Hydroxyapatite Nanocrystal Deposition on Plasma Modified Titanium Surface

Yeong-Mu Ko[1], Kyo-Han Kim[2], Byung-Hoon Kim[1,*]

[1] *Department of Dental Materials, School of Dentistry, MRC center, Chosun University,*
309 Pilmun-daero, Donggu, Gwangju, 501-759, South Korea
[2] *Department of Dental Materials, School of Dentistry, Kyungpook National University,*
2177 Dalgubeol-daero, Jung-gu, Daegu, 700-412, South Korea

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) is materials mainly known for its special ability to contact bone tissue. Nanostructures on implant surfaces, a coating composed of nano-HAp particles on Ti, have aroused increasing research interest in the biomedical field. In this study, we prepared HAp nanocrystal coated Ti surface by plasma surface modification and wet chemical method and then evaluated biological behavior of MC3T3-E1 on the HAp coated on plasma modified Ti surface. Nano-size crystals of sintered HAp were uniformly coated on polyacrylic acid (PAA) deposited Ti surface through the ionic interaction between calcium ions on the HAp nanocrystal and carboxyl groups on the PAA/Ti. *In vitro* cell tests revealed surface modification of Ti surface with HAp nanocrystal significantly improved the proliferation and growth of the osteoblastic MC3T3-E1 cells and induced them to differentiate at an enhanced level.

1. INTRODUCTION

Commercial pure titanium (CP-Ti) and Ti alloys are widely used as load-bearing dental and orthopaedic implant materials due to their superior biocompatibility, appropriate mechanical properties and high corrosion resistance in physiological environment [1, 2]. However, being bio-inert metallic implant, they cannot bond to living bone directly after implantation into host body [3]. To overcome this drawback, hydroxyapatite (HAp) has been applied as a coating material on Ti implant for hard tissue applications because of its chemical similarity to the inorganic component of human bone, capability of conducting bone formation and strong affinity to the surrounding bone tissue [4-6]. The plasma spray technique is currently the only method commercially available for coating metallic surface [7, 8].

Recent studies have shown that cells in the human body are predisposed to interact with nanostructured, such as surface of nanoscale roughness [9] and surface with immobilized nanoparticle [10]. Thus nanostructures on implant surfaces, a coating composed of nano-HAp particles on Ti, have aroused increasing research interest in the biomedical field.

To improve the biological properties of HAp coating on biomaterials, several studies have explored and examined the approach of SAM (self-assembled monolayer)–assisted HAp coating of Ti implant surfaces [11]. Plasma polymerization is a polymer thin film-forming process and the growth of low-molecules into high molecules occurs with the assistance of the plasma energy [12]. Puleo et al. found that surface modification of Ti surface by plasma polymerization of allyl amine is useful for the immobilization of bioactive molecules such as BMP-4 [13]. In particular, hydrophilic coatings with a tunable surface density of COOH groups have been investigated as cell adhesives [14] and bone like apatite formation in simulate body fluid (SBF) solution [11].

In this study, we prepared HAp nanocrystal coated on Ti surface by plasma surface modification and then evaluated biological behavior of MC3T3-E1 on the HAp/Ti surface. HAp nanocrystal coated layers were examined at the surface of plasma polymerization modified Ti with acrylic acid (AA).

2. MATERIALS AND METHOD

2.1 Materials

Commercially Ti disks (NSC, Japan) with grade 2 (10mm diameter) were used substrates and mechanically polished by utilizing 100 grit emery paper down to 1200 grit emery paper. After polishing, the discs were washed thoroughly with distilled water and sonication for 10 min in ethyl alcohol and then drying in vacuum oven at 60 ℃.

2.2 Synthesis of nanocrystal hydroxyapatite

The HAp nanocrystal were prepared by wet chemical method using $Ca(NO_3)_2 \cdot 4H_2O$ (98.5%, Samchon Chemical, Korea) and $(NH_4)_2HPO_4$ (99.0%, Samchon Chemical, Korea) Ca and P precursors, respectively. To precipitates stoichiometric HAp, 15 mM aqueous solution was slowly added drop by drop to 25 mM aqueous solution of $Ca(NO_3)_2$. The minimum pH was adjusted to 10 by adding concentered NH_4OH using an injection syringe. The rotation speed of stirrer was adjusted to 500 rpm and reaction temperature was 25 ℃. The resultant precipitate was aged for 24 h under stirring at the same speed. After aging, the obtained white precipitate was filtered, washed four to five

* Kim5055@chosun.ac.kr

times with distilled water until the aqueous medium was reduced to almost 7.0 and then drying in vacuum oven at 60℃. As synthesized powder was calcined at 800℃ for 1 h in stagnant air uisng a heating rate of 5℃/min.

2.3 Polymeric thin films onto the Ti surface by plasma polymerization

Ti surface modification was carried out by depositing an ultra-thin polymeric layer containing functional groups on the film surface through plasma polymerization using acrylic acid and using radio frequency (RF) plasma device (MINI PLASMA STATION, Korea). The Ti substrates were pretreated with Ar gas at a flow rate of 50 sccm and a plasma power of 200 W for 5min. After the pre-plasma treatment, plasma polymerization carried out at a discharge power of 50 W for 1 min and 200 mtorr working pressure with monomer (AA) evaporation.

2.4 HAp nanocrystal coating on PAA deposited Ti surface

Polyacrylic acid (PAA) deposited Ti surface were treated with alkali (pH, 10: adjusted with 25% ammonia solution) for 1h at room temperature in order to introduce carboxyl groups on the PAA deposited Ti (PAA/Ti) surface, washed with water and then dried reduced pressure. The alkali-treated and dried PAA/Ti were washed with ethanol and immersed in a 1.0% HAp ethanol dispersion for 1 h at room temperature under stirring. The HAp-coated PAA/Ti was washed five times with ethanol under sonication for 3 min and dried under reduced pressure.

2.5 In vitro cell test

MC3T3-E1 (ATCC CRL-2593) cells, a clonal pre-osteoblastic cell line derived from newborn mouse calvaria, were cultured in α-modified minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and a penicillin–streptomycin solution (100 units/ml penicillin and 100 units/ml streptomycin). All procedures were carried out at 37 ∘C in a CO_2 incubator (MCO-15 AC, Sanyo Electric Co. Ltd.) containing 5% CO_2 mixed gas.

The proliferation of osteoblast cells on substrates was examined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to a purple formazan product, Sigma-Aldrich Co.) assay after 1, 4, and 7 days of culture. All samples were placed into a 24-well plate and seeded with a density of 2×10^5 cells/ml. MTT assay was described previously [15].

The level of osteogenic differentiation induced by the ALP (alkaline phosphatase) activity of the MC3T3-E1 cells plated at 2×10^5 cells/well in 12-well plates at 6 and 12 days on each sample was assayed using a standard ALP test procedure. The ALP activity in the cell lysates was determined by measuring the level of p-nitrophenol (p-NP) released form disodium p-nitropheny phosphate (p-NPP). ALP assay was described previously [16].

3. RESULTS AND DISCUSSION

3.1 Characterization of Hap nanocrystals

The precipitation reaction for HAp nanocrystals was carried out following an idealized stoichiometric equation as described below:

$$10Ca(NO_3)_2 + 6(NH_4)_2HPO_4 + 8NH_4OH \rightarrow$$
$$\rightarrow Ca_{10}(PO_4)_6(OH)_2 + 6H_2O + 20NH_4NO_3$$
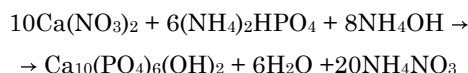
The HAp nanocrystal were prepared by the wet chemical process, and used after sintering at 800 ℃ for 1h. Fig 1 shows the XRD pattern of the nanoparticle after sintered high crystalline HAp. The peak are clearly indicative of HAp were seen throughout the pattern with 2θ values the that correspond closely to those observed in the International Centre Diffraction Data (ICCD) file # 09-0432 for HAp. The four strongest peaks are observed 25.9, 31.7, 32.1 and 32.8 ˚ 2θ dominates the diffraction pattern. Other calcium phosphate phases and thermally decomposed products such CaO and $Ca(OH)_2$, were not detected. The number-average size measured for SEM observations were 75 nm. The Ca/P ratio of the HAp nanocrystal (Ca/P = 1.7), measured with an energy dispersive spectrometer, was larger than that of stoichiometric HAp (Ca/P = 1.67).
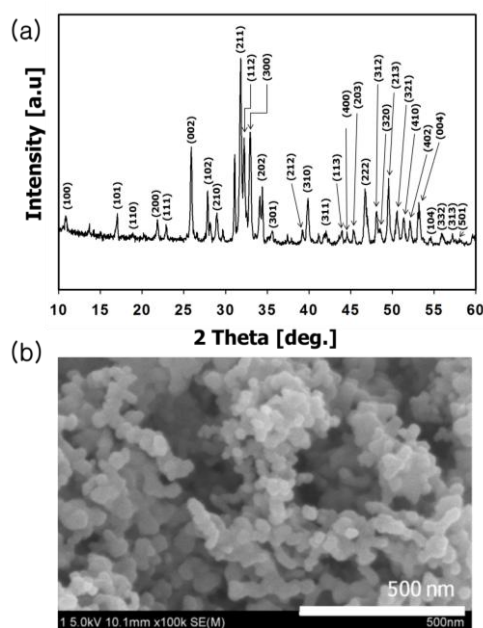

Fig. 1 – Powder XRD pattern and (b) FE-SEM image of HAp nanocrystal after sintering at 800 ℃ for 1h

3.2 Characterization of HAp nanocrystals

In general, the morphology of polymeric thin films deposited by plasma polymerization can be controlled by main parameters of deposition such as monomer and RF power. The surface morphologies of the HAp coating on the PAA/Ti surface are shown in Fig 2. The polished Ti surface had some machining grooves (Fig. 2-a). After plasma polymerization of the Ti at 50 W, a globular shape polymer layer was formed the Ti surface

(Fig. 2 -b). Fig. 2 c-d shows the HAp nanocrystal coated PAA/Ti surface after ultrasonic washing in ethanol. PAA/Ti surface was uniformly covered with spherical nanoparticle. The HAp coating method developed here can finely control the surface morphology by using pre-synthesized HAp nanocrystal having controlled morphologies as the coating agent.
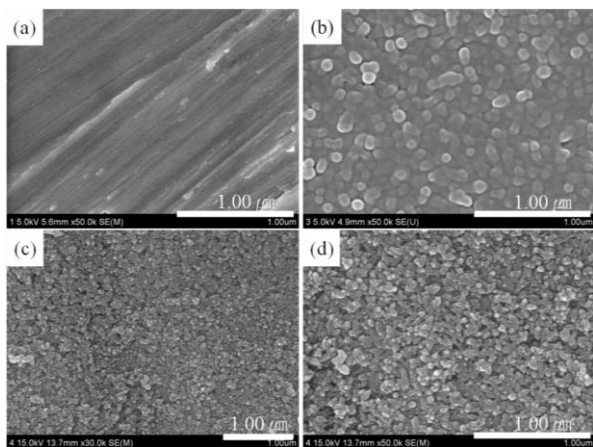

Fig. 2 – FE-SEM images of (a) untreated Ti, (b) PAA/Ti, (c and d) HAp/PAA/Ti surface.

The thin film (TF)-XRD pattern of the CP-Ti and HAp coated PAA/Ti surface were depicted in Fig 3. All of the diffraction peaks were similar to Fig. 1 (a) and no other impurity phase was detected. However, there was obvious broadening and overlap of these diffraction reflections in the XRD pattern. For example, the (211), (121) and (300) peaks of apatite were merged in to one broad peak centered at about 32˚. These results implied that the as-coated HAp was poorly crystallized and that their coated layer was very thin.
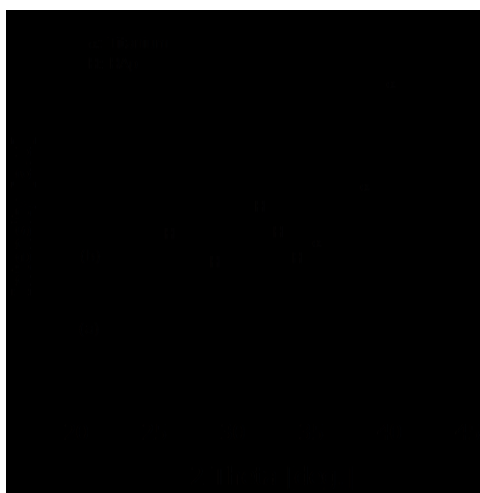

Fig. 3 – TF-XRD patterns of (a) CP-Ti and (b) HAp/PAA/Ti surfaces.

The carboxyl group interacts with the calcium ions on HAp through ionic interactions [18]. In order to bond the HAp nanocrystal on the PAA on Ti, carboxyl group were introduced on the PAA deposited Ti surfaces by alkaline hydrolysis of the PAA main chains, because the number of carboxyl groups on the surface of PAA is small.

The ionic interaction between PAA and HAp was estimated from FT-IR measurement of the PAA molecules which adsorbed on the HAp nanocrystal (Fig. 4). Although direct measurement of the interaction between solid-state PAA deposited Ti surface and HAp nanocrystal was also conducted, the interaction was not clearly observed, which should be because the ratio of carboxyl end groups on the surface of the PAA deposited Ti to those layer is too thin film. The spectrum of PAA layer showed a major peak at 1757 cm^{-1}, attributed to C-O stretching vibrations of the ester groups in its main chain. In spectrum of the HAp coated PAA/Ti sample, a new broad peak appeared the P-O stretching vibrations 960 and 1030 cm^{-1}. The broad absorption band peak from 1600 to 1650 cm-1, attributed to stretching vibrations of ionized carboxyl group interacting with Ca ions on the HAp on the surface.
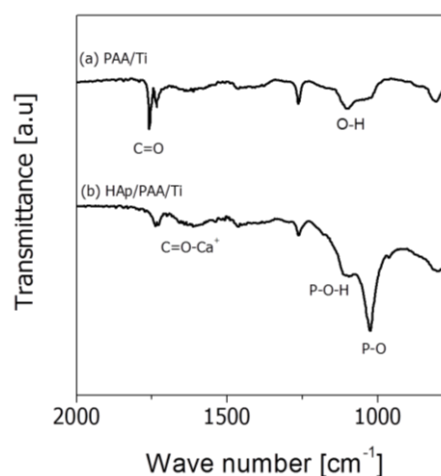

Fig. 4 – FT-IR spectra of (a) PAA/Ti and (b) HAp/PAA/Ti surfaces
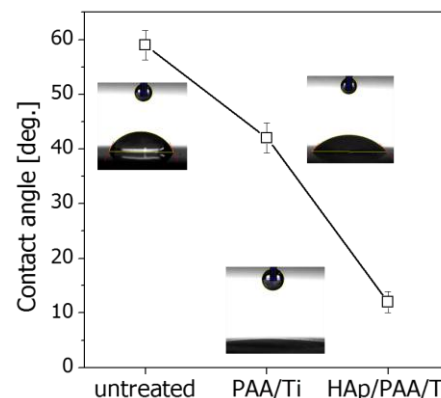

Fig. 5 – Contact angle values of water droplets measured on the different surface treatment.

In the case of biomaterials, the wettability (hydrophobicity/hydrophobicity) of its surface is considered to be one of the critical factors determining tis biological performance. The appearance of the water droplet on different samples surfaces and contact angle results are shown in Fig. 5. The untreated Ti had a contact angle of $59 \pm 2.6°$. PAA itself is known to have good hydrophilic properties, showing a contact angle of $47 \pm 2.7°$. The HAp coated on PAA/Ti samples, with contact angle of less than 15° are more hydrophilic than the others.
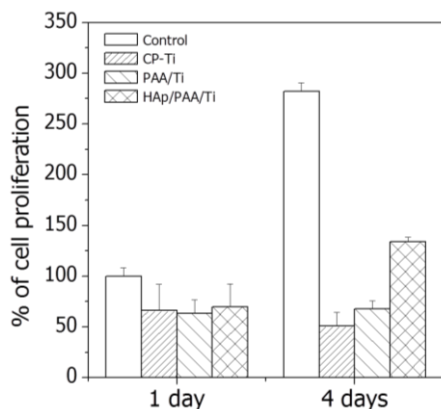
Fig. 6 – The results of MTT assay for MC3T3-E1 seed on different surface conditions
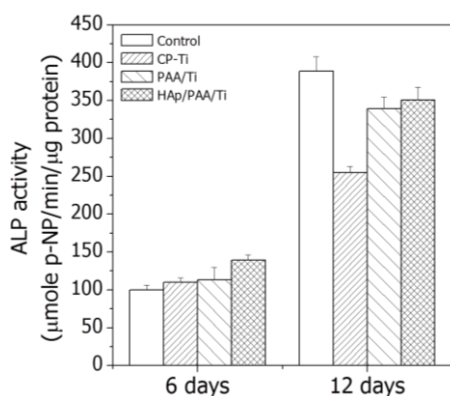

Fig. 7 – ALP activity of MC3T3-E1 cells cultured on different surface conditions for 6 and 12 days

Fig. 6 shows cell proliferation measured by MTT assay after 1 and 4 days of culture on the untreated CP-Ti (negative control), PAA/Ti and HAp coated PAA/Ti surfaces in comparison with cell culture plastic dish (positive control). For control and all experimental groups, the results show an increase in cell proliferation indicating cell proliferation with culture time. After 1 day of culture, the cell proliferation of PAA/Ti and HAp/PAA/Ti surface were slightly different compared with untreated CP-Ti surface. After 4days of culture, cell proliferation appeared similar for untreated CP-Ti and PAA/Ti groups significantly superior for HAp/PAA/Ti groups compared with experimental groups. This was probably due to improvement in the surface wettability afforded by the incorporation of HAp nanocrystal, as demonstrated in Fig. 5.

To investigate the effect of the HAp coated surface on the cell differentiation behavior, the ALP activity was measured by culturing MC3TC-E1 cell on the different experiment groups in comparison with cell culture plastic dish (positive control). ALP activity of control and all experimental groups increased with culture time. After 6 days of culture, ALP activities were slightly higher on HAp/PAA/Ti than on controls and PAA/Ti groups. After 12 days, HAp/PAA/Ti surface exhibited a significantly higher ALP level than the untreated CP-Ti and PAA/Ti surface.

Based on these cellular results, we confirmed that the surface modification of Ti surface with HAp nanocrystal significantly improved the adhesion and growth of the osteoblastic MC3T3-E1 cells and induced tem to differentiate at an enhanced level.

4. CONCLUSION

A novel technique for coating plasma polymerized Ti with sintered HAp nanocrystal was developed. The coating involved three steps: (1) preparation of sintered HAp nanocrystal; (2) PAA deposited on Ti by plasma polymerization; (3) adsorption of the HAp nanocrystal on the PAA/Ti surface. The surfaces of the PAA/Ti were uniformly coated with HAp nanocrystal through the ionic interaction between the sintered the calcium ions on the HAp nanocrystal and the carboxyl groups on the PAA/Ti.

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