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Biocompatibility of Hydroxyapatite Films Formed on Various Metals by a Surface Treatment Using an Enzyme Reaction

J. Nozaki, H. Uchida, K Itatani, I. Okada, and S. Koda
Sophia University, 7-1 Kioi-cho, Chiyoda-ku, Tokyo, 102-8554, Japan
M. Aizawa
Meiji University, 1-1-1 Higashi-Mita, Tama-ku, Kawasaki-shi, Kanagawa, 214-8571, Japan
Fax: 81-44-934-7906, e-mail: mamorua@isc.meiji.ac.jp
M. Matsumoto, H. Matsumoto, and Y. Toyama
Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan

Hydroxyapatite (HAp) films were directly formed on various metals from the simulated body fluid (SBF) by a simple surface treatment using an enzyme reaction of urea and urease. Pure Ti, Ti6Al4V alloy, stainless steel (SUS316L), and Co-Cr alloy were selected as substrates to the present investigation. From the results of TF-XRD and FT-IR, it could be confirmed that all the substrates were coated with carbonate-containing apatite. The SEM observation revealed that the morphologies of HAp particles covering substrates depend on the kinds of the metals. Osteoblastic cells (MC3T3-E1) seeded on all the substrates showed good proliferations in similar manners to the control, polystyrene plates for cell culture. No inflammatory reaction was detected for HAp-coated Ti rods when implanted in subcutaneous tissue of rats. A large amount of newly formed bone was observed around the HAp-coated Ti rods implanted in rabbits' tibiae, which bonded directly to the implants. Both *in vitro* and *in vivo* evaluations demonstrate that the present HAp-coated Ti substrate has excellent biocompatibility. The other metals with HAp also showed good biocompatibility from the results of cell-culture tests using osteoblastic.
Key words: Hydroxyapatite(HAp), Coating, Biocompatibility, *In vitro* evaluation, *In vivo* evaluation

1. INTRODUCTION

Composites of hydroxyapatite (HAp) with high-strength materials have been attempted for an improvement of mechanical properties of HAp ceramics as biomedical devices. Coating is one of the most popular processes to solve this problem.

A plasma-spray technique has widely been applied to artificial roots of tooth and joints [1]; however, it is known that formation of by-product make a controversial impact on human bodies. Therefore, development of the various types of coating processes has been reported as follows: sputtering [2], electrophoresis [3], biomimetic coating using the A-W glass and the simulated body fluid [4], dipping-pyrolysis [5], and spray-pyrolysis [6-8].

Kokubo et al. have reported that materials which precipitate HAp when soaked into a simulated body fluid (SBF), have an excellent bioactivity and directly bond to living bones [9]. Therefore, the SBF has been widely used for evaluations of bioactivities of different materials. We have also reported that HAp films could be formed on pure Ti substrates by a simple surface treatment utilizing SBF containing urea and urease under very mild conditions similar to that of human bodies [10,11].

The aim of the present investigation is to clarify whether if the above-mentioned coating process can be applied to the other metals or not, and to examine its biocompatibility using both MC3T3-E1 cells as an osteoblast model and rats/rabbits as animal models.

2. METHODS

2.1 Metals

Commercially available pure Ti (CP-Ti; the Nilaco Corpolation, Tokyo, Japan), Ti-6Al-4V, stainless steel (SUS316L; the Nilaco Corporation, Tokyo, Japan) and Co-Cr alloy (Japan Dental Metal Co., Ltd, Osaka, Japan) were selected as the substrate in the present investigation. All the metals were cut into disks of 10 mm in diameter and 1 ± 0.1 mm thick. The disks were ground with #240 abrasive paper, and then ultrasonically washed with acetone for 3 minutes. The disks were air-dried at room temperature for a night to proceed to the next procedure of HAp coating.

2.2 HAp coating

In order to form HAp films on the substrates, 3 kinds of solutions were used. First of all, SBF(1.5), 1.5 tumes as concentrated as that of the standard SBF proposed by Kokubo *et al.* [12] was prepared. Surface-treatment solution (ST solution) was prepared by dissolving urea into SBF(1.5) to be 2.0 mol·dm^{-3}. Urease aqueous solution was prepared by dissolving urease (Urease, from Jack Bean activity 108 units/mg Wako Pure Chemicals Industries, Ltd.) to be in 0.01 mass %.

HAp films were formed by immersing the disks heated at 200 °C into ST solution, 5 cm^3 each for a disk; 0.03 cm^3 of urease solution was added into each plastic

vessel with ST solution just before putting the disk in. The immersed disks were kept in the vessels at 50 °C for 1 d, and then the ST solution was replaced by new SBF(1.5) not containing urea or urease. SBF(1.5) was renewed every other day for 1 week. Then the disks were taken out and washed with deionized water for 3 times and air-dried at room temperature for a night.

2.3 Characterization

TF-XRD and SEM were used for phase identification and morphology observation of the resulting films, respectively. An EDX apparatus attached to SEM was used to make the compositions of the coatings clear. FT-IR measurement was used to detect functional groups. ESCA was used to clarify the mechanism of the HAp-coating process. Tape tests using Scotch #810 were performed to examine the adhesion property of the coatings qualitatively

2.4 *In vitro* evaluations

In vitro evaluations involved observing the behavior of MC3T3-E1 cells cultured on untreated materials (NT-X, X: metal), surface-treated materials (ST-X), and HAp coated materials (HA-X). ST-Xs were prepared by immersing metal substrates heated at 200 °C into ST solution for 10 min. Polystyrene plates were used as the control samples. The temperature and atmosphere of cell culture were kept at 37 °C and 5% CO_2, respectively; α-minimum essential medium (α-MEM) containing 10% of a fetal bovine serum (FBS) was used as a medium. Sixty thousands cells were seeded on each specimen in order to examine the initial cell-attachment efficiency (culture time: 5 h) and the cell proliferation (culture time: 1 to 7 d).

2.5 *In vivo* evaluations

Pure Ti rods (ϕ2 mm x 8 mm) were selected as specimens for *in vivo* evaluations. The HA-Ti, ST-Ti, and NT-Ti prepared by the above-mentioned process were implanted into the back of wister rats and the tibia of Japanese white rabbits. The animals were sacrificed after the desired experimental periods (2, 4, 8, 12, and 24 w) and undecalcified sections were prepared from the rabbit bones and rat soft tissues. Hematoxylin-Eosin (HE) and Villanueva-Goldner's stains were applied to histological evaluations of sections from rats and rabbits, respectively.

3. RESULTS

3.1 Characterization of each material

3. RESULTS

3.1 Formation of HAp film and its characterizations

Figure 1 shows the TF-XRD patterns of the surfaces of typical specimens. As seen from Fig. 1(a), (d), (g), and (j), some peaks newly appeared after soaking into SBF(1.5), in contrast to the XRD patterns of untreated substrates (Fig.1(c, f, i and l)). These peaks were assigned to crystalline apatite phase. As the (002) peak appearing at 2θ=25.877° was higher than that of typical HAp pattern, the present apatite formed on the metal substrates had a preferred orientation along the (002) plane. As seen from Fig. 1(b, e, h and k), neither surface-treated metals nor untreated substrates have peaks other than those of the substrates. Additionally, FT-IR results indicated that the coated HAp contained carbonate ions.

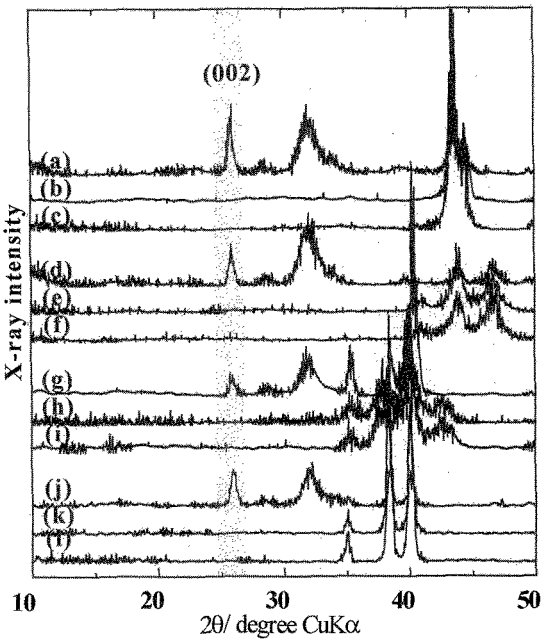

Fig. 1 TF-XRD patterns of each specimen
(a)HA-Ti (b)ST-Ti (c)NT-Ti
(d)HA-Ti6Al4V (e)ST-Ti6Al4V (f)NT-Ti6Al4V
(g)HA-CoCr (h)ST-CoCr (i)NT-CoCr
(j)HA-SUS316L (k)ST-SUS316Lr(l)NT-SUS316L

The microstructures of the surface of above-mentioned specimens are shown in Fig 2. Fig. 2 (a, d, g and j) show the microstructure of various metal substrates coated with HAp films. The SEM observations revealed that the crystals uniformly covered all the tested substrates. The microstructures of untreated and surface treated metals were relatively flat except for the scratches formed during the polishing

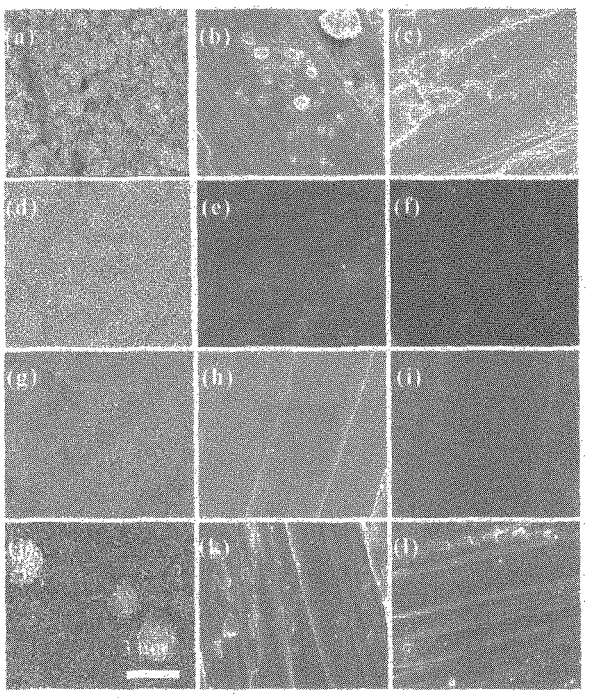

Fig. 2 Microstructure of each specimen
(a)HA-Ti (b)ST-Ti (c)NT-Ti
(d)HA-Ti6Al4V (e)ST-Ti6Al4V (f)NT-Ti6Al4V
(g)HA-CoCr (h)ST-CoCr (i)NT-CoCr
(j)HA-SUS316L (k)ST-SUS316L (l)NT-SUS316L

process. However, morphologies of the HAp crystals were different depending on the metal substrates. In the case of CP-Ti and Ti6Al4V substrates, the HAp films were composed of platelet-shaped particles on a scale of sub-micrometer.

EDX results revealed that all the substrate coated with HAp, independentlyof the kinds of metal substrates, contained a trace amount of Mg. The presence of Mg may be due to the substitution for Ca in the HAp crystals. The other elements derived from the substrates, , except Ca and P were not detected. ICP results reveals that the composition of the HAp films on various metal substrates has lower Ca/P ratios than 1.67 which corresponds to the stoichiometric HAp. ESCA spectra indicate that the Ca and P elements are detected from all the surface-treated substrates. This fact shows that nucleation of HAp have occurred during the surface treatment process. From the results of the tape test, HAp-coated Ti-6Al-4V and Co-Cr have good adhesiveness; however, the HAp film on SUS316L is easily peeled off.

Thus, we have found that the HAp films could be formed on various metal substrates by a simple surface-treatment using SBF containing urea and urease.

3.2 *In vitro* evaluations

The results of the relative initial cell-attachment efficiency after seeding for 5 h are shown in Fig. 3. In the case of CP-Ti group, the control was 100 ± 13.36%, NT 86.75 ± 10.54%, ST 84.47 ± 11.61%, and HA 83.50 ± 8.51%. In the Ti-6Al-4V group, the control was 100 ± 13.29%, NT 96.26 ± 12.91%, ST 88.77 ± 9.38%, HA 87.70 ± 11.98%. In the Co-Cr group, the control was 100 ± 13.56%, NT 86.75 ± 10.54%, ST 84.47 ± 11.61%, and HA 83.50 ± 8.51%. Finally, the control 100 ± 6.13%, NT 90.15 ± 4.30%, ST 91.56 ± 4.10%, and HA 81.70 ± 5.05% in the case of the SUS316L group. Cell numbers of all the HAp-coated materials were significantly inferior to those of the controls. This may be based on the surface roughness of the HAp-coated substrates.

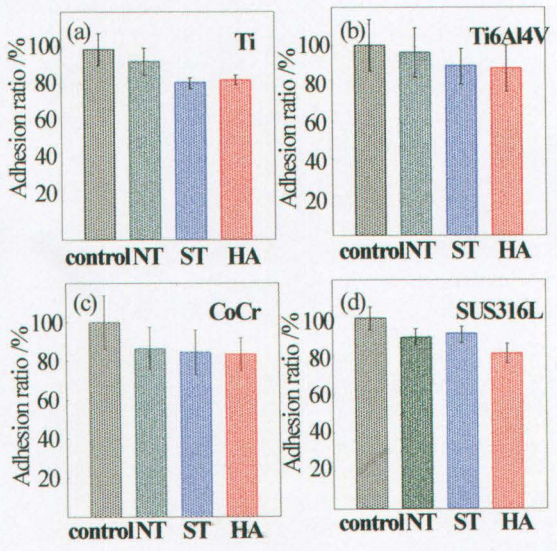

Fig. 3 Initial Attachment of each specimen
(a) Ti (b) Ti6Al4V (c) CoCr (d) SUS316L

Although the numbers of cells after 1 day of culture varied from specimen to specimen due to the differences in the initial-attachment efficiency, the cells cultured on all the specimens were proliferated very similarly to the case of the control as seen from Fig. 4. The calculated values of the doubling time (D.T.) of each specimen are given in Table 1. The D.T. of MC3T3-E1 is reported to be 15-18 h [13]; therefore, those results are thought to be appropriate.

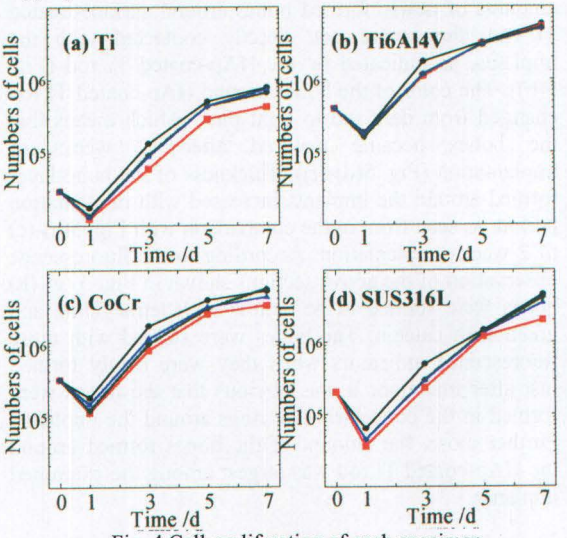

Fig. 4 Cell proliferation of each specimen
(a) Ti (b) Ti6Al4V (c) CoCr (d) Ti6Al4V

3.3 *In vivo* evaluations

In vivo evaluations were carried out using rats and rabbits models; rats were used to examine the reactions of each specimen with the soft living tissue and rabbits with the hard living tissue. Throughout the whole implant period, all the animals were healthy and no inflammation or infection was observed. In the experiment with rat models, undecalcified sections were prepared from specimens implanted in the back of the rats, and then stained with HE. All the specimens were surrounded with fibrous tissue and no inflammation reaction was observed during the experimental period. The thickness of the fibrous tissue was independent the materials and the implanted period.

In the case of rabbit models, undecalcified sections were also prepared from implants retrieved with the

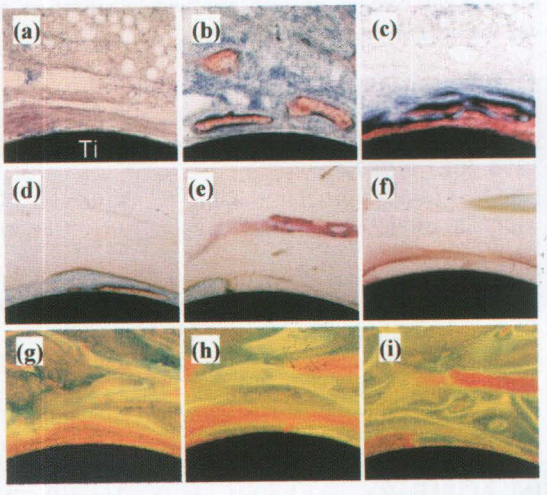

Fig. 5 Histological evaluation of each specimen
(a) HA-Ti2 w (b) ST-Ti 2 w (c) NT-Ti 2 w
(d) HA-Ti 24 w (e) ST-Ti 24 w (f) NT-Ti 24 w
(g)HA-Ti 24 w fl. (h)ST-Ti 24 w fl. (i)NT-Ti 24w fl

surrounding bone, and then stained with Villanueva-Goldner's method. Fig. 5(a)-(f) shows the optical microscopic observation of the sections. Newly formed bone was observed around the HAp-coated Ti rod, but not around the untreated rod after 2 weeks implantation (Fig. 5(a), (c)). Although there was a few amounts of newly formed bones around surface-treated Ti rod, they were not directly contacted with the implants, as indicated by the HAp-coated Ti rod (Fig. 5(b)). The color of the bones around HAp-coated Ti rod changed from dark red to light pink, which means that the bones became matured after 12 weeks of implantation (Fig. 5(d)-(f)). Thickness of the bone-layer formed around the implants increased with implantation period, as seen from in the comparison with Fig. 5(a)-(c) of 2 weeks implantation. According to the fluorescence observation of the above sections shown in Fig. 5 (g)-(h), bones were stained to be yellow with tetracycline and green with calcein. The bones were stained with these fluorescence indicators when they were newly formed just after injection. It was obvious that the bones were formed in the concentric-like rings around the implants. Further more, the amount of the bones formed around the HAp-coated Ti rod was largest among the examined implants.

4. DISCUSSIONS AND CONCLUSION

HAp films were formed by a simple surface-treatment utilizing an enzyme reaction of urea and urease on CP-Ti, Ti-6Al-4V, Co-Cr, and SUS316L substrates. The resulting films consisted of carbonate-containing HAp with the preferred (002) surfaces and low crystallinity. The microstructures and adhesive properties of HAp on the materials were different from sample to sample. The mechanism of the nucleation and the crystallization of HAp seems to depend on the kinds of substrates. The cells seeded on all the coated materials showed good proliferations proving no problem with their biocompatibility. *In vivo* evaluation using rats and rabbit models showed there was no negative reaction by implantation of the CP-Ti specimens. HAp-coated Ti rods directly bonded to bones to form a large amount of new bones around the implant. It can be concluded that the present coating process has wide application to coating of HAp on metal substrates.

5. REFERENCES

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