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Evaluation of Biopolymers Effect on In vitro Biocompatibility Property of Nano Hydroxyl Apatite Composites

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ABSTRACT

In this work, we report the effect of biopolymers (starch and gelatin) on in vitro biocompatibility property of nano hydroxyapatite (nHAp) composites. Cell culture and MTT assays were performed for in vitro biocompatibility. They show that nHAp can affect the proliferation of cells and the nHAp-starch and nHAP-gelatin biocomposites have no negative effect on the cell morphology, viability and proliferation. By evaluation of MTT results it seems that starch composite is more biocompatible compare to gelatin one.

Keyword: Biocompatibility; Biocomposite; Organic templating; Bioactivity.

1. INTRODUCTION

The main composition of the extra cellular matrices of hard tissues are included both organic and inorganic phases. The inorganic phase consist of hydroxyapatite (HAp) crystals and the organic phase consist of type-I collagen and small amounts of glycosaminoglycans, proteoglycans and glyco-proteins [1]. So, in recent years, many interests have been attracted by inorganic nanoparticles which are embedded in polymeric matrixes. Various methods have been carried out to prepare this type of composite to obtain required properties and structures. Template - based synthesis is one of the most common methods [2]. This technique is based on the interaction between organic template and the inorganic filler, affecting controlled nucleation and crystal growth of the inorganic part or to the orientation of the organic components to form a higher order hierarchical structure [3]. HAp by Ca_{10} $(PO_4)_6$ $(OH)_2$ formula, which has compositional and biological similarities to the mineral phase of natural bone and biodegradable polymers such as collagen, gelatin, chitosan, starch, etc are the best candidates for preparing biomaterials for use in bone tissue regeneration. Gelatin and starch

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are natural biopolymers by functional groups such as amino acids and hydroxyl, respectively. Both of them are biodegradable, biocompatible, water soluble and inexpensive in comparison to other biodegradable polymers. Gelatin can be obtained by thermal, physical or chemical denaturation of collagen [2], [4-7]. Gelatin due to its hydrophilisity has great affinity with HAp and can be homogenized well with it in aqueous solution; in other side the polar nature of starch facilitates strong adhesion between the HAp and starch. So, we have decided to synthesize nHAp using gelatin and starch as a matrix and evaluate the biopolymer effect on composites in vitro biocompatibility property.

2. EXPERIMENTAL

2.1. Materials

Extra pure water soluble wheat starch and food grade gelatin were obtained from Merck. All the chemicals needed for synthesis of hydroxyl apatite; $CaCl_2.2H_2O$, NaH_2PO_4, NH_4OH were also supplied from Merck and used without any further purification.

2.2. Experimental Procedures

In situ synthesis of hydroxyapatite in the matrix of wheat starch and gelatin were carried out as reported before [8,9]. Pure nHAp also was prepared in the absence of biopolymers for comparison. Briefly, 0.8 g of starch in 70 ml water, 5.6 g gelatin in 70 ml water,0.5 M solution of calcium chloride and 0.3 M solution of dihydrogen phosphate, using double distilled water were prepared separately. For preparation of nHAp composites, the solution of calcium chloride was slowly added to the prepared biopolymers solutions and after stirring slowly for 3 h, appropriate amounts of sodium dihydrogen phosphate solution was added little by little to the stirring mixture. NH_4OH solution was added to control the solution pH at 10-10.5.

Obtained solutions by this manner (with and without biopolymers) were kept overnight at room temperature, centrifuged, decanted, washed with double-distilled water and dried (starch biocomposite at 60°C for 6 h in vacuum oven and gelatin one at room temperature). As dried products were characterized using a Fourier Infrared spectroscopy (FT-IR) Thermo Nicolet Nexus 870, X-ray Powder diffraction (XRD) Seisert Argon 3003 PTC using nickel-filtered XD-3a Cu Kα radiations (λ = 0.154 nm), Scanning Electron Microscopy (SEM) Philips and Transmission electron microscope (TEM) Phliips EM208 operated at 100 kV was used.

2.3. Cell Culture Experiments

2.3.1. Cells and Matrix Seeding

The human Bone Marrow Stem Cells (BMSCs) maintained from the Pastor Institute (Iran) were used as a test model in this study. Defreeze BMSCs was transferred into culture flasks with Dulbecco's Modified Eagles Medium (DMEM) low glucose containing 10% fetal bovine serum and 1% antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin). The medium was changed every 3 days. The biocomposites were sterilized by incubation in an autoclave at 121°C temperature and 2 bar pressure for 15 min. After sterilizing, samples incubated in the culture media before cell seeding. The biocomposite samples were seeded with BMSCs (5×10^3 cells/cm^2) by direct pipetting of the cell suspension onto the biocomposites and incubated at 37°C/5% CO_2 in 1 ml of cell culture medium in 96-well dishes. The cell culture medium was changed every 4 days. BMSCs cultured without biocomposites were used as a control group.

2.3.2. MTT Assay

The proliferation of BMSCs cultured with and without nHAp biocomposites was measured by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenylte-2H-tetrazoliumbromide) assay. After seeding for 1, 3 and 7 days, cells were incubated in 100 µl MTT solution (0.5 mg/ml, 37°C and 5% CO_2) for 3 h. After removal of supernatants, 100 µl/well of dimethyl sulfoxide (DMSO) was added and mixed. After complete solubilization of the MTTformazan, the absorbance of the contents of each well was measured at 570 nm with a

spectrophotometer (Perkin Elmer Co.).

3. RESULTS AND DISCUSSIONS

3.1. Fourier Transform Infrared Spectroscopy (FT-IR)

Figure 1 represents the FT-IR spectra of the amylopectin-rich starch and gelatin in comparison with the synthesized HAp in the absence and presence of 0.8 g starch and 5.6 g gelatin from top to bottom, respectively. In the starch spectrum, the wide band observed at 3348 cm^{-1} is attributed to the O-H stretching of the amylopectin and its width ascribed to the formation of inter and intra molecular hydrogen bonds. The bands at 2935 and 2887 cm^{-1} are attributed to the asymmetric stretching of C-H, while the band at 1656 cm^{-1} is due to the adsorbed water. The band at 1015 cm^{-1} is assigned to the C-O alcohol bond and the bands at 1421 and 1357 cm^{-1} may concern to the angular deformation of C-H bonds in starch molecule [10, 11]. The FTIR spectra of gelatin shows peaks at 3450 cm^{-1} and 3423 cm^{-1} due to -NH stretching of secondary amide, C=O stretching at 1700 cm^{-1} and 1640 cm^{-1}, -NH bending between 1550 cm^{-1} and 1500 cm^{-1}, -NH out-of-plane wagging at 670 cm^{-1}, and C-H stretching at 2922 cm^{-1} and 2850 cm^{-1} [12]. In the spectrum of HAp, the characteristic bands of ν_4 (PO_4^{3-}) is observed at 560-604 cm^{-1}. The weak band at 470 cm^{-1} is due to the υ_2 of the phosphate. ν_1 (PO_4^{3-}) can be seen at 960 cm^{-1} and the bands at 1030-1100 cm^{-1} assigned to the ν_3 (PO_4^{3-}). In phosphate network, bending and stretching modes of P-O vibrations are present as bands around 600 cm^{-1} and 1049 cm^{-1}, respectively. Besides of these spectra, a broad band concerning to the main vibration of ν OH^- at 3566 cm^{-1}, joined with the bands at 3400 and 1629 cm^{-1} (H-O-H) of water absorption in the products are observed [13, 14]. Comparison of the gained results show that, all the peaks obtained from composites spectra are matched well with the pure synthesized HAp as well as starch and gelatin spectra. The slight shift in the position of absorption bands for the HAp prepared in the presence of biopolymers is indicative of dissociation and interaction of polymer with the nucleating crystals [15].

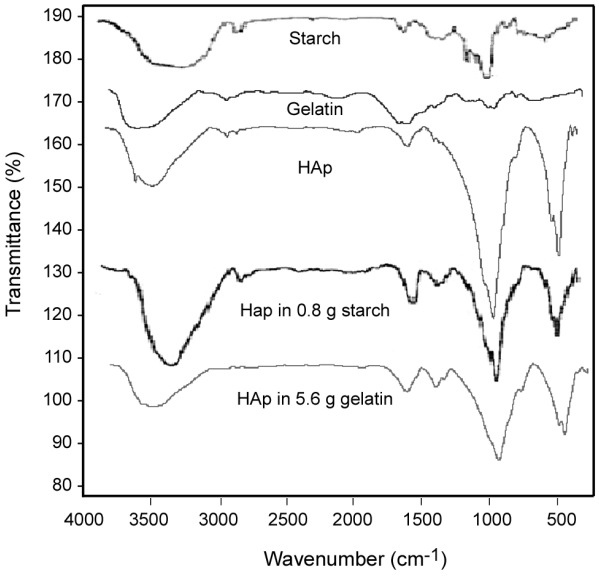


Figure 1: *FT-IR spectra of the amylopectin-rich starch and gelatin in comparison with the synthesized HAp in the absence and presence of 0.8 g starch and 5.6 g gelatin from top to bottom, respectively.*

3.2. X-ray Diffraction Results (XRD)

Figure 2 shows the X-ray diffraction patterns of the synthesized nano HAp in the presence and absence of the biopolymers. As shown in this figure, pure HAp and HAp in biopolymers matrix have similar XRD patterns. All the diffraction peaks can be assigned to monophase low crystalline HAp nanocomposite. It indicates that, using biopolymers have no effect on the change of crystallographic

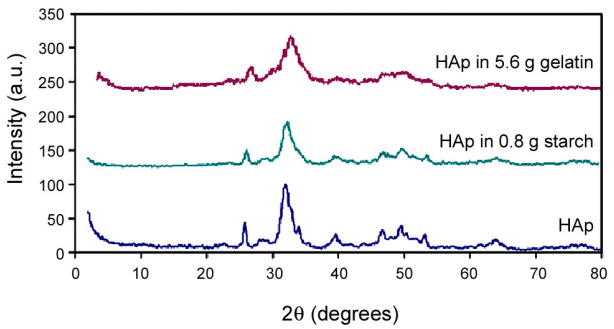
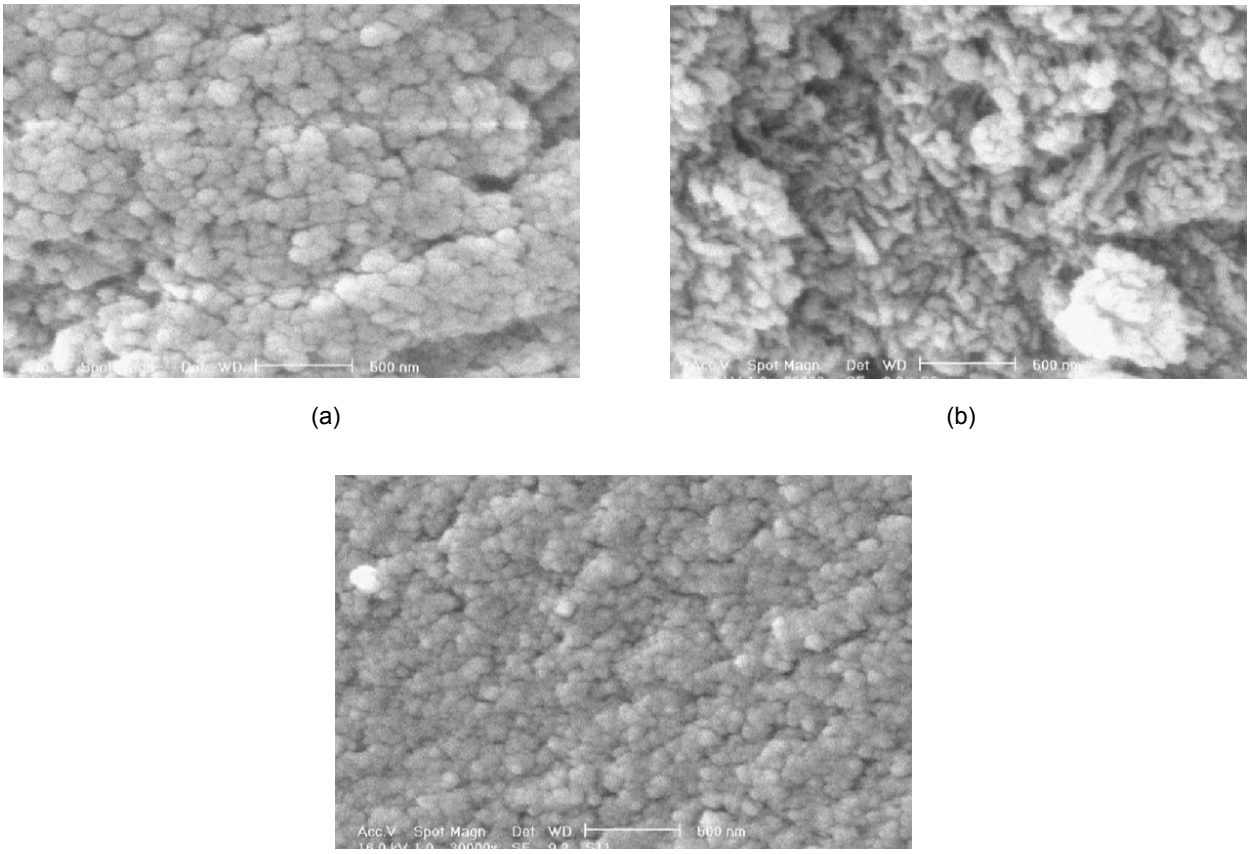


Figure 2: *X-ray diffraction patterns of the synthesized nano HAp in the absence and presence of the starch and gelatin biopolymers.*



(a) (b)

(c)

Figure 3: *SEM micrographs of nHAp prepared in the (a) absence and presence of (b) starch and (c) gelatin matrix.*

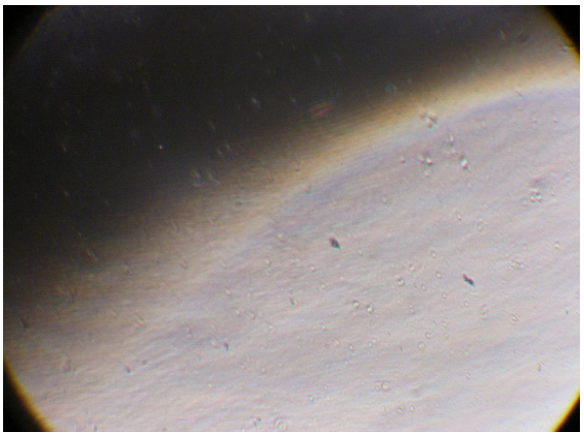
structure of the synthesized HAp in the polymeric matrixes. Broadening of the peaks in XRD patterns points out the small size and low crystallinity of the HAp. The poor crystalline nature of the prepared HAp may be due to the low temperature process operated in this work. The peaks of nanocomposites are slightly broader than pure HAp which can be a sign for decreasing the HAp size and crystallinity in the presence of biopolymer matrix [16].

3.3. Scanning Electron Microscopy (SEM)

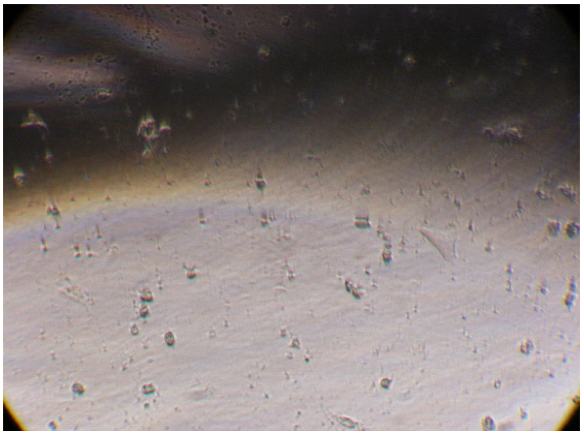
Figure 3a-c represents the SEM micrographs of nHAp prepared in the absence and presence of starch and gelatin matrix, respectively. Overall morphology for nHAp in Figure 3a shows regular distribution of spherical nanoparticles for the samples prepared in the absence of starch biopolymer. But for the nHAp samples synthesized at the presence of starch biopolymer (Figure 3b), irregular aggregates of spherical and rice form nanoparticles can be observed. This observation suggests that, the presence of starch has influence on the morphology of the product due to; the interaction of starch OH-groups with Ca^{2+} ions in the solution and finally formation of HAp nanoparticles occurred on the surface of starch matrix. In Figure 3c, the overall morphology of the samples seems to be regularly distributed spherical particles like nHAp in the absence of biopolymer. So, it seems that Gelatin has no effect on the morphology and crystal structure of formed HAp.

3.4. Cell Experiment

The morphology and behavior of BMSCs cultured in vitro with the n-HAp composites are observed under phase-contrast microscope and evaluated by MTT assays. Figures 4(a-c) and 5 (a-c) presents phase-contrast micrographs of cell attachment on



(a)



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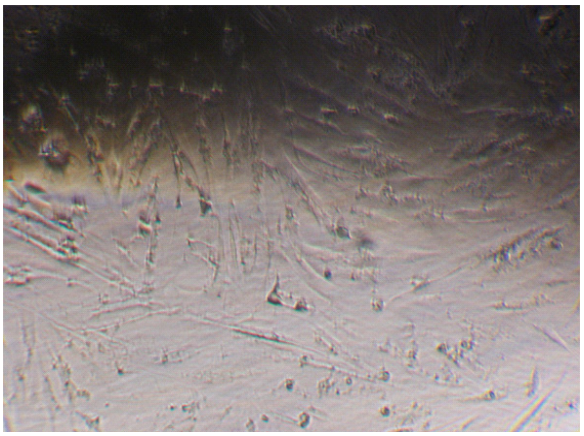
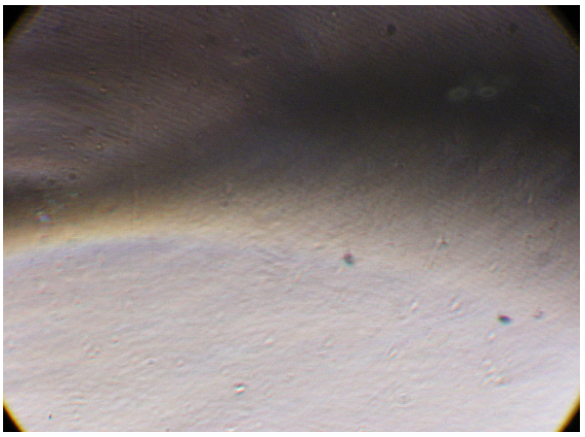
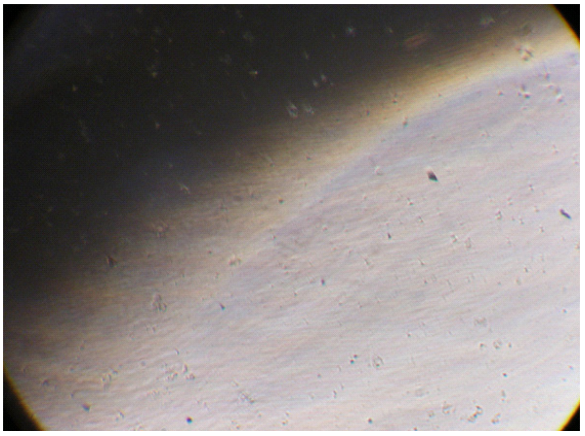


(c)

Figure 4: *Phase-contrast micrographs of cell attachment on the nHAp-0.8 g starch biocomposite after culture for (a) 1, (b) 3 and (c) 7 days.*



(a)



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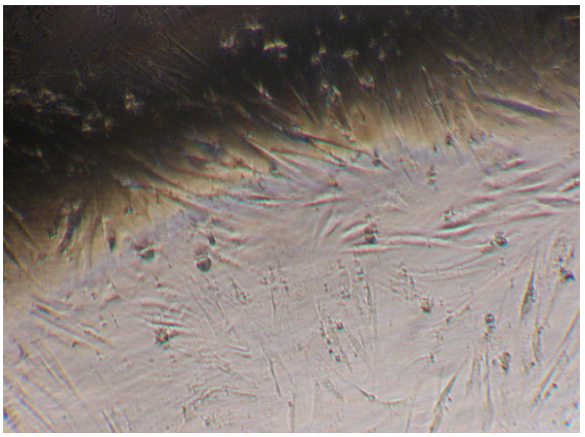

(c)

Figure 5: *Phase-contrast micrographs of cell attachment on the nHAp-5.6 g gelatin biocomposite after culture for (a) 1, (b) 3 and (c) 7 days.*

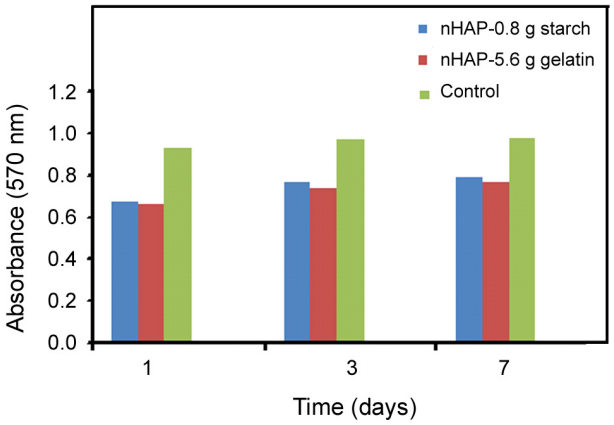

Figure 6: *MTT assays for proliferation of BMSCs combined with nHAp-0.8 g starch and nHAp-5.6 g gelatin cultured for 1, 3 and 7 days,compared with the control under the same culture condition.*

the nHAp-0.8 g starch and nHAp-5.6 g gelatin biocomposites after culture for 1, 3 and 7 days, respectively. As it can be seen at the first day, recognition of elongated fusiform shape BMSCs is difficult. At 3 days, a few BMSCs cells are present. At 7 days, a large amount of cells proliferate, forming a cell colony and fully attached to the biocomposite. Obviously, the nHAp-starch and nHAp-gelatin composites have no negative effect on the cell morphology, viability and proliferation. So, we can conclude that there is no remarkable difference between starch and gelatin from biocompatibility point of view.

In MTT assays nHAp-0.8 g starch and nHAp-5.6 g gelatin composites are used to culture with BMSCs for 1, 3 and 7 days, therewith a culture without biocomposite is used as a blank control group. From the data in Figure 6, the cell number increases with the culture time on all tested groups. At the first and third day, there are no significant differences between absorbance values of samples. The cells on biocomposites and control group starts to proliferate rapidly from 7 days. As can be seen, BMSCs cultured on of starch biocomposite have a little more proliferation compared with gelatin one in all time periods. We can conclude that both starch and gelatin composites are biocompatible.

4. CONCLUSIONS

We conclude that:
- nHAp composite synthesis can be performed at room temperature by a mimetic method using biopolymers as a templatting agent.
- Cell culture and MTT assays show that nHAp-starch and nHAp-gelatin composites have no cytotoxic effect on cells and possess good biocompatibility and there is no significant difference between these two polymers from biocompatibility point of view.

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