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High Biological performance of Silicon Substituted Nano Hydroxyapatite Synthesized in Simulated Body Fluid at 37°C

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ABSTRACT

In this work, we report high biological performance of silicon substituted nano hydroxyapatite (nHA) prepared by immersion of calcium phosphate and sodium silicate as precursors in Simulated Body Fluid (SBF) solution for 24, 36, 48 and 72 hrs at 37°C. Characterization and chemical analysis of the synthesized powders were performed by Fourier transform, infrared spectroscopy (FT-IR), X-ray powder diffraction (XRD) and Dispersive x-ray analysis (EDAX). In vitro biocompatibility test was then carried out by using Bone Marrow Stem Cells (BMSCs) as seeding cells. The MTT assays revealed that, silicon substituted nHA enhance proliferation of the cells and cell cultures showed no negative effect on the cell morphology, viability and proliferation.

Keyword: Nano hydroxyapatite; Simulated body fluid; In vitro biocompatibility.

1. INTRODUCTION

Due to the high demand for synthetic biomaterials to assist and replace skeletal tissues, and the high failure rate of these medical implants, a great deal of research focused on improvement of the strength of implant-tissue interface, and in the design of implants that degrade in concert with the natural healing process [1]. To day, much attention has been paid to hydroxyapatite (HAp) because of its chemical and crystallographic characteristic similarity to the inorganic component of natural bone and has been extensively investigated due to its excellent biocompatibility, bioactivity and osteoconductivity [2]. The major improvement in the bonding between the implant and growing bone

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on the calcium hydroxyapatite (HAp) surface layers has been demonstrated in many studies [3,4]. In one of these works, important HAp in addition to these surface layers and species for promotion of bone growth in some form of silica/silicate species has been shown [5]. This kind of substitutions have influence on the solubility, surface chemistry, morphology of the material and demonstrate markedly increased biological performance in comparison to stoichiometric counterparts [6].

In this work, synthesis and characterization of the silicon substituted nHAp were performed using calcium phosphate and sodium silicate as precursors after immersion in Simulated Body Fluid (SBF) solution for 24, 36, 48 and 72 hrs at 37°C.

2. EXPERIMENTAL

Materials and methods

2.1. Materials

All the chemicals needed for synthesis of hydroxyl apatite and SBF solution; calcium phosphate $Ca_3(PO_4)_2$, sodium silicate Na_2SiO_3, NaCl, $NaHCO_3$, KCl, $K_2HPO_4.3H_2O$, $MgCl_2.6H_4O$, Na_2SO_4, $(CH_2OH)_3CNH_2$ and HCl were supplied from Merck and used without any further purification.

2.2. Methods

The SBF solution was prepared by dissolving appropriate amounts of reagent grade chemicals; NaCl, $NaHCO_3$, KCl, $K_2HPO_4.3H_2O$, $MgCl_2.6H_2O$, Na_2SO_4 and $(CH_2OH)_3CNH_2$ in distilled water and buffered with HCl to pH 7.4 at 37°C. Ionic composition of the prepared SBF solution was very close to the human blood plasma which has been given in table 1. [7-9].

Preparation of silicon substituted nHAp was carried out by suspending appropriate amount of calcium phosphate, $Ca_3(PO_4)_2$ (93.71 g) in above prepared SBF solution and added drop wisely 61.3 ml of sodium silicate solution, Na_2SiO_3 in 30 min. The mixture was stirred for 1 h and incubated for 24, 36, 48 and 72 hrs at 37°C. The samples obtained were finally filtered washed by double distilled water and then were dried. Characterization of the samples were performed by 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) and X-ray dispersive analysis (EDAX).

2.3. Cell culture experiments

2.3.1. Cells and matrix seeding

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

2.3.2. MTT assay

The proliferation of BMSCs cultured on silicon substituted nHAp samples after 24 h, 36 h, 48 h and 72 h aging in incubator at 37°C as well as BMSCs

Table 1: *SBF and human blood plasma ion concentration (10^{-3} mol L^{-1}).*

	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	H_2PO^{4-}	SO_4^{2-}
SBF solution	142.1	5.0	1.5	2.5	124.91	27.0	0.0	1.1	0.5
Blood plasma	142.0	5.0	1.5	2.5	103.00	27.0	1.0	0.0	0.5

cultured without any samples were measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylte-2H-tetrazolium bromide) assay. After seeding for 1, 3 and 7 days, the cells were incubated in 100 μL of MTT solution (0.5 mg/mL, 37°C/5% CO_2) for 3 hrs and removal of supernatants, 100 μL/well dimethyl sulfoxide (DMSO) was added and mixed. After complete solubilization of the MTT, formazan absorbance of the contents of each well was measured at 570 nm with a spectrophotometer (Perkin Elmer Co.).

3. RESULTS AND DISCUSSION

3.1. Fourier transform infrared spectroscopy (FT-IR)

Figure 1 represents FTIR spectra of the samples

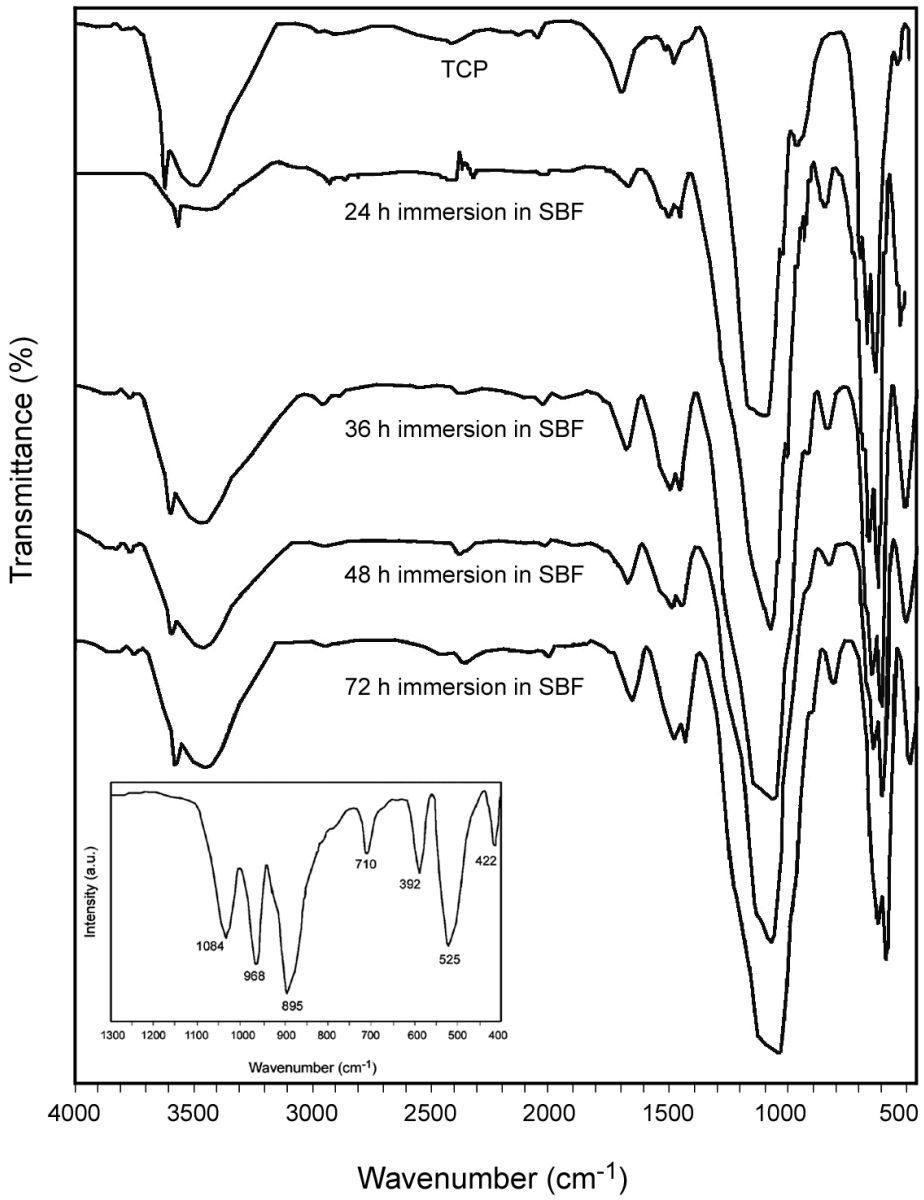

Figure 1: *FTIR spectra of: a) calcium phosphate precursor, b) nHAp obtained: after 24 h, c) 36 h, d) 48 h, e) 72 h soaking of TCP in SBF solution in an incubator, at 37°C compared with inset: FTIR spectrum of* Na_2SiO_3.

prepared after 24 h, 36 h, 48 h and 72 h soaking in SBF solution in comparison with the pure TCP. These spectra show the presence of functional groups such as phosphate and silicate groups. The characteristic absorption bands of phosphate appearing at 574, 607 and 962-1100 cm^{-1} in addition to the silica group characteristic can be observed for all immersed specimens. The peaks at 3571 and 630 cm^{-1} are due to vibrational bands of hydroxy group [10-12]. After immersion in SBF solution the spectrum of specimens show three new small peaks at 471 cm^{-1} (υ_2), 798 cm^{-1} (υ_4), and 1200 cm^{-1} (υ_3), which belongs to the bone-like apatite stretching bands of silicate. The bands at 471 cm^{-1} and the shoulder at 1200 cm^{-1} can be assigned to Si-O-Si bending mode and the shoulder at 798 is related to the Si-O-Ca vibration band [13-14]. These results show that, the apatite formed in SBF solution, is silicated apatite (Si-HAp) [15]. Note that, absorption bands observed in the range of 1300-1650 cm^{-1} are due to the stretching and bending modes of C-O and P-O bonds and air carbonate (CO3)2- ions [16-18].

3.2. X-ray diffraction results (XRD)

Figure 2 represents X-ray diffraction patterns of the calcium phosphate base and the prepared samples after 24, 36, 48 and 72 hrs immerssion of calcium phosphate and sodium silicate in SBF solution at 37°C. The diffraction peaks using PDF card # 00-009-0432, have been assigned to HAp. There were a few minor peaks which are corresponded to $Na_2Si_4O_9$ (PDF card # 39-0382). These results confirm co-existing formation of silica-hydroxyapatite (Si-HAp) in all of the cases after immersion of the samples in SBF solution. The diffraction peaks at 2θ values of 25.9° corresponding to (002) Miller plane, was selected for calculation of the crystalline size, as (002) peak is an isolated sharp peak with relatively high intensity. The mean crystallite size (D) of the particles was calculated from the XRD line broadening measurement using Scherrer

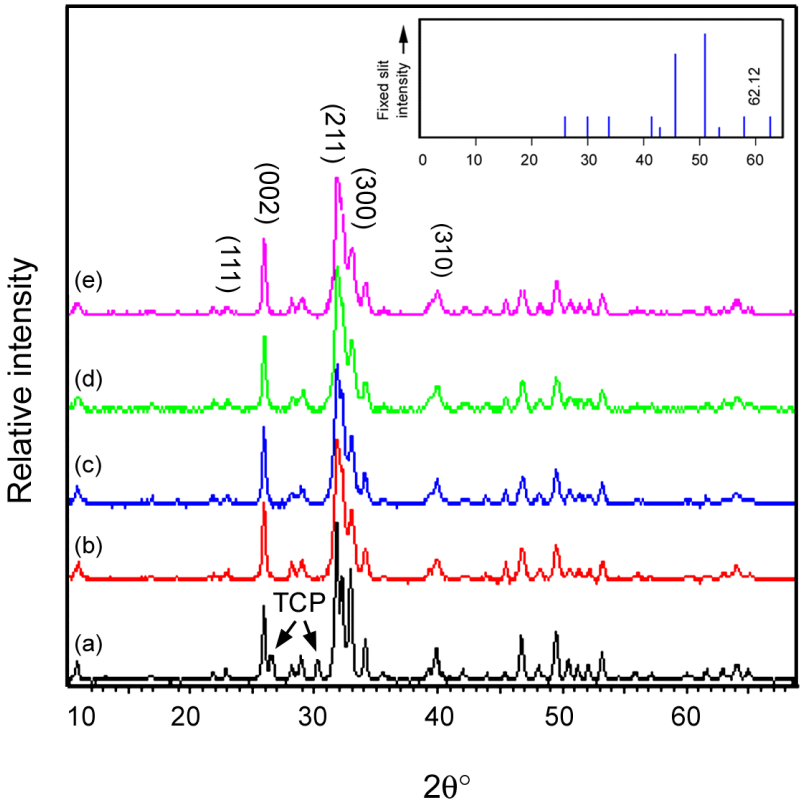

Figure 2: *XRD patterns of : (a) calcium phosphate precursor, formed nHAp after (b) 24 h (c) 36 h, (d) 48 h and (e) 72 h soaking in SBF solution in an incubator, at 37°C. Inset: $Na_2Si_4O_9$ pattern (PDF card # 39-0382).*

Table 2: *EDAX results of nHAp and nHAp after aging for 24 h, 36 h, 48 h and 72 h in incubator, at 37°C.*

Sample	P (%W)	O (%W)	Ca (%W)	Na (%W)	Si (%W)	Ca/P
HAp	26.354	21.727	51.919	0.050	0.0	1.9
After 24 hrs	18.720	38.879	33.493	1.298	7.610	1.7
After 36 hrs	16.994	46.257	28.166	1.545	7.039	1.65
After 48 hrs	18.113	31.689	42.017	1.155	7.026	2.3
After 72 hrs	17.228	43.977	30.790	1.049	6.956	1.7

equation [19]:

$$D = \frac{0.89\lambda}{\beta \cos\theta}$$

Where, λ is the wavelength (Cu; Kα), β is the full width at the half maximum of the HAp (002) line and θ is the diffraction angle. The average crystallite size calculated for the samples before and after soaking of the samples in SBF solution were 17.3nm, 18.1nm, 16.1 and 16.9 nm and 16.5 nm respectively. These periodic increase and decrease of calculated D values confirm bioactivity of the prepared samples and formation of a bone-like apatite layer on the surface of TCP or HAp and successive interactions between surface of the prepared HAp samples with SBF solution [4].

3.3. Dispersive X-ray analysis (EDAX) and TEM micrographs

Table 2. shows EDAX data of the above cited samples before and after soaking of n-HAp in SBF solution. Decreasing of Ca/P ratio in the samples obtained after soaking for 24 hrs and 36 hrs to 1.7 nm and 1.65 nm respectively from 1.9 nm and its consecutive increase and decrease of the Ca/P ratio confirm bioactivity and biodegradability of the prepared HAp in the presence of SBF solution containing silica ions. This result also shows also that, the best condition for formation of a bioactive and biodegradable HAp samples with an appropriate Ca/P ratio with hydroxyappatite can be realized by soaking n-HAp and silica ions in BSF solution after 36 hrs only. This results was in conformity with our earlier reported TEM micrographs of HAp nanocrystals formed after 24 h soaking of TCP in silica-containing SBF solution (Figure 3). The HAp grain size was about 35 nm width and 80 nm length [4].

3.4. Cell experiments

The morphology and behavior of BMSCs cultured in vitro with the TCP and silicon substituted n-HAp formed after aging for 36 hrs were observed under phase-contrast microscope and evaluated by MTT assays. Figure 4a-c presents phase-contrast micrographs of the cell attachment on TCP after culturing for 1, 3 and 7 days. Recognition of elongated fusiform shape of BMSCs, in the first day was so hard. After 3 days, a few BMSCs cells and after 7 days a relatively large number of proliferated cells that form cell colony were attached to TCP.

Figure 4d-f shows phase-contrast micrographs of cell attachment on the formed nHAp aged for 24 h for 1, 3 and 7 days. At the first day, recognition of elongated fusiform shape BMSCs is so hard. At 3 days, a few BMSCs cells were present and form cell colony and after 7 days a large amount of cells profilated and fully attached to the formed nHAp.

For MTT assays, TCP and silicon substituted nHAp formed after soaking for 24 h, 36 h, 48 h and 72 h were used to culture of BMSCs for 1, 3 and 7 days in an incubator at 37°C, therewith a culture without anything is used as blank control group. The cell number which increases with the culture time have been observed for all the tested groups based on the results obtained (Figure 5).

This Figure also shows also that, the number of cells for nHAp obtained after soaking for 36, 48 and 72 hrs have been increased with the culture time compared to TCP. But the cells on silicon sub-

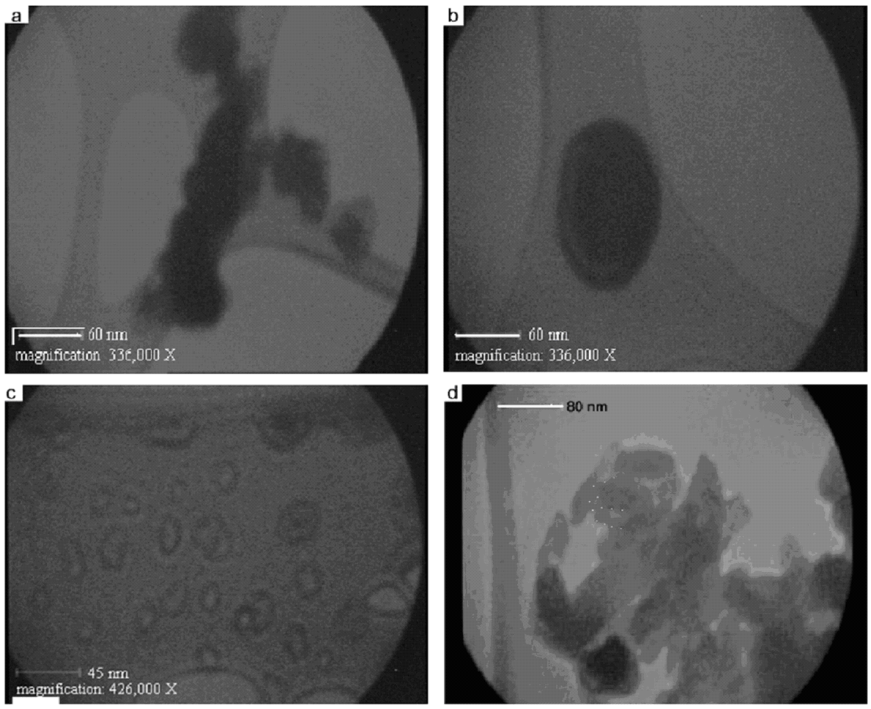

Figure 3: *TEM photographs of HAp nanocrystals formed after 24 h soaking of TCP in silica-containing SBF solution. The HAp grain size was about 35 nm width and 80 nm length.*

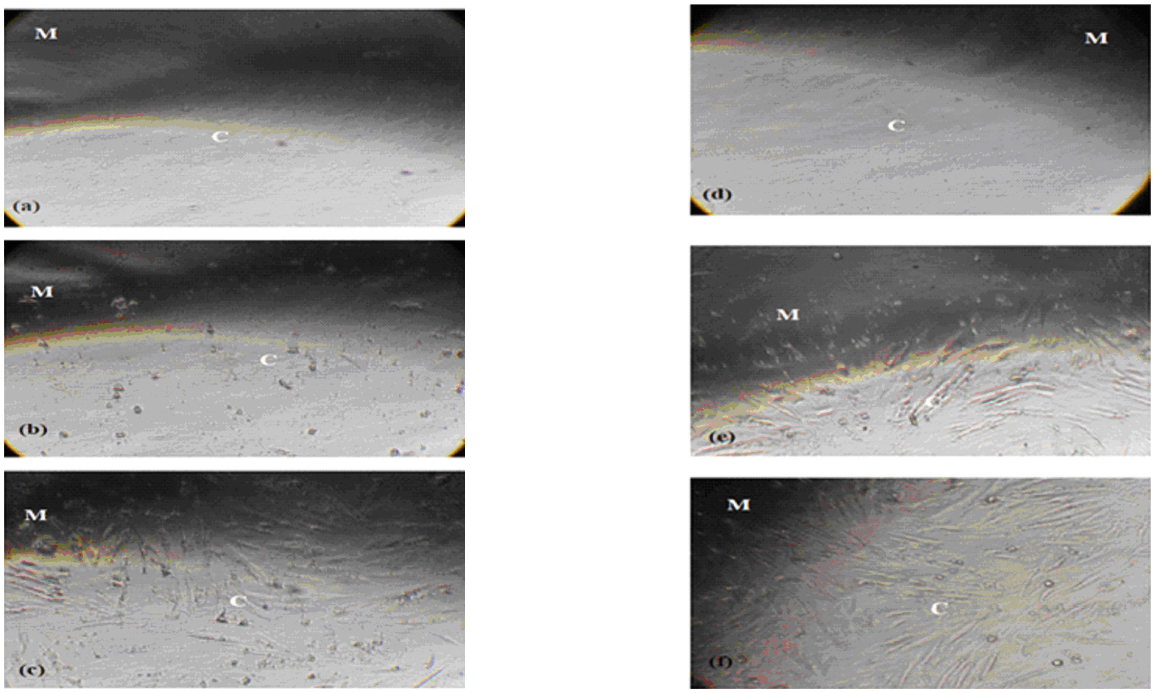

Figure 4: *Phase-contrast micrographs of the BMSCs (denoted as C) attached to TCP (denoted as M) after in vitro culture for 1day (a), 3 days (b) and 7 days (c) and BMSCs (denoted as C) attached to the nHAp obtained by aging for 24 h (denoted as M) after in vitro culture for 1day (d), 3 days (e) and 7 days (f).*

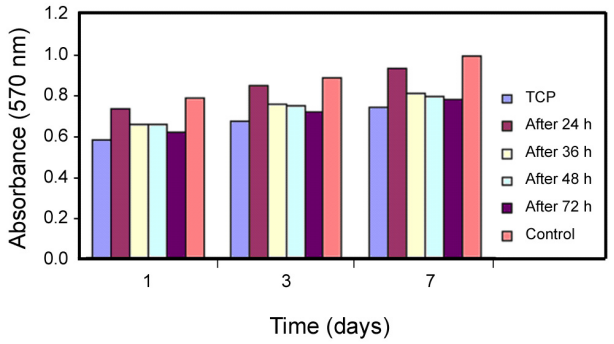

Figure 5: *MTT assays for proliferation of BMSCs combined with TCP and the silica containing nHAp after aging for 24 h, 36 h, 48 h and 72 h in incubator at 37°C, cultured for 1, 3 and 7 days, compared with the control under the same culture condition.*

stituted nHAp obtained after soaking for 24 h proliferate rapidly compare to the remained items in all time periods. This result shows that, increasing of the silicon content in the prepared samples enhance proliferation of the cells and cell cultures showed no negative effect on the cell morphology, viability and proliferation on BMSCs.

4. CONCLUSIONS

- Nano-sized hexagonal and bioactive silicon substituted HAp crystals can be prepared successfully in SBF solution at 37°C, using calcium phosphate, $Ca_3(PO_4)_2$ and sodium silicate, Na_2SiO_3 as precursors.
- Bioactivity and biodegradability enhancement of the synthesized HAp samples can be enhanced in the presence of silica ions.
- Biocompatibility enhancement of the silica substituted HAp samples can be related to the silica contents increase.

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