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ORIGINAL RESEARCH PAPER

Synthesis, characterization and biocompatibility evaluation of hydroxyapatite - gelatin polyLactic acid ternary nanocomposite

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ABSTRACT

Objective(s): The current study reports the production and biocompatibility evaluation of a ternary nanocomposite consisting of HA, PLA, and gelatin for biomedical application.

Materials and Methods: Hydroxyapatite nanopowder (HA: $Ca_{10}(PO_4)_6(OH)_2$) was produced by burning the bovine cortical bone within the temperature range of 350-450 °C followed by heating in an oven at 800. Synthesis of the ternary nanocomposite was carried out in two steps: synthesis of gelatin-hydroxyapatite binary nanocomposite and addition of poly lactic acid with different percentages to the resulting composition. The crystal structure was determined by X-ray diffraction (XRD), while major elements and impurities of hydroxyapatite were identified by elemental analysis of X-ray fluorescence (XRF). Functional groups were determined by Fourier transform infrared spectroscopy (FTIR). Morphology and size of the nanocomposites were evaluated using field emission scanning electron microscope (FE-SEM).Biocompatibility of nanocomposites was investigated by MTT assay.

Results: XRD patterns verified the ideal crystal structure of the hydroxyapatite, which indicated an appropriate synthesis process and absence of disturbing phases. Results of FTIR analysis determined the polymers' functional groups, specified formation of the polymers on the hydroxyapatite surface, and verified synthesis of nHA/PLA/Gel composite. FESEM images also indicated the homogeneous structure of the composite in the range of 50 nanometers. MTT assay results confirmed the biocompatibility of nanocomposite samples.

Conclusion: This study suggested that the ternary nanocomposite of nHA/PLA/Gel can be a good candidate for biomedical application such as drug delivery systems, but for evaluation of its potential in hard tissue replacement, mechanical tests should be performed.

Keywords: *Biocompatibility, Gelatin, Hydroxyapatite, Nanocomposites, Polylactic Acid*

INTRODUCTION

Hydroxyapatite has been developed as a bio-material and is applied in various medical applications due to its high biocompatibility. Hydroxyapatite (HA) has important features such as bioactivity and osteoconductivity [1]. Calcium phosphate has recently been considered one of the potential materials for bone drug delivery systems owing to its physical and chemical properties as well as biological features [1, 2].Using a bioactive matrix, this type of drug delivery systems can release a therapeutic agent to provide osteoconductivity [3-5].

Numerous studies have been conducted on methods of preparing HA, which include deposition [6], hydrolysis [7], and hydrothermal methods [8]. One of the most widely used methods is wet precipitation, where chemical reactions take place between calcium and phosphorus ions under a controlled pH and temperature of the solution. An alternative method for

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the preparation of hydroxyapatite can be its extraction from natural resources. In fact, some researchers have attempted to synthesize hydroxyapatite from biological materials such as marine crustacean shell [9], eggshell [10] and bovine cortical bone ash [11]. Today, many studies have been performed on composites in the field of tissue engineering and bone regeneration in order to simulate the composition and structure of human hard tissues, which are natural nanocomposite composed of hydroxyapatite nano- crystals and collagen proteins [12-15]. Due to their biocompatibility and ability to be used in bone grafting, many composite materials have been developed for medical applications, including ceramic or polymer matrix composites [16, 17]. There are different biocomposites in nature in which an organic matrix is combined with a mineral component [18].

These composites provide mechanical properties required for specific applications such as the skeleton, teeth, or cells of living organisms [19, 20]. Many attempts have been made to develop new alternative materials to bone, among which are hydroxyapatite/polymer composites that have attracted great attention [21-23]. A large variety of biodegradable polymers have been developed and used in medical applications, among these, PLA is preferred in biomedical applications due to its biodegradability, biocompatibility, and non-toxicity [24]. Degradation of pure PLA implants in the body is known to produce intermediate acidic products that often lead to adverse inflammatory responses; addition of HA could also help to buffer such products [25].

Gelatin, a protein based material derived from the hydrolysis of collagen, has been well utilized in this area on account of its biodegradable, biocompatible nature and its commercial availability at low cost. It has been shown to have advantages over its parent protein, which include lower immunogenicity [26]. Poor mechanical properties which reveal its successful use in soft tissue engineering and not for load bearing applications. Therefore, a binary gelatin and HA composite is therefore expected to show increased osteoconductivity and biodegradation together with sufficient mechanical strength [27-29].

Composite systems such as powders, dense structures, and porous scaffolds have been manufactured from hydroxyapatite and collagen-based proteins such as gelatin. [30-32].Studies have demonstrated that composite systems increase the cellular responses of osteoblasts and capability of in vivo osteogenesis [14, 33]. Compared to micro–scale composites, nano- composites show better mechanical and biological properties. Gelatin-nanohydroxyapatite composite can be used as filler, either alone or in combination with another polymer matrix in bone tissue engineering applications [33]. In addition, this composite can be loaded with bioactive and biological materials and can be used in the controlled delivery during the bone remodeling process [34-36].

So far, hydroxyapatite-gelatin and hydroxyapatite-poly lactic acid binary composites have been synthesized by researchers and their properties have been studied. The main purpose of this study was the synthesis and characterization of a ternary nanocomposite consisting of HA, PLA, and gelatin for biomedical applications.

MATERIALS AND METHODS

Materials

Poly Lactic Acid, (3251D injection grade PLA) was prepared from Nature Works LLC (Nebraska, USA). Gelatin (Type B bovine) were obtained from Aldrich (St. Louis, Missouri, USA). Solvents used include chloroform and acetic acid with a purity of over 99.9% and NaCl, KCl, KH_2PO_4, Na_2HPO_4 for preparation of phosphate buffered saline (PBS) were purchased from Merck (Darmstadt, Germany).

Preparation of nHA/PLA/Gel nanocomposite

In this study, the bovine cortical bone was used as a biological source for hydroxyapatite production. First, cancellous parts of the bone as well as bone marrow and meat and fat pieces were separated from the dense bone and, then, the dense bone was heated by the torch until its organic compounds were burned and removed. The resulting material was black due to the carbon content resulting from the burning process. In order to remove the carbon, the black ash was heated for 60min at800! in the air, which produced a white powder called natural hydroxyapatite [37].

Gelatin was dissolved in acetic acid and stirred for 3hat the room temperature.

Then, hydroxyapatite nanopowder was added and stirred for 8h in the water bath at 36. Polylactic acid granules were dissolved in chloroform at the ambient temperature for 3 h. The PLA solution was added to gelatin and hydroxyapatite mixture. The resulting mixture was stirred for 36h in a water bath to form a jelly-like material. The gel was stored for 12 h at the ambient temperature and, then, for 24h in the oven at 60^{o}C to completely dry up. nHA/

PLA/Gel nano- composite was manufactured based on various formulations which are presented in Table 1.

Table 1. Various formulations of nHA/PLA/Gel nanocomposite

	A	B	C	D	E
% Gelatin	30	30	30	30	30
% nHA	70	60	50	40	30
% PLA	0	10	20	30	40

Characterization of nHA/PLA/Gel nanocomposite

Phase and morphology analysis

The phase analysis of natural hydroxyapatite and nanocomposite samples was performed by the X-ray diffraction (XRD) device manufactured by BRUKER Company(D8-Advance)with Cu Kα radiation at the wavelength of 1.5418Å within the range of $2\theta=5^{o}$-70^{o} and step size of 0.01.Basic constituent elements of the hydroxyapatite and its impurities were identified by X ray fluorescence spectroscopy analysis using a device manufactured by Philips Company (PW400).In order to study structure of nanohydroxyapatite and nanocomposites and also investigate the functional groups, the infrared spectrometer device (FTIR, Shimadzu 8300) was used. Shape, particle size, and morphology of the nanoparticles were examined by field emission scanning electron microscope (FE-SEM) manufactured by Hitachi Company (S-4160).

Biocompatibility evaluation

In order to use a material as a drug carrier or other biological applications, its biocompatibility and lack of cytotoxicity must be ensured. Toxicity of nanocomposites depends on numerous factors including dosage, chemical composition, applied method, biodegradability, solubility, pharmacokinetic, biological distribution, surface chemistry, structure, etc. Generally, size, surface area, shape, composition, and nanocomposites coating are among the most important characteristics related to cytotoxicity.

The human osteosarcoma cell lines (MG-63) were obtained from National cell bank of Iran and cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Scotland) supplemented with 10% fetal calf serum (FCS) (Nano Bio Array, Iran), 100 µg /ml penicillin and 100 µg/ml streptomycin (Sigma, USA). A number of 1×10^{4} cells per 100 µl for each well were seeded in a 96 well plate and incubated at 37°C under a humidified atmosphere with 5% CO_2. After 24 hours, 100µl of each sample (200 µg/ml in culture medium) was added and incubation proceeded for the next 24 hours. Subsequently, the cell medium was discarded and replaced by 100 µl of 3-(4,5- dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) solution (0.5 mg/ml in phosphate buffer saline) followed by incubation for 5 hours at 37°C. The purple crystals dissolved by addition of 100 µl of isopropanol (Merck, Germany) and incubation for 15 minutes. The absorbance of each well was measured by a micro plate reader (STATFAX2100, USA) at 545 nm and normalized to control (culture medium without sample in the same condition). The viability was calculated according to the following equation:

Viability % = (sample absorbance) × 100 / (control absorbance)

RESULTS AND DISCUSSION

XRD curves for HA, gelatin and binary nanocomposite are shown in Fig. 1.According to Fig. 1(A) which is related to X-ray diffraction pattern for hydroxyapatite nanoparticles, the process product was a single–phase hydroxyapatite with no disturbing stable phase. Also, there was an appropriate agreement between diffraction peaks in our product (natural hydroxyapatite) and those provided by the standard. The average size of the manufactured hydroxyapatite nanocrystals was calculated using Debye- Scherrer equation.

$$D = \frac{K\lambda}{\beta.\mathrm{Cos}2\theta}$$

Where K is Scherrer constant (0.9), λ is X-Ray wavelength (0.154nm), θ is peak width at half of its height, and β is angle of diffraction. Size of these crystallites was calculated approximately as 40 nanometers. In Fig. 1(B), X-ray diffraction curve of gelatin is shown, where there is no sharp peak in XRD curve of gelatin, indicating that this is an amorphous polymer material. Intensity of hydroxyapatite peaks was reduced in nHA/Gel nanocomposite, as shown in Fig. 1(C).

This issue represents that the surface factors between hydroxyapatite and gelatin changed the crystalline structure and reduced its crystallization [38].

In Fig. 2, X-ray diffraction pattern of the composite samples A to E can be seen. Because hydroxyapatite surface was covered with gelatin and poly lactic acid, gelatin is amorphous, and poly lactic acid is semi-crystalline, there was a decrease in the crystallinity of ternary nanocomposites compared to pure hydroxyapatite nanoparticles and binary nano-composites. Reduction level of crystallinity increased by increasing the percentage of poly lactic acid and simultaneously reducing percentage of the hydroxyapatite nanoparticles. Also, the peak observed at 2θ=16.652 in the ternary nanocomposites was related to the poly lactic acid. The peak intensity increased by increasing the weight fraction of PLA in nanocomposite. Results of XRF analysis of natural hydroxyapatite nanoparticles are presented in Table2. These results demonstrate that Ca and P were the main components, magnesium and sodium were impurities, and the potassium and strontium were negligible.

The presence of various elements in hydroxyapatite nanoparticles structure is because of the ion exchange in their structure. In the other words, Ca^{2+}, OH^{-}, and PO_4^{3-} ions in the hydroxyapatite structure can be easily substituted by other ions.Results of FTIR for pure hydroxyapatite nanoparticles, binary and ternary nanocomposites are shown in Fig. 3.The bands appearing at 470.59, 570.89, 601.74, 946.3, 1049.2, and 1087.87 cm^{-1}, were related to apatite ion[39]. These peaks were seen with less intensity in hydroxyapatite-gelatin binary nanocomposite (sample A) and hydroxyapatite-gelatin-poly lactic acid ternary nanocomposite (sample C, D, E). The peaks at 879.57, 1411.79 and 1458.08, were related to groups in

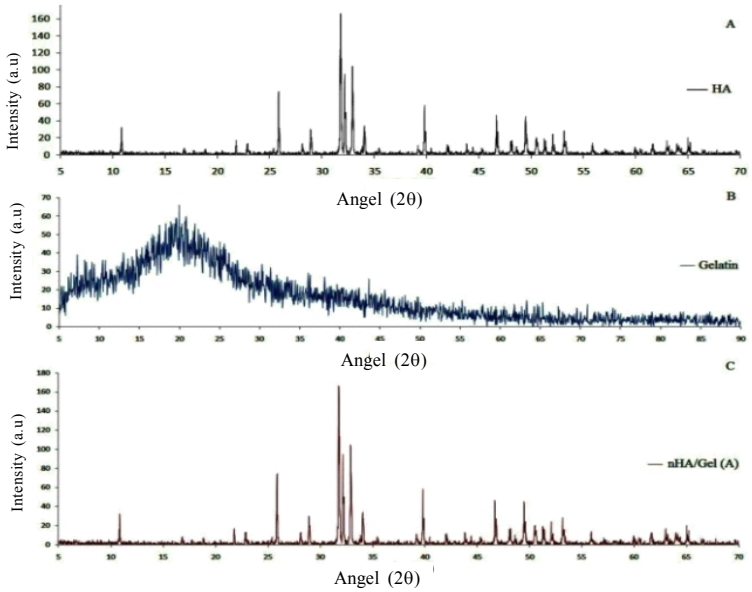

Fig. 1. XRD curves for (A) pure hydroxyapatite nanoparticles, (B) pure gelatin, (C) nHA /Gel nanocomposite

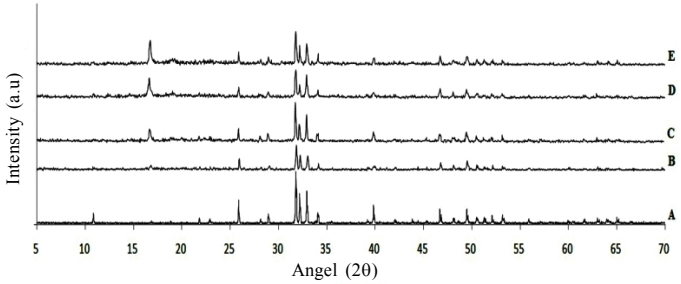

Fig. 2. XRD curves of samples A-E

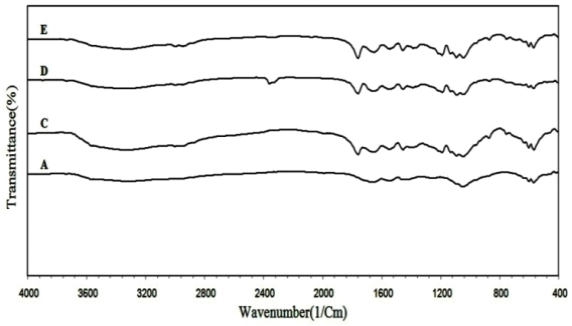

Fig. 3. FTIR spectrum of hydroxyapatite, sample A and samples C-E

Table 2. Elemental analysis performed by XRF method on natural hydroxyapatite powder sample

Chemical Formula	Concentration (Wt %)	Error (%)
CaO	53.698	0.08
P_2O_5	42.701	0.06
MgO	1.2	0.01
Na_2O	1.11	0.02
K_2O	0.072	0.002
SrO	0.063	0.001

Table 3. Summary of mean peaks in FTIR spectrum of nHA, nHA/Gelatin, and nHA/Gel/PLA

	nHA(cm^{-1})	nHA / Gelatin(A)(cm^{-1})	nHA/Gel/PLA(cm^{-1})		
			C	D	E
PO^{3-}_4	470.59	478.31	---	478.31	478.31
	570.89	570.89	570.89	570.89	570.89
	601.74	601.75	601.74	601.74	601.74
	964.34	---	---	---	---
	1049.2	1049.2	1049.2	1049.2	1049.2
	1087.78	1087.77	1095.49	1087.78	1095.49
-OH	3571.92	3571.92	3564.2	3564.2	3564.2
	632.608	632.608	632.608	640.32	632.608
CO^{2-}_3	879.47	---	871.76	871.76	871.76
	1411.79	1411.8	1427.22	1427.22	1427.22
	1458.08	1450.36	1458.08	1458.08	1458.08
C = O		1666.38	1658.67	1650.95	1650.95
N - H		3309.62	3301.91	3325	3309.62
C - O					1188.07

hydroxyapatite. These peaks in gelatin-hydroxyapatite binary nanocomposite were located at 1411.8 and 1450.36, respectively. The band at, disappeared in gelatin-hydroxyapatite binary nanocomposite whereas appeared in gelatin-hydroxyapatite-polylactic acid ternary nanocomposite in 871.76.

In Fig.4 (a), FE-SEM image of HA nanoparticles are presented. Morphology of the particles was relatively spherical, which provided the highest specific surface area. Also, size of the prepared hydroxyapatite particles was in nanometer scale and the relatively homogeneous and narrow (uniform) distribution of the particle size can be clearly seen in these images.

According to the microscopic images of 30%Gel-70%nHA binary composite shown in Fig. 4(b), it can be observed that gelatin covered the surface of the hydroxyapatite nanoparticles.

Figs 4(c) and (d), which are related to samples B and E respectively, represent the surface coverage of the nanoparticle porosities by the polymers and indicate that the composites' particle size was still within the nanometer scale; the shapes also verified accumulation and clustering to some extent. MTT assay results for samples A-E are shown in Fig. 5.

According to this test, if durability percentage of the cells inside the culture medium were more than 80%, the material would be considered nontoxic.

As can be seen in this figure, the resulting

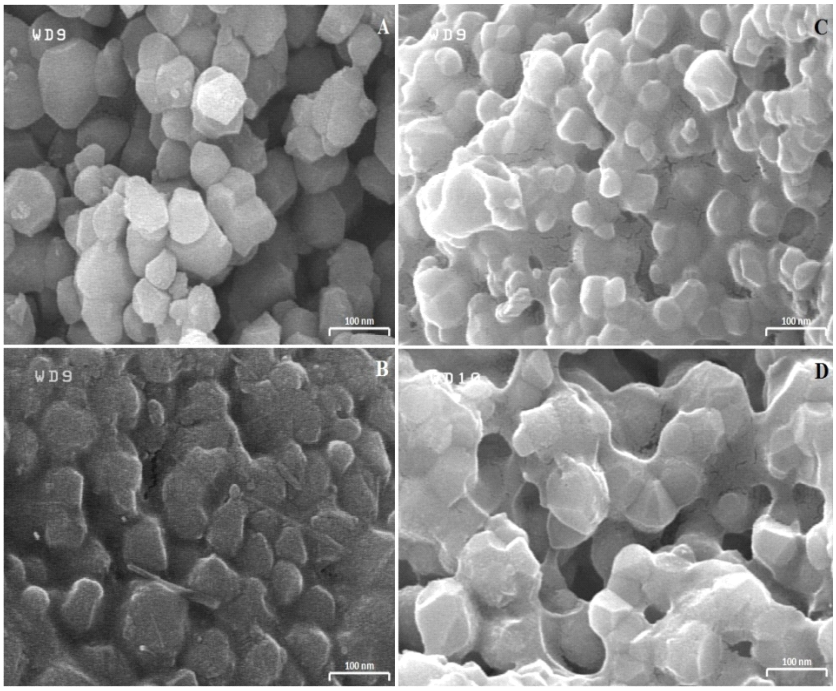

Fig. 4. FESEM image of the (A) hydroxyapatite nanoparticles, (B) Sample A , (C) sample B, (D) sample E

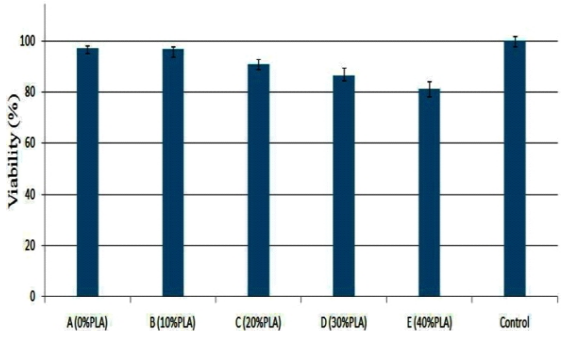

Fig. 5. MTT assay results as a function of PLA weight fraction in nanocomposites

nanocomposites are nontoxic, which can be used for biological applications.

CONCLUSION

nHA nanoparticles were prepared based on natural synthesis method. The crystalline structure of the nanoparticles was verified by XRD analysis. Also, investigating results of the XRF analysis of the nanoparticles showed that Ca and P were among the main components of the structure, while elements of magnesium, sodium, potassium, and strontium were in trace level.

Therefore, the appropriate quality of the synthesized material and the ideal synthesis process were confirmed. Also, the average size of the hydroxyapatite crystallites was evaluated as 40 nanometers by Debye-Scherrer method. XRD results of the composites also indicated a decrease in intensity of the characteristic peaks. Results of FTIR spectroscopy not only verified the presence of hydroxyapatite structural bands, but also indicated the appropriate incorporation of polymers and hydroxyapatite nanoparticles. FESEM images not only determined the size of hydroxyapatite particles to be smaller than 50 nanometers, but also confirmed the spherical morphology with high surface to volume ratio.

MTT assay results also confirmed the biocompatibility of nanocomposites. In summary it seem that our nanocomposite can be a good candidate for biomedical application such as drug delivery systems, but for evaluation of its potential in hard tissue replacement, mechanical tests should be performed.

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