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Investigation of biphasic calcium phosphate/gelatin nanocomposite scaffolds as a bone tissue engineering

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Abstract

The porous scaffold of nanobiphasic calcium phosphate (n-BCP) and gelatin from bovine skin type B was prepared by freeze-drying method. The porogen which used was Naphthalene. EDC (N-(3-dimethyl aminopropyl)-N′-ethyl carbodiimide hydrochloride) for stabilization of gelatin by cross-linking method was used. The scaffold was characterized by SEM, XRD and FTIR. As a result, a biocompatible scaffold with good cell attachment, facility in formation in desired shapes and simplicity in production were prepared for bone tissue engineering.

Keywords: A. Sintering; B. Nanocomposite; B. Porosity; E. Biomedical application; Scaffold

1. Introduction

Nowadays tissue engineering is one of the important ways to achieve tissues for repair or replacement applications. Its goal is to design and fabricate reproducible, bioactive and bioresorbable 3D scaffolds with tailored properties that are able to maintain their structure and integrity for predictable times, even under load-bearing conditions [1]. Materials and fabrication technologies are critically important for tissue engineering in designing temporary, artificial extra cellular matrixes (scaffolds), which support three-dimensional tissue formation [2].

In the tissue engineering approach, the temporary 3D scaffold serves an important role in the manipulation of the functions of osteoblasts and a central role in the guidance of new bone formation into desired shapes [3–7].

Currently, composites of polymers and ceramics are being developed with the aim to increase the mechanical scaffold stability and to improve tissue interaction. Many suitable materials have been used as scaffolds, including bioactive ceramics (e.g. hydroxy apatite (HA), TCP, BCP), bioactive glass (BG) and polymers. Totally there are two types of biodegradable polymers: The natural-based materials are one category, including polysaccharides (starch, alginate, chitin/chitosan, hyaluronic acid derivatives) or proteins (soy, collagen, fibrin gels, silk) and, as reinforcement, a variety of biofibers such as lignocellulosic natural fibers and on the second category, synthetic biodegradable polymers such as saturated poly-a-hydroxy esters, including poly(lactic acid) (PLA) and poly(-glycolic acid) (PGA), poly(lactic-coglycolide) (PLGA) copolymers. Synthetic polymers can be produced under controlled conditions and therefore exhibit in general predictable and reproducible mechanical and physical properties such as tensile strength, elastic modulus and degradation rate [8].

Natural polymers possess several inherent advantages such as bioactivity, the ability to present receptor-binding ligands to cells, susceptibility to cell-triggered proteolytic degradation and natural remodeling [9].

The fragility of the ceramics and the inflammatory response induced by the degradation products of the synthetic polymers are problems which have been encountered regarding the use of ceramic and polymer materials [10].

Nowadays, biphasic calcium phosphate (BCP) ceramics (HA/β-TCP) were developed as scaffolding materials. This matter can be explained by effective role of biphasic calcium phosphates in

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bone repair and regeneration in comparison with pure HA or β-TCP, and their controllable degradation rate [11].

Gelatin is a natural material derived from collagen by hydrolysis and has almost identical composition as that of collagen. Since is a denatured biopolymer, the selection of gelatin as a scaffolding material can circumvent the concerns of immunogenicity and pathogen transmission associated with collagen. The collagen-based gelatin has a high degree of biological functional groups, and has some potential for application in tissue scaffolds. In practical terms, gelatin is currently used in pharmaceuticals, wound dressings and adhesives in clinics due to its good cell viability and lack of antigenicity. Its shape availability and cost-efficiency can facilitate the selectivity and mass-producibility [12–14].

Some researches in preparation of gelatin scaffolds such as phase separation with porogen leaching techniques [15], gel-casting and polymer sponge methods [16], co-precipitation of HA within a gelatin sol and further freeze-drying [17] ceramic–gelatin assembly (CGA) and precipitation of hydroxyapatite nanocrystals in aqueous solution of gelatin [18] were studied before.

There are some difficulties for preparation of a nanocomposite which is similar to the nanostructure of real bone. The biggest practical problem with type-I COL is its cost, solubility and the poor definition of commercial sources of this material which makes it difficult to follow up on well controlled processing. Therefore in the present study, COL type-1 was replaced by a gelatin (GEL) precursor. The commercial sources of GEL materials show good water solubility, and well-defined physical and chemical properties. Our objective in this study is to achieve a biomimetic ECM (extra cellular matrix) similar to the natural bone with a novel technique for using in orthopedic application. Advantages of this method are biocompatibility, simplicity in production and facility in formation in desired shapes.

2. Experimental procedures

2.1. Synthesis of nano-BCP powder

The nano-sized BCP powder with 70% hydroxy apatite (HA) and 30% β-tricalcium phosphate (β-TCP) were prepared by precipitation method. Calcium hydroxide ($Ca(OH)_2$, Acros Organics, 98% purity) and orthophosphoric acid (H_3PO_4, Merck, 85% purity) were used as starting materials. For obtaining a homogenous suspension, calcium hydroxide solution was prepared by magnet stirrer for 1 h and then 1 M orthophosphoric acid solution was added with a rate of 15–20 ml/min. For controlling the PH of the solution in the range of 10–12, hydrogen nitride (NH_3, Merck) was used. To obtain high homogeneity and gel, the prepared sol was aged at room temperature (25 °C) for 24 h. The gel was dried at 110 °C for 48 h and then calcined at 700 °C for 2 h to achieve mixture of hydroxy apatite (HA) and β-tricalcium phosphate

2.2. Scaffold fabrication

Naphthalene powder was used as porogen material with particle sizes under 500 μm. Naphthalene and BCP powder were mixed with a ratio of 2:3, respectively. To supply adhesion, the prepared batch and 5% polyvinyl alcohol (PVA, Acros Organics, 88% purity) solution were mixed (weight ratio of 10:1). The batch was aged for 24 h and then prepared powder was pressed by uniaxial press with 50 MPa pressure and then sintered at 1400 °C for 1 h with a heating rate of 10 °C min^{-1}

Thereafter, the BCP scaffold was immersed in gelatin from bovine skin type B (Sigma–Aldrich) in distilled water (6 mg/ml and pH ∼7) for 4 h, so that pores could be filled by the gelatin solution. Immersing up to 4 h could be result in gelling formation and decreasing flow ability of gelatin into the pores, so it caused making some difficulties during sublimation process. The BCP scaffold was kept in freezer (Ultra Low Temperature Freezer, New Brunswick Scientific) at −70 °C for 18 h and then freeze-dried in freeze-dryer (Christ Alpha 2–4, German) at −80 °C for 7 h to form a gelatin network matrix on the pores and surface of the scaffold. During the freezing, the gelatin solution which filled the pores were frigid and by freeze-drying and sublimation of distilled water, gelatin network matrix was formed on the pores and surface of the walls but it did not fill the entire pores. Then, the gelatin network matrix immersed in 1% EDC (N-(3-dimethyl aminopropyl)-N′-ethyl carbodiimide hydrochloride) (Merck) solution in distilled water for cross-linking and stabilizing the gelatin. Then the samples washed by a 0.1 M $Na_2HPO_4{\cdot}12H_2O$ (Merck) and deionized water and finally dried in air and again freeze-dried at −80 °C for 4 h.

Phase characterization of nano-BCP powder was carried out using X-ray diffraction (XRD: JDX-8030, Jeol, Japan). The surface morphology of the BCP powder and scaffolds was investigated by scanning electron microscopy (SEM: XL30, Philips).

In order to further illustration of the formation mechanism of HA and β-TCP, Fourier-transformed infrared spectroscopy (FTIR: Shimadzu, 8400s) before and after calcination was used. FTIR spectra were recorded in a spectral range of 400–4000 cm^{-1}.

The osteoblast cell line (G292) was obtained from National Cell Bank of Iran (NCBI, Iran). The cells were cultured in HAMS-DMEM medium with 10% FBS (fetal bovine serum) as seeding material. After incubating in a humidified atmosphere of 5% CO_2 at 37 °C, the cells were washed with trypsin 1× solution in PBS (phosphate-buffered saline) and centrifuged and resuspended for in vitro tests.

The scaffolds were sterilized by gamma irradiation with 2.5 KGy dose [19]. Cell adhesion test was carried out by osteoblast cells. 10^4 cells were poured on the surface of the steriled samples. After 24 h seeding, cells were stabilized by Karnovsky's fixative and then were characterized by SEM. Cell adhesion test was repeated three times for samples.

3. Results and discussion

3.1. Characterization of nano-BCP powder

Fig. 1 shows the XRD patterns of pre-calcined and calcined synthesized powders at different temperatures. By calculation

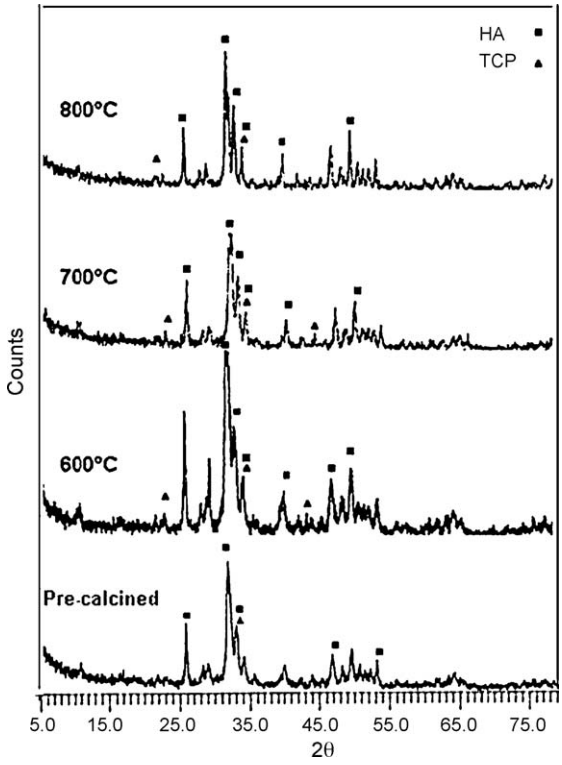

Fig. 1. XRD patterns of pre-calcined and calcined synthesized powders at different temperatures.

the quantitative analysis of the XRD patterns, it was confirmed that 70% HA and 30% β-TCP was acquirable at 700 °C.

Fig. 2 shows the microstructure of the BCP powder after calcination at 700 °C. As this figure shows the particles are agglomerated with size in the range of 20–50 nm.

Figs. 3 and 4 show the FTIR spectra of the non-calcined and calcined powders. As FTIR spectra in Figs. 3 and 4 show, both spectra have similar FTIR bands but with different intensities. The characteristic of the OH^{-1} bands in HA were observed at 3448.49 and 3421.48 cm^{-1} in non-calcined sample (Fig. 3) and 3429.20 cm^{-1} in calcined one (Fig. 4). $CO_3{}^{-1}$ groups were observed at 1400–1500 cm^{-1}, which was commonly found in synthetic HA and natural bone [20]. Carbonate bands in BCP

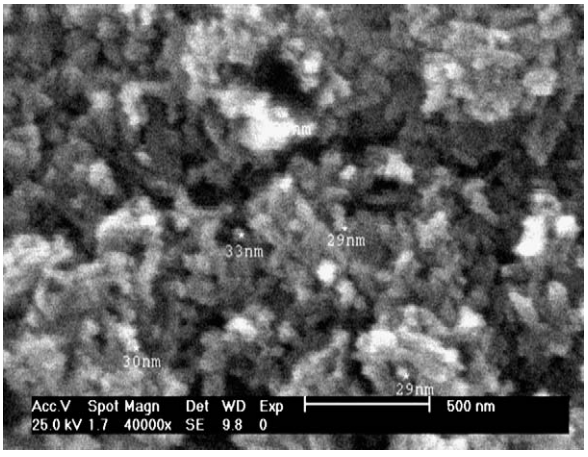

Fig. 2. SEM micrograph of the BCP powder.

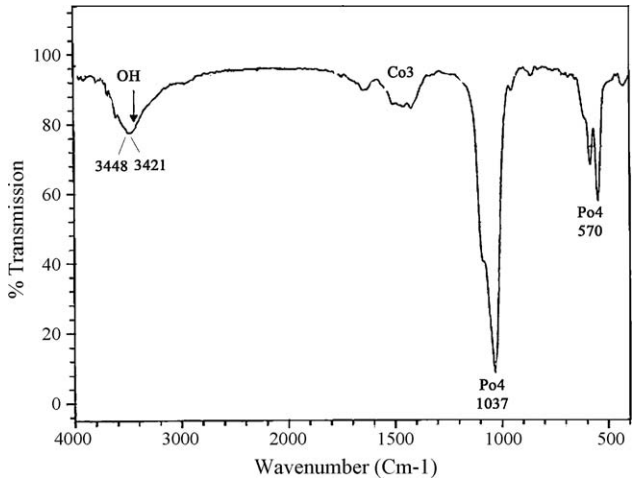

Fig. 3. FTIR spectra of HA powder before calcination.

powder (calcined sample) were more intense than non-calcined sample, which could be explained by forming HA crystals after calcination. The strong bands at 1041.46, 570.86 cm^{-1} for non-calcined sample (Fig. 3) and 1037.63, 580 cm^{-1} for calcined one (Fig. 4) associated with $PO_4{}^{-3}$ groups.

3.2. Structure of nano-BCP scaffold

SEM observation of the nano-BCP scaffold showed a 3D interconnected porous structure with 65% porosity. In the other hand calculated degradation rate of scaffold was 0.008 g in 24 h. Scaffolds fabricated from biomaterials with a high degradation rate should not have high porosities (>90%), since rapid depletion of the biomaterial will compromise the mechanical and structural integrity before substitution by newly formed bone. In contrast, scaffolds fabricated from biomaterials with low degradation rates and robust mechanical properties can be highly porous, because the higher pore surface area interacting with the host tissue can accelerate degradation due to macrophages via oxidation and/or hydrolysis [21]. Fig. 5 shows cross-section of nano-BCP scaffold with pore size in the range of

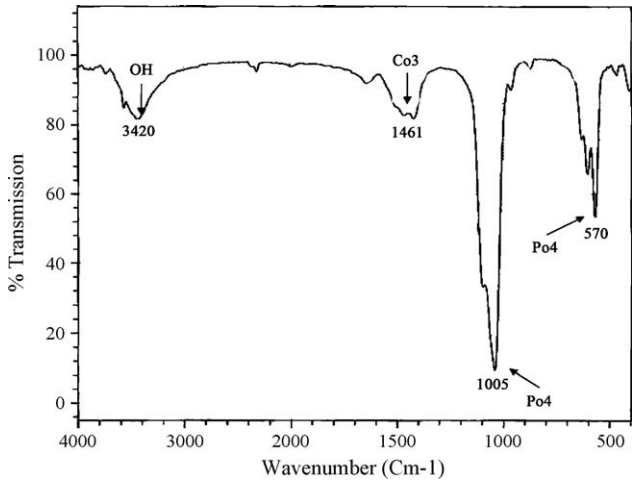

Fig. 4. FTIR spectra of HA powder after calcination.

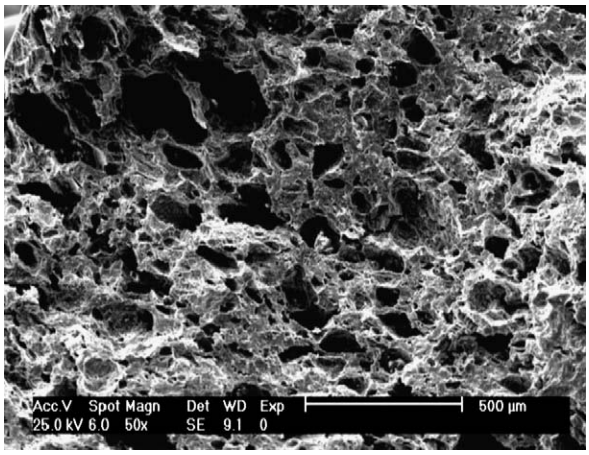

Fig. 5. Cross-section of nano-BCP scaffold.

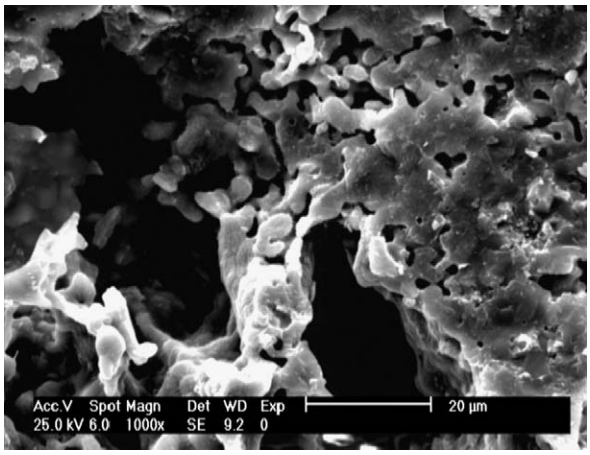

Fig. 7. SEM micrograph of pores on the wall of the BCP/GEL scaffold.

100–200 μm. Pores which made by exhausting the naphthalene powder through sintering, can be observed.

3.3. Structure of nano-BCP/GEL scaffold

Freezing the immersed nano-BCP scaffold in gelatin solution, could be resulted in the formation of ice crystals that force and aggregate the gelatin molecules into the interstitial spaces [22]. The ice crystals were then removed by freeze-drying so that scaffold with homogen distribution of porosities was obtained. In the other hand, using the sphere-like naphthalene particles as porogen before freeze-drying, pre-pores could be formed. Because of the high degradation rate and low biomechanical stiffness of gelatin in vivo, cross-linking is necessary to reduce biodegradation and enhance the biomechanical properties of the biomaterial for tissue repair. In this study, EDC cross-linking method was carried out. SEM micrograph of the nano-BCP/GEL scaffolds show a 3D porous structure with interconnected porosities similar to natural bone in the range of 100–200 μm (Fig. 6). As Fig. 7 shows, there are some macropores and micropores, whose pore size was less than 50 μm. Roughness and micro-holes on the walls of the BCP/GEL scaffold fabricated by freeze-drying method could be resulted in good cell attachment.

Fig. 8 shows SEM micrograph of gelatin stabilized in BCP powder and nanocomposite structure of scaffold.

Fig. 9 shows FTIR spectra of BCP, gelatin and BCP/GEL scaffold. As the results show, PO_4^{-3} groups in BCP/GEL scaffold were more intense than BCP powder. For the BCP powder resonances associated with the stretching mode of $(PO_4)^{3-}$ ion, were observed at 1041 and 570 cm^{-1}, respectively. Resonances associated with the stretching mode of the $(CO_3)^{2-}$ ion, were also observed at 1400–1500 cm^{-1}. The resonances around 1445 cm^{-1} were the result of carbonate stretching vibrations. The FTIR spectrum of gelatin showed the typical amides I (C–O), II (N–H) and III (C–N) peaks at 1691, 1250 and 950 cm^{-1}, respectively. The spectrum of GEL/BCP composite sample was characterized by absorption bands arising from BCP and gelatin, determined by analogy with FTIR spectra of pure BCP and gelatin standard samples. Absorption bands at 561–609 cm^{-1} associated with the $(PO_4)^{3-}$ groups of BCP. Bands at 3400, 3000, 1700 and 1200 cm^{-1} in BCP/GEL scaffold associated with formation of amide A, B, I and II due to adding gelatin in BCP/GEL spectra. The appearance of an amide I mode at 1664 cm^{-1} indicates that BCP–GEL composites adopt a predominantly

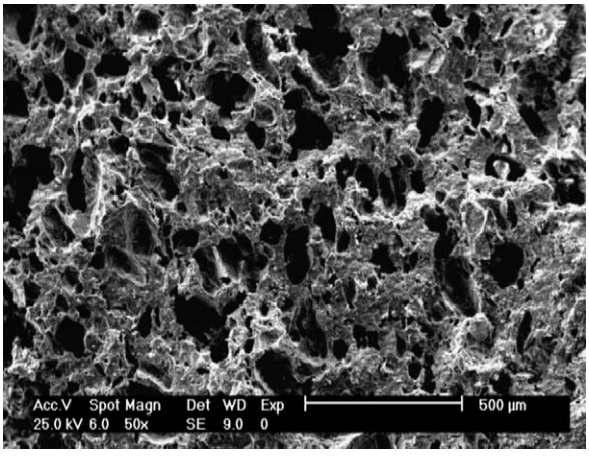

Fig. 6. Cross-section of nano-BCP/GEL scaffold.

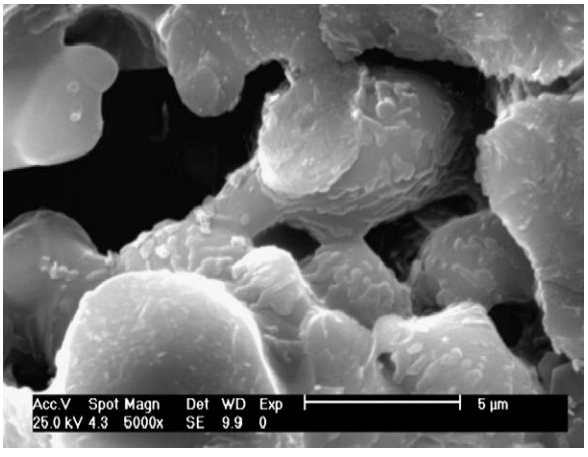

Fig. 8. SEM micrograph of BCP/GEL nanocomposite.

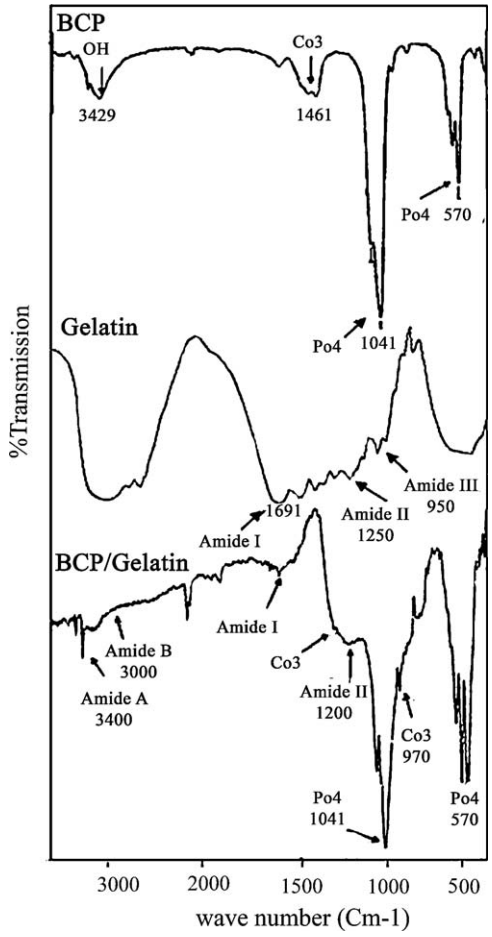

Fig. 9. FTIR spectras of BCP, gelatin and gelatin/BCP scaffold.

a-helical configuration and this is confirmed by the appearance of amide II mode at 1537 cm^{-1} [23].

3.4. Cell adhesion

An important objective of bone tissue engineering is to develop improved scaffold materials or arrangement to control osteoblast behavior which significantly affecting its response. Osteoblastic cells on biphasic calcium phosphate exhibited unique attachment and subsequent behavior in vitro, which may explain why mineralized tissue formation is better on BCP. Fig. 10 shows that osteoblast cells could attach and proliferate on the BCP/GEL scaffold which prepared by freeze-drying method.

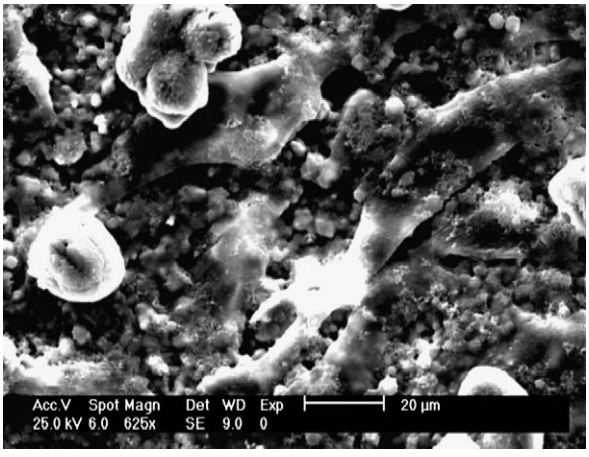

Fig. 10. Osteoblast adhesion on scaffold surface after 24 h seeding.

4. Conclusions

An ideal tissue engineering implant should be biocompatible, highly porous with adequate mechanical properties. In this study, scaffolds of BCP/GEL were used as a skeleton implant to hold and growth osteoblast cells. Effective role of nano-BCP powder as basic material for preparing of this scaffold was in bone repair or regeneration. By preparing this gelatin network matrix of BCP/GEL scaffold, forming the desired shapes were obtainable. A cross-linked gelatin network matrix and BCP as primary components of bone tissue with good cell attachment was achieved. The other in vitro tests of BCP/GEL nanocomposite scaffolds are in progress.

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