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Biocompatibility Evaluation of Nano Hydroxyapatite-Starch Biocomposites

M. Meskinfam[1,*], M. A. S. Sadjadi[2], H. Jazdarreh[1], and K. Zare[3]

[1] *Department of Chemistry, Lahijan Branch, Islamic Azad University, Lahijan 4416939515, Iran*
[2] *Department of Chemistry, Science and Research Branch, Islamic Azad University, Tehran 14778, Iran*
[3] *Department of Chemistry, Faculty of Science, Shahid Beheshti University, Tehran 61357831351, Iran*

In this work, we report the synthesis and characterization of nano hydroxyapatite (nHAp) in the presence of starch matrix via a biomimetic process for *in vitro* biocompatibility evaluation. Characterization of the samples was carried out using X-ray diffraction (XRD) and Fourier Transform infrared spectroscopy (FT-IR). The Size and morphology of the nHAp samples were determined using scanning and transmission electron microscopy (SEM and TEM). The results show that, the shape and morphology of nHAp is influenced by the presence of starch as a template agent. It leads to formation of rod-like nHAp. Cell culture and MTT assays were performed for *in vitro* biocompatibility. They show that n-HAp can affect the proliferation of cells and the n-HAp/starch biocomposites have no negative effect on the cell morphology, viability and proliferation.

Keywords: Starch, Biocomposite, Nano hydroxyapatite, Biocompatibility, Template.

RESEARCH ARTICLE

1. INTRODUCTION

The biomaterial requirements for clinical orthopaedics are being biodegradable and inducing and promoting new bone formation. They should provide temporary mechanical support. Both organic and inorganic phases are the main composition of the extra cellular matrices of hard tissues. The inorganic phase consists of hydroxyapatite (HAp) crystals and the organic phase consists of type-I collagen and small amounts of glycosaminoglycans, proteoglycans and glycoproteins.[1] HAp with the $Ca_{10}(PO_4)_6(OH)_2$ formula has compositional and biological similarities to the mineral phase of natural bone but its brittleness limits its use. One of the best methods to solve the problem is combination of the HAp with different biodegradable polymeric materials such as collagen, gelatin, chitosan and starch. Achieving different properties such as the capability of carrying functional groups, chelating to metal ions via functional groups and hydrophilicity can be obtained by tailoring the surfaces of organic materials.[2] So, in recent years, much interest has been directed to inorganic nanoparticles that are embedded in polymeric matrixes. Various methods have been carried out to prepare this type of composite to obtain the required properties and structures. Template—based synthesis is one of the most common methods.[3] This technique is based on the interaction between an 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.[4] One of the biopolymers with biomedical application is starch. This natural polymer is biodegradable, biocompatible, water soluble and inexpensive in comparison to other biodegradable polymers[5,6] and seems to be a good candidate in this subject. The nature of starch facilitates strong adhesion between the HAp and starch.

HAp and its composites owing to their structure and chemical compositions are suitable for attachment, proliferation and differentiation of stem cells. However, HAp also has some disadvantages, instability of the particulate HAp is often encountered when the particles are mixed with saline or patient's blood and hence migrate from the implanted site into surrounding tissues causing damage to healthy tissue.[7] In order to obtain intelligent biomaterials, a great deal of attention has been focused on the composites of HAp in conjugation with some synthesized and natural polymers via different methods, such as biocomposites,[8] biomineralization,[9] HA crystalline surface patterning,[10] surface modification[11] and biological self-assembly.[12]

In this work, bone-like nano HAp using different concentration of starch matrix via biomimetic method is

*Author to whom correspondence should be addressed.

prepared. Their characterization and *in vitro* biocompatibility using bone marrow stem cells are reported.

2. MATERIALS AND EXPERIMENTAL PROCEDURES

2.1. Materials

Extra pure water soluble wheat starch was obtained from Merck. All the chemicals needed for synthesis of HAp; $CaCl_2 \cdot 2H_2O$, 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 rods was carried out in the wheat starch matrix and pure nHAp was prepared in the absence of starch for comparison according to our previous report with a slight modification.[13] Briefly, different concentrations of starch (0.5 and 0.8 g of starch in 70 ml water) were prepared. For preparation of nHAp in the presence of starch, 10 ml calcium chloride 0.5 M was slowly added to the prepared biopolymer solutions and after stirring slowly for 4 h, 10 ml sodium dihydrogen phosphate 0.3 M was added little by little to the stirring mixture for 2 h. NH_4OH solution was added to control the solution pH at 10–10.5. HAp was synthesized in this manner by omission of the biopolymer step. Solutions obtained by this method (with and without biopolymer) were kept overnight at room temperature, filtered on a buchner funnel, and then washed with doubly-distilled water. The resultant precipitate was dried at 60 °C for 6 h in a vacuum oven.

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 n-HAp-starch biocomposites was measured by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenylte-2H-tetrazolium bromide) 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 MTT formazan, the absorbance of the contents of each well was measured at 570 nm with a spectrophotometer (Perkin Elmer Co.).

3. RESULTS AND DISCUSSION

3.1. X-Ray Diffraction Results (XRD)

Figure 1 shows the X-ray diffraction patterns of the synthesized nano HAp in the presence and absence of the starch. As shown in this figure, pure HAp and HAp in starch matrix have similar XRD patterns. All the diffraction peaks can be assigned to monophase low crystalline HAp. It indicates that using starch has no effect on the change of crystallographic structure of the synthesized HAp in the polymeric matrix. The peaks of nanocomposites are slightly broader than pure HAp which can be a sign of decreasing the HAp size and crystallinity in the presence of biopolymer matrix similar to natural bone mineral.[14]

3.2. Fourier Transform Infrared Spectroscopy (FT-IR)

Figure 2 represents FT-IR spectra of starch, the synthesized HAp in the absence and presence of 0.5 g, and 0.8 g starch from top to bottom, respectively. In the starch spectrum, the wide band observed at 3348 cm^{-1} is attributed to the O–H stretching 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

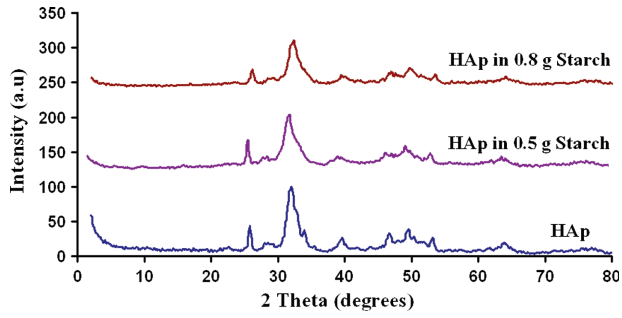

Fig. 1. XRD patterns of synthesized HAp in the absence of polymer, in the presence of 0.5 and 0.8 g Starch.

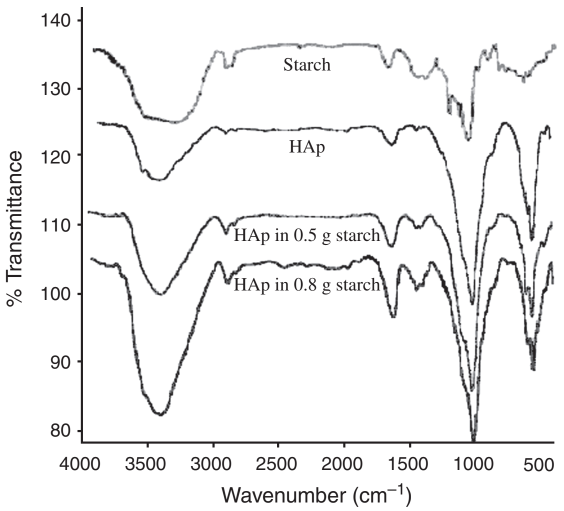

Fig. 2. FT-IR spectra of Starch, synthesized HAp in the absence of polymer, in the presence of 0.5 g and 0.8 g starch.

of C–H bonds in starch molecule.[15,16] In the spectrum of HAp, the weak band at 470 cm^{-1} is due to the υ_2 of the phosphate. The characteristic bands of $\upsilon_4(PO_4^{3-})$ are observed at 560–604 cm^{-1} while υ_1 (PO_4^{3-}) can be seen at 960 cm^{-1} and the bands at 1030–1100 cm^{-1} assigned to the $\upsilon_3(PO_4^{3-})$. In the phosphate network, bending and stretching modes of P–O vibrations are present as bands around 600 cm^{-1} and 1049 cm^{-1}, respectively. Besides these spectra, a broad band concerning the main vibration of υ 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.[17,18] All the peaks obtained from the HAp and starch samples (remained spectra) are matched well with the pure synthesized HAp as well as starch spectra. In fact, using biocompatible and biodegradable wheat starch as a template agent facilitates *in situ* precipitation of the nano HAp.

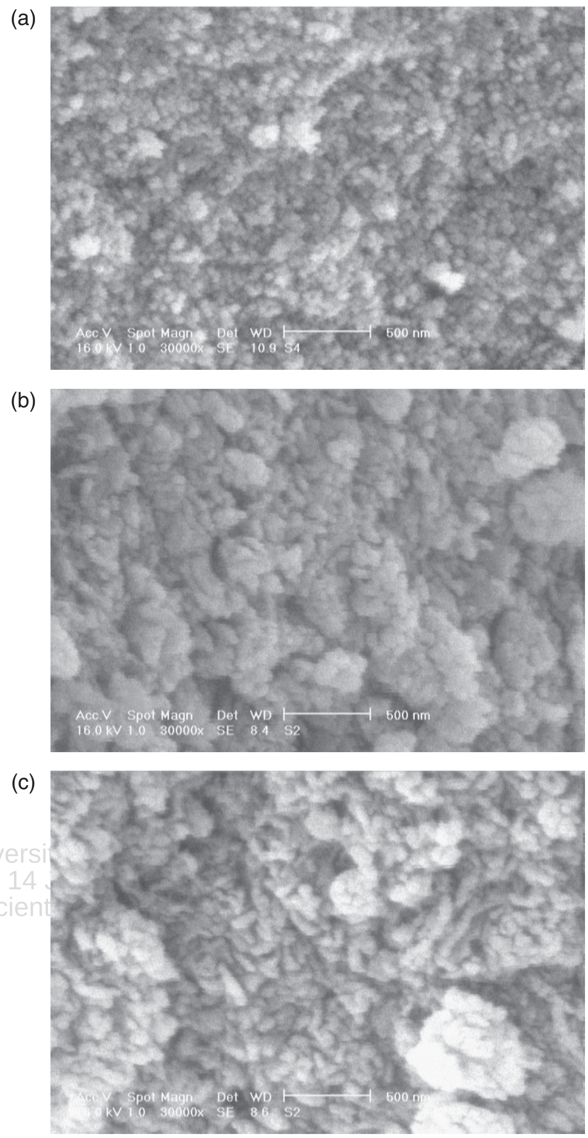

Fig. 3. SEM micrographs of synthesized HA (a) in the absence of polymer, in the presence of (b) 0.5 g starch and (c) 0.8 g starch.

3.3. Scanning Electron Microscopy (SEM)

Figures 3(a–d) represents the SEM micrographs of nHAp prepared in the absence and presence of starch matrix. Samples prepared in the absence of starch biopolymer shows a regular distribution of spherical nanoparticles of nHAp. But for the samples synthesized in the presence of starch biopolymer, 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 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.

3.4. Transmission Electron Microscopy (TEM)

Figure 4(a) shows the TEM images of HAp prepared in the absence of starch biopolymer. In this figure a mixture of some regular rods and hexagonal crystal forms of nHAp can be distinguished.

Figure 4(b) shows the morphology of HAp particles synthesized in the presence of 0.8 g starch template. This image, in comparison to Figure 4(a), confirms the influence of the starch template on the final morphology of nHAp. Rod like nHAp with about 6–12 nm width and 45–85 nm length similar to natural bone has been formed in the presence of starch.

3.5. Cell Experiment

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

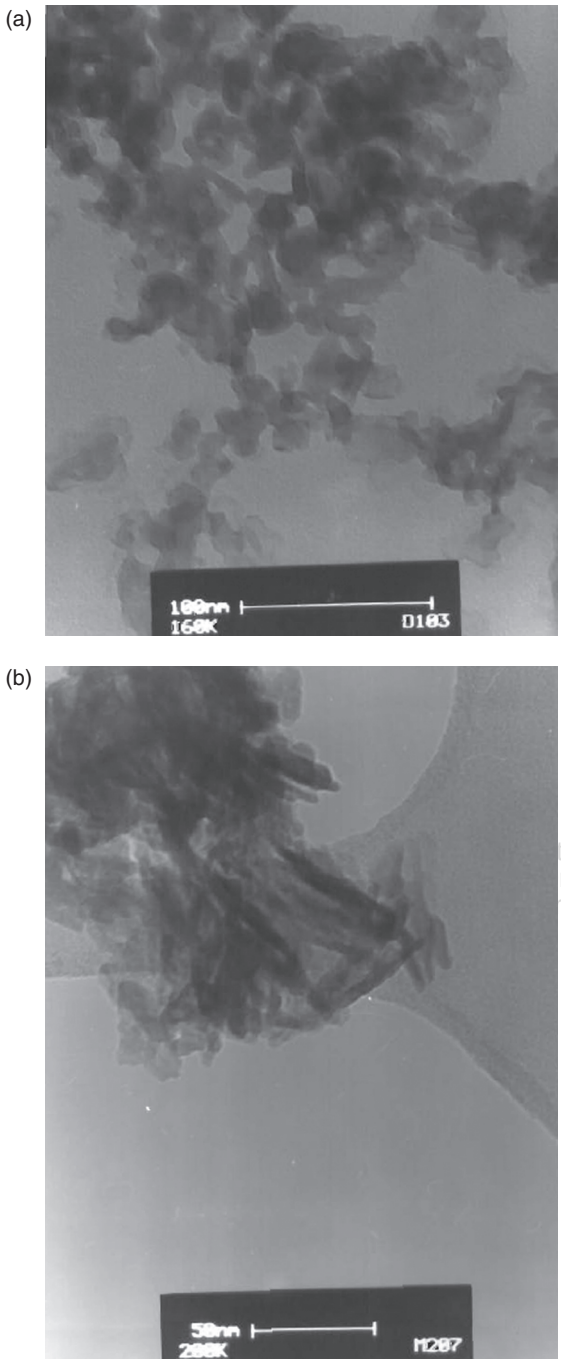

Fig. 4. TEM micrographs of synthesized HA (a) in the absence of polymer, and (b) in the presence of 0.8 g starch.

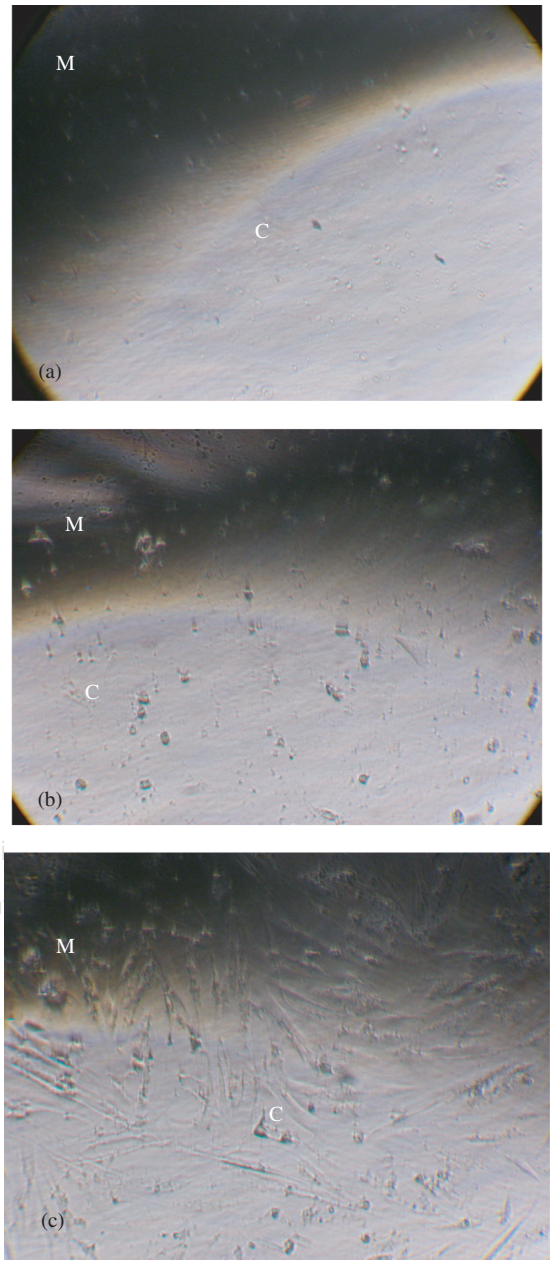

Fig. 5. Phase-contrast micrographs of the BMSCs (denoted as C) attached to n-HAp- 0.8 g starch (denoted as M) after *in vitro* culture for (a) 1, (b) 3 and (c) 7 days.

attachment on the n-HAp-0.8 g starch biocomposite after culture for 1, 3 and 7 days. 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 n-HAp-starch composite have no negative effect on the cell morphology, viability and proliferation.

In MTT assays starch, n-HAp-0.5 g starch and n-HAp-0.8 g starch 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 biocomposite have much more proliferation compared with starch in all time periods. The

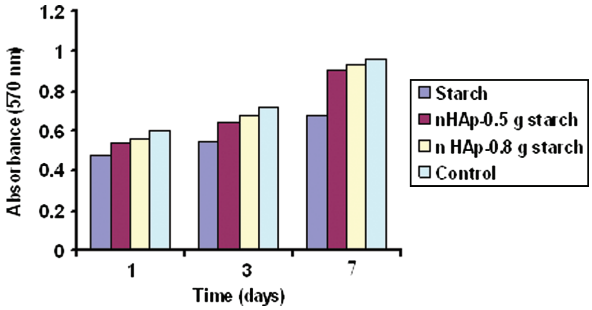

Fig. 6. MTT assays for proliferation of BMSCs combined with starch, n-HAp-0.5 g starch and n-Hap-0.8 g starch cultured for 1, 3 and 7 days, compared with the control under the same culture condition.

result shows that n-HAp has an obvious effect on the proliferation of BMSCs and there is no difference in cell proliferation of biocomposites because their nHAp content is the same. Investigation on the proliferation of BMSCs is an important technique to evaluate the biocompatibility of biomaterials *in vitro*.[19]

4. CONCLUSION

We conclude that

- nHAp composite synthesis can be performed at room temperature by a mimetic method using wheat starch as a templating agent.
- Controlling of the size and shapes of nHAp is possible by using starch as a template.
- Cell culture and MTT assays show that n-HAp-starch composite have no cytotoxic effect on cells and possess good biocompatibility.

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