 7-day culture. These results indicate that the addition of alginate in nHAC composite does not bring any evident negative effects on the excellent biological properties of nHAC composite, which the high biocompatibility of nHAC composite was reported in former reports [7–9].

SEM observation of osteoblasts cultured on glass and nHAC/alginate composites shows good cell affinity to nHAC/alginate composites. Fig. 4 shows the characteristic morphology of osteoblasts cultured on glass and nHAC/alginate composites. Osteoblasts attach and proliferate well on the composites. Their shapes are similar to those cultured on glass. They are well spread, exhibiting a relatively flat configuration. The cell adhered to the membrane with processes and multiple filopodia. Much fibrillar bundles of extra cellular matrix were found on the composite and cell surface. Comparing these results with those of our previous work [7–9], it seems that nHAC/alginate composite is biologically comparable to nHAC composite.

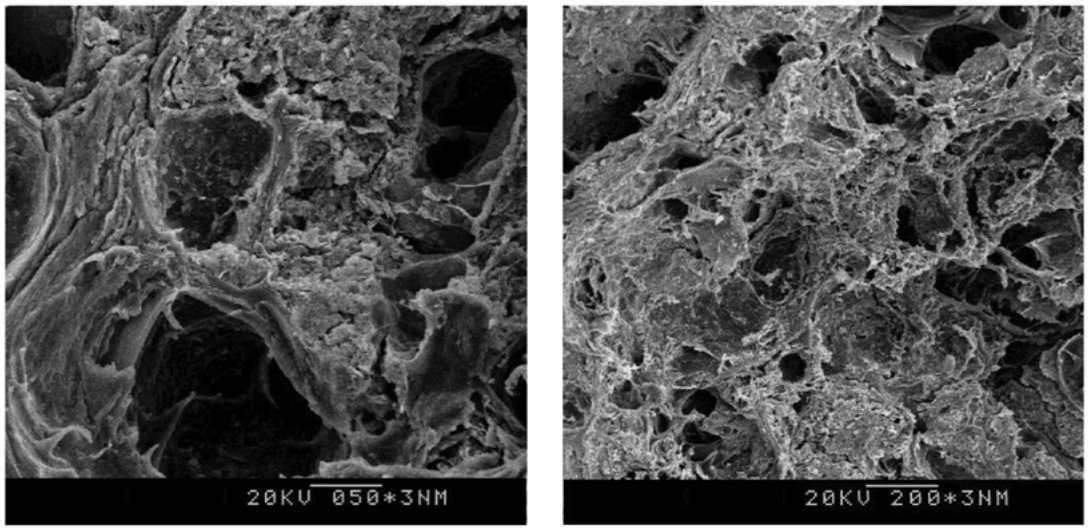


*Figure 2* SEM images of the cross-section of nHAC/alginate composite.

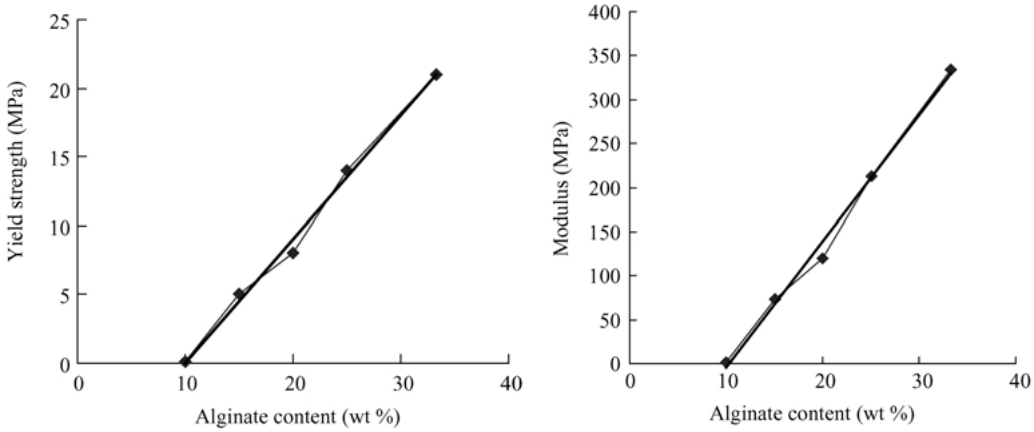


*Figure 3* The changing pattern of compressive modulus and yield strength of nHAC/alginate composite with alginate content.

Fig. 5 shows the osteoblast viability of the nHAC/alginate samples and controls at 2, 4, 5 days culture. Compared to the DMEM controls, osteoblasts cultured in the nHAC/alginate composite containing medium possessed satisfied organism competence after a period of culture. The percentage response is 82.4%, 92.7% and 77.4% respectively at 2, 4 and 5 days culture. The growth trend at the composite in a week culture was similar to that of control group both in the absolute value and the percentage. The light absorbance in the samples was significantly $(P < 0.01)$ greater than that in the phenol controls (data not shown).

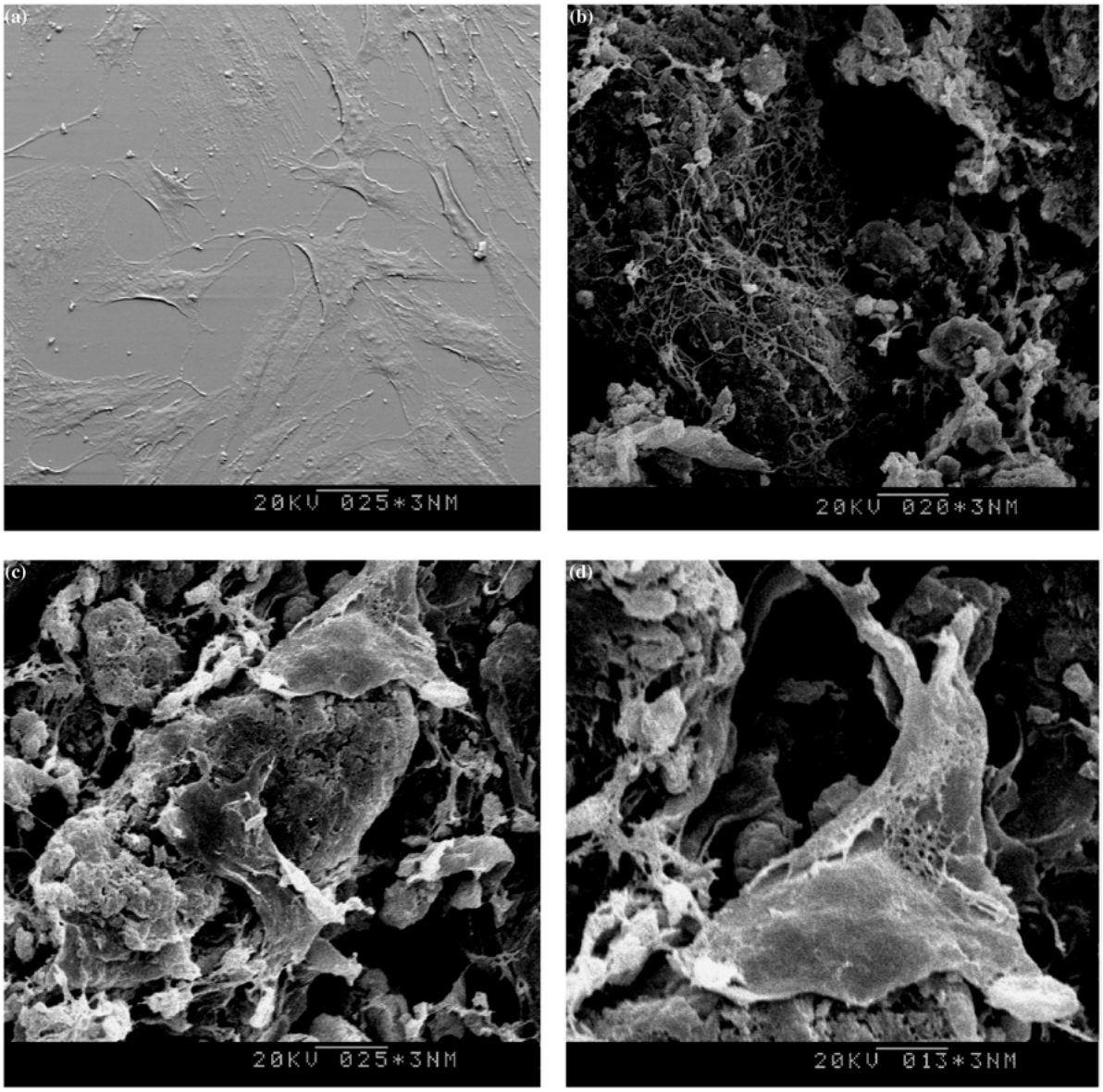


*Figure 4* SEM images after one week of osteoblasts co-culture with nHAC/alginate (a) control group – osteoblasts on glass; (b) fibrillar-like extra cellular matrix on nHAC/alginate composite; (c) oseoblasts on nHAC/alginate surface; (d) magnification of (c).

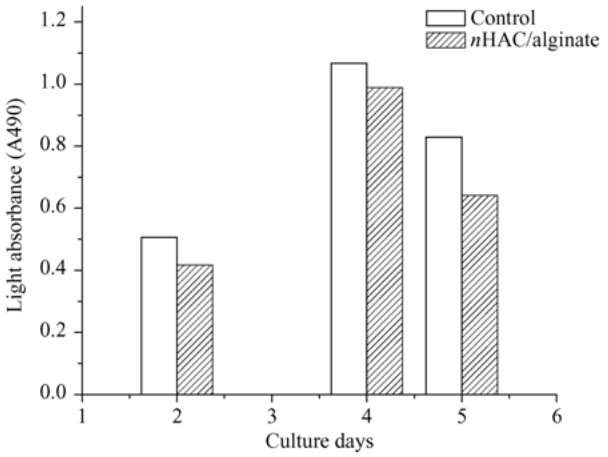


*Figure 5* MTT assay, formazan absorbance was expressed as a measure of cell viability from osteoblasts cultured in the nHAC/alginate samples and controls. Initial seeding density was 4000 cells/cm$^2$, $n = 3$.

Alkaline phosphatase is a diagnostic marker enzyme of osteoblast. It is about 80% to DMEM control in the samples group in 5 days. Compared to results of the MTT, this value is due to no direct inhibition of ALP enzyme activity by nHAC/alginate composite.

## 4. Discussion

Understanding why it is possible to improve the mechanical properties of nHAC composite by adding Ca-crosslinked alginate maybe brought to light if we consider the structure of alginate. Alginate is a linear polysaccharide composed of mannuronic acid (M) and guluronic acid (G). These monomers can be organized in blocks of consecutive G, M or alternating M and G. And two consecutive G-blocks of more than six residues each can be crosslinked by divalent cations (e.g. $Ca^{2+}$) making possible of a strong scaffold structure.

Fibroblasts and osteoblasts co-culture with nHAC/alginate composite proved excellent biological properties of nHAC/alginate composite. For orthopaedic materials it is essential to determine the interaction with bone forming cells. Osteoblasts have been shown to be more sensitive to biomaterial interactions than fibroblasts. After the co-culture experiments with fibroblasts, the co-culture with osteoblasts and quantitatively biochemical measures were processed. The high biocompatibility was shown not only at the well adhesion morphology of osteoblasts, but also at high percentage response on the three points MTT assay and one point ALP activity. This is not surprising, because alginate is one kind of polysaccharides, and it is one of the first materials employed in tissue engineering. In recent years alginate has been used for encapsulation of cells and enzymes [17]. It also has been successfully adapted to culture of cartilage cells (chondrocytes) to form cartilage tissue for *in vitro* and *in vivo* study of cartilage behavior [18–22]. Some researchers have successfully integrated inorganic HA granules for bone fixation or gap filling materials using alginate as cohesive additive [23, 24]. All of these results agree with our results well and imply that the addition of alginate into nHAC composite would not bring unfavorable effect. Thus nHAC/alginate composite could be a potential candidate for bone substitute materials.

## 5. Conclusions

Porous nHAC/alginate composite with good mechanical properties was developed through integrating nHAC powder with Ca-crosslinked alginate. The mechanical properties could be increase by the increase addition of alginate in the composite. The high biocompatibility of the composite is assured by cell culture *in vitro*. It is a promising candidate of scaffold material for bone tissue engineering.

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