JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **16** (2005) 985–991

Effects of hydrothermal treatment with $CaCl_2$ solution on surface property and cell response of titanium implants

M. NAKAGAWA*, L. ZHANG, K. UDOH, S. MATSUYA, K. ISHIKAWA
Department of Biomaterials, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan
E-mail: nakagawa@dent.kyushu-u.ac.jp

In order to obtain early and good osteointegration after implantation of a titanium implant in the human body, the surface modified treatments using NaOH or H_2O_2 etc. were reported. In this study, titanium was hydrothermally treated with $CaCl_2$ solutions at 200 °C for 24hr ($CaCl_2$-HT). Scanning electron microscope (SEM) observation clearly showed apatite deposition on the surface of $CaCl_2$ HT treated titanium faster than other chemical treated titanium immersion in simulated body fluid. X-ray photoelectron spectroscopy (XPS) analysis demonstrated that Ti–O–Ca bonding was formed on titanium surface by hydrothermal treatment with $CaCl_2$ solution. And it was revealed that thickness of TiO_2, which was known to play important roles for the formation of bone-like apatite, became approximately three times thicker than as-polished titanium. The amount of initial attached MC3T3-E1 cells on as-polished and NaOH, H_2O_2 and this $CaCl_2$ HT treated titanium were almost the same values. After 5 days incubation, the growth rate of MC3T3-E1 cells on $CaCl_2$-HT treated titanium was significantly higher than that on other chemical treated titanium. The hydrothermal treatment with 10–20 mmol/L $CaCl_2$ solution at 200 °C was an effective method for the fabrication of titanium implant with good bioactivity and osteoconductivity.

1. Introduction

Titanium has been used as implant materials in dentistry and orthopedics, because of their good mechanical properties, high corrosion resistance and excellent biocompatibility. Bioactive materials such as sintered hydroxyapatite, bioactive glasses and calcium phosphate bio-ceramics form bioactive bonding with a living bone by forming a bone-like apatite layer on their surface after they are implanted in living bone. However, the ability to bond with living bone of titanium is not enough as compared with that of other bioactive materials. The combination with the bone occurs slowly and the bonding strength is relatively weak. When titanium implant is implanted in a bone, new bone is formed around the implant and bonded to the existing bone. The process usually takes more than 1–2 months. Since bone formation is sensitive to micro movement and infection (especially in dental implant), it is important to avoid unnecessary force to the implant and to beware of infection. As faster osteointegration of titanium implant is beneficial to the patients, various surface modification techniques have been developed in order to improve the bioactivity. Though apatite coating is a quite popular technique [1–6], its application is restricted because the coated apatite is easily removed from the metal surface [7, 8]. It was reported that faster new bone formation was obtained on the surface of titanium implant when titanium was treated with NaOH or H_2O_2 solution [9–19]. In addition the titanium implant treated with NaOH or H_2O_2 bonded directly to the bone without the intermediate fibrous connective tissue. In other words, if titanium is given a suitable surface treatment, the titanium implant can be endowed with a good osteointegration. On the other hand, it is believed that formation of so-called bone-like apatite plays an important role for the osteoconductivity. The formation of bone-like apatite is usually evaluated by using the simulated body fluid (SBF) that has the same inorganic ion concentration with human blood plasma. Takadama *et al*. reported that Ca^{2+} ion in the body fluid forms bonding with Ti-OH on the chemically treated surface of titanium implant and then reacts with PO_4^{3+} and OH^- to form amorphous calcium phosphate which gradually transformed to bone-like apatite [20, 21]. It is reported that titanium surface modified with solutions containing calcium ion promoted an apatite precipitation on the surface [22].

*Author to whom all correspondence should be addressed.

Hamada *et al*. performed surface modification of titanium with hydrothermal treatment in calcium solutions at 121 °C for 2 h [24]. They reported that hydrothermal modification in CaO solution enhanced the precipitation of apatite on the titanium surface. In this study, the titanium was subjected to hydrothermal treatment in $CaCl_2$ aqueous solution to evaluate whether or not hydrothermal treatment is effective for the fabrication of titanium implant with calcium bonded on its surface. Also the titanium subjected to hydrothermal treatment was immersed in simulated body fluid (SBF), to evaluate the bioactivity of titanium implant. And initial cell attachment and cell growth rate of chemical treated specimens were examined using osteoblast-like cell.

2. Materials and methods

2.1. Materials

Pure titanium (Kobe Steel Ltd., Kobe, Japan) was used in this study. The surface was polished with #1500 waterproof abrasive paper followed by washing with distilled water and ethanol, and dried. All specimens were cut to the same size of $8 \times 8 \times 1$ mm^3.

2.2. Surface modification treatments

Titanium plate was placed in a Teflon crucible containing 5, 10, 20 and 100 mmol/L of $CaCl_2$ aqueous solution and then the Teflon crucible was put in a stainless jacket. The vessel was placed in a temperature-controlled bath kept at 200 °C for 24 h for hydrothermal treatment. For comparison, two types of chemical treatments were also performed in the same way as reported previously [9, 10, 19]. One is NaOH treatment; immersion in 5 mol/L NaOH solution at 60 °C for 24 h and another is H_2O_2 treatment; immersion in 8.8 mol/L H_2O_2 + 0.1 mol/L HCl at 80 °C for 30 min followed by heating at 400 °C for 1 h.

2.3. Immersion of the treated specimen in simulated body fluid (SBF)

A simulated body fluid (Na^+ 142 mmol/L, K^+ 5.0 mmol/L, Mg^{2+} 1.5 mmol/L, Ca^{2+} 2.5 mmol/L, Cl^- 147.8 mmol/L, HCO_3^- 4.2 mmol/L, HPO_4^{2-} 1.0 mmol/L, SO_4^{2-} 0.5 mmol/L) was used to estimate bioactivity *in vitro* [23]. The pH value of test solutions was adjusted at pH 7.41 by adding HCl. The temperature of the test solution was kept at 37 ± 0.1 °C in the temperature controlled bath. The surface-treated specimens and no treated control specimen were immersed in SBF at 37 °C for various times up to 7 days (168 h).

2.4. Analysis of Calcium and Phosphate concentration in SBF

Changes in calcium and total PO_4 ions with immersion time were determined by atomic absorption analysis (Analyst 300, Perkin Elmer, Wellesley, MA, USA) and Spectrophotometry (U-best 50, Jasco, Tokyo, Japan). The immersion test was performed five times under the same conditions. Results were statistically analyzed by ANOVA with Scheff's test at a significance level of 1%.

2.5. Characterization of the surface after treatment and immersion in SBF

The surfaces of the specimens were analyzed by X-ray diffractometer (RINT-2500V, Rigaku Co., Tokyo, Japan) and by X-ray photoelectron spectrometer (XPS) (ESCA 750, Shimadzu Co., Kyoto, Japan). In the XPS analysis, argon ion etching was performed at 2 kV, 20 mA under 5×10^{-4} Pa. The surface morphology of specimens was observed by scanning electron micrographs (SEM) (JSM-5400LV, JEOL Ltd., Tokyo, Japan).

2.6. Cell culture

The MC3T3-E1 osteoblast-1ike cells [25] were used. The cells were incubated in alpha modified Eagle's medium (α-MEM, SIGMA-ALDRICH, Inc., USA) supplemented with 10% fetal bovine serum (FBS, Thermo Trace Co., Australia), 1% penicillin and streptomycin (10 mg/ml) in an atmosphere containing 5% CO_2 at 37 °C. The culture medium was changed every 2 days. Cell culture specimen size was 15 mm $\phi \times 1$ mm. Five specimens of each type of non-treated and treated Ti that were sterilized on both sides of specimen in an ultraviolet sterilizer for 2 h and placed individually into the 24-well plate were each inoculated with 5×10^3/cm^2 of MC3T3-E1 osteoblast-like cells.

2.7. Initial cell attachment and proliferation

After 5 h incubation, the cells were rinsed with phosphate buffer saline (PBS, SIGMA-ALDRICH, Inc., USA) and were detached from specimens with 0.25% trypsin and 0.02% EDTA (EDTA, SIGMA-ALDRICH, Inc., USA) for 5min incubated at 37 °C. The amount of initial attached cells on Ti plates was by a hemocytometer. The cell proliferation on each specimen was measured by MTT assay [26]. The MC3T3-E1 cells incubated for 1, 3, 5 and 7 days with the culture medium being changed every 2 days. They were on each specimen were gently washed with PBS and were measured by MTT assay using 3-(4, 5-dimethyl-thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Wako Chemical Co., Japan). The MTT solution was added to each specimen and the cells were incubated for 3 h at 37 °C and the medium was replaced with dimethylsulfoxide. Absorbance of the solution was measured by a plate reader (MODEL550, BioRAD, USA) at 570 nm. Results were statistically analyzed by ANOVA with Scheff's test at a significance level of 1%.

3. Results

3.1. Changes in calcium and phosphate concentration in SBF

Fig. 1 shows the changes in calcium and phosphate concentrations in SBF with immersion time of specimens. A non-treated Ti specimen was taken as a control.

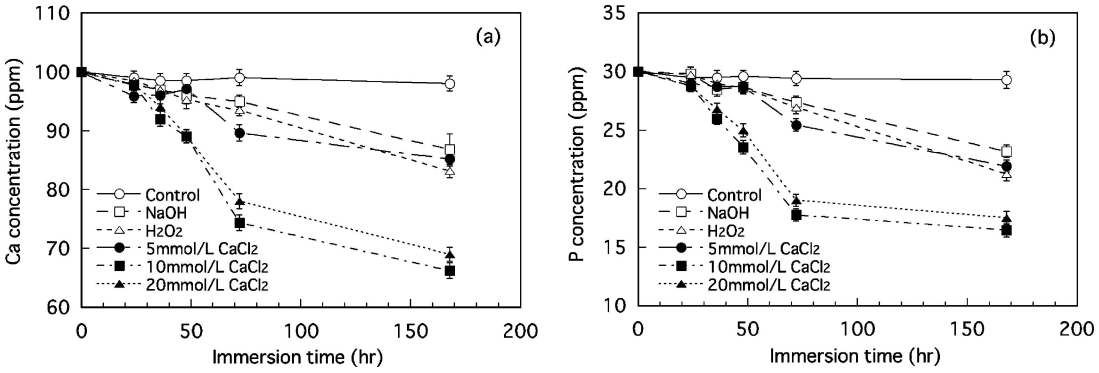

Figure 1 Changes in (a) Ca and (b) P concentrations in the SBF with time during immersion of the Ti treated chemically an hydrothermally.

Calcium and phosphate concentrations of the control specimen were unchanged during immersion for 7 days (168 h). It is seen that the specimens chemically treated with NaOH or H_2O_2 solution, calcium and phosphate concentrations in SBF decreased with the immersion time. A specimen treated hydrothermally with 5 mmol/L $CaCl_2$ solution showed the same tendency with the chemical treatments. The result of 100 mmol/L $CaCl_2$ treatment was the same tendency with that of 5 mmol/L $CaCl_2$ treatment. It should be noted that the calcium and phosphate concentrations decreased were rapidly with the specimens hydrothermally treated with 10 or 20 mmol/L $CaCl_2$ solution. The decrease in concentration after 48 h immersion was equal to the same level as that after 7 days immersion for the chemical treatment with NaOH or H_2O_2 solution. It is thought that the decrease of calcium and phosphate concentrations in SBF was due to the precipitation of apatite on the Ti specimen, because Ca/P ratio is between 1 and 2.

3.2. X-ray diffraction profiles and SEM observation

Fig. 2 shows the X-ray diffraction patterns of the titanium specimen treated hydrothermally with 10 mmol/L $CaCl_2$ solution after immersion in SBF up to 7 days. It was seen that the apatite diffraction peaks were detected after 48 h (2 days) immersion. After 7 days immersion, intensities of the apatite peaks became quite strong. The result of the titanium treated hydrothermally with 20 mmol/L $CaCl_2$ solution was the same tendency to that of 10 mmol/L $CaCl_2$. The apatite diffraction peaks of the specimen treated hydrothermally with 5 mmol/L $CaCl_2$, NaOH and H_2O_2 solutions were detected after 7 days immersion in SBF.

SEM observation showed no appreciable change in the specimen surfaces after before immersion for 24 h. However, apatite precipitates wee seen on the specimen surfaces hydrothermally treated after immersion in SBF for 36 h, as shown in Fig. 3. Many apatite crystals can be observed on the surfaces of specimens treated with 10 and 20 mmol/L $CaCl_2$ solution. On the other hand, no apatite precipitates on the surface of specimen treated with 5 and 100 mmol/L $CaCl_2$ solutions. Fig. 4 shows comparison among the typical SEM images of the specimen treated by NaOH, H_2O_2 and 10 mmol/L $CaCl_2$ after immersed in SBF for 48 h. There was no deposition on the surface of non-treated control specimen (Fig. 4(a)). It is seen that a small amount of the deposit on the surface of specimen treated with NaOH solution (Fig. 4(b)). In the case of titanium treated with H_2O_2 solution, the amount of deposit increased (Fig. 4(c)), but it was not too much to cover the whole specimen surface. In the specimen hydrothermally treated with 10 mmol/L $CaCl_2$ solution, there was the largest amount of deposit on the surface of specimen, and whole surface was covered with the deposit (Fig. 4(d)).

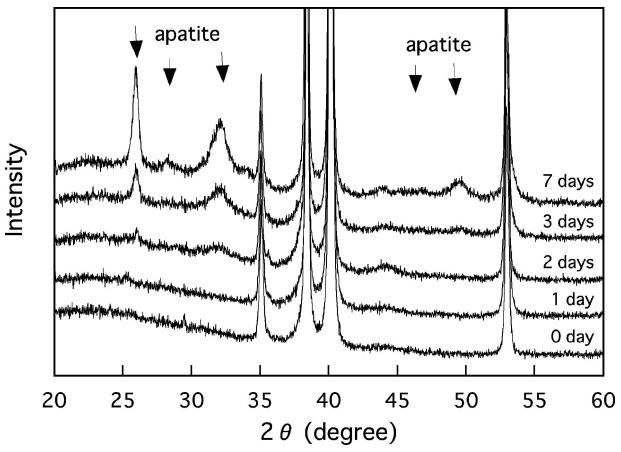

Figure 2 X-ray diffraction pattern of the Ti specimen hydrothermally treated with 10 mmol/L $CaCl_2$ after immersed in SBF for various periods up to 7 days.

3.3. Surface analysis by XPS

Fig. 5 shows the results of XPS analysis on the surface of the non-treated specimen and the hydrothermally treated specimen with 10 mmol/L $CaCl_2$ solution at 200 °C for 24 h. The Ti2p spectra show the existence of TiO_2 on the surfaces of the both specimens. Peaks corresponding to metallic titanium with Ar^+ ion etching gradually replace the TiO_2 peaks. It takes 300 sec to detect metallic titanium peak in the case of non-treated titanium (Fig. 5(a)). On the other hand, in the specimen hydrothermally treated with 10 mmol/L $CaCl_2$ solution, it takes 900 sec until the TiO_2 layer completely disappears (Fig. 5(b)). As shown in Fig. 5(c), calcium is present in the surface layer of the specimen treated hydrothermally with 10 mmol/L $CaCl_2$ solution. The intensity of the Ca2p peaks decreased with the Ar^+

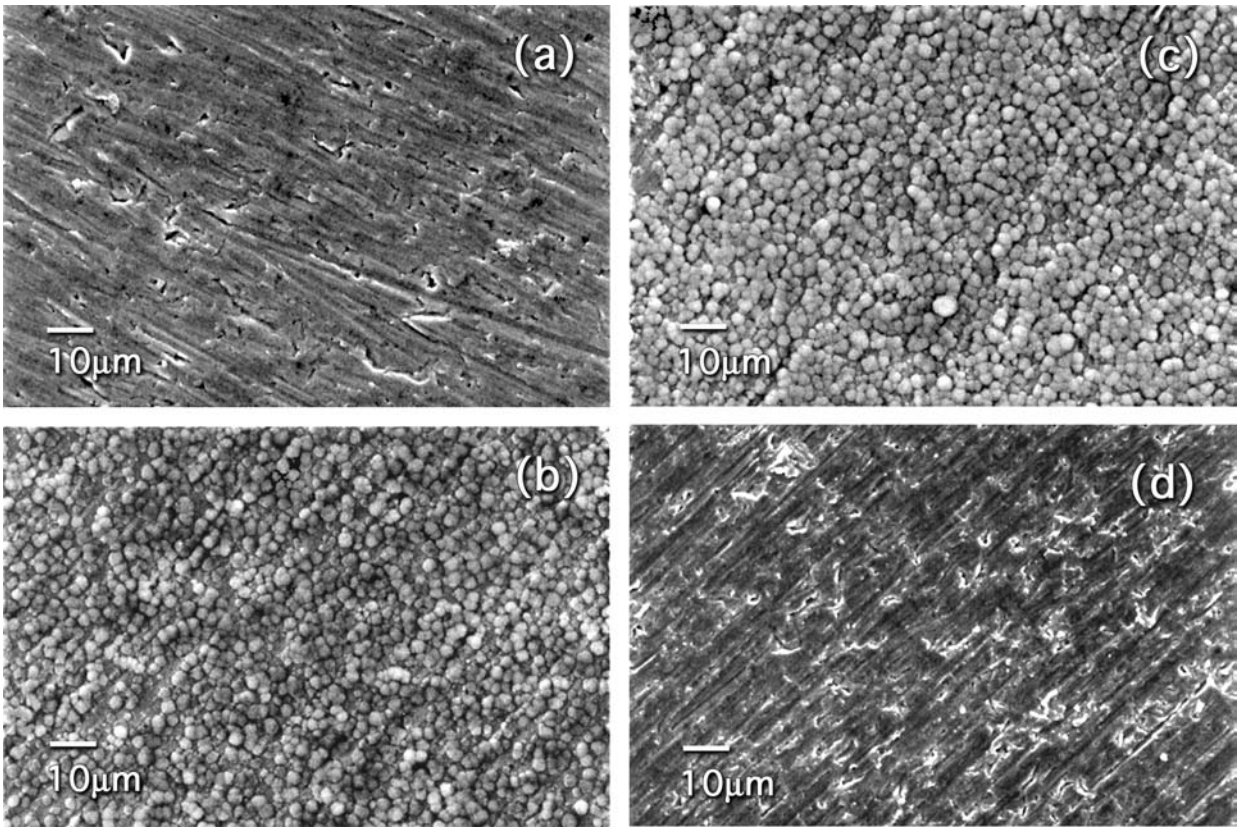

Figure 3 SEM micrographs of Ti treated hydrothermally after immersion in SBF for 36 h. (a) 5 mmol/L, (b) 10 mmol/L, (c) 20 mmol/L, (d) 100 mmol/L $CaCl_2$ hydrothermal treatment at 200 °C for 24 h.

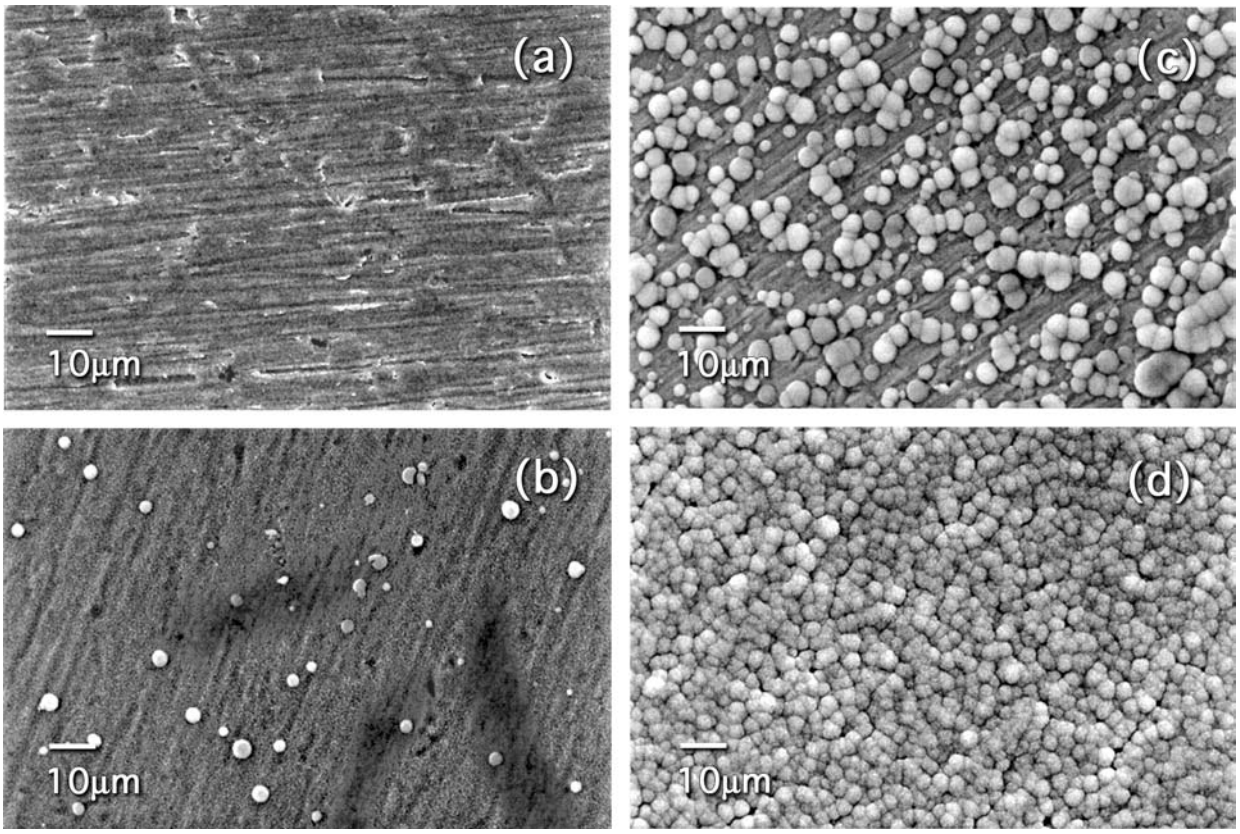

Figure 4 SEM micrographs of Ti treated chemically and hydrothermally after immersion in SBF for 48 h. (a) as-polished (non-treated Ti), (b) NaOH, (c) H_2O_2, (d) 10 mmol/L $CaCl_2$ hydrothermal treatment at 200 °C for 24 hrs.

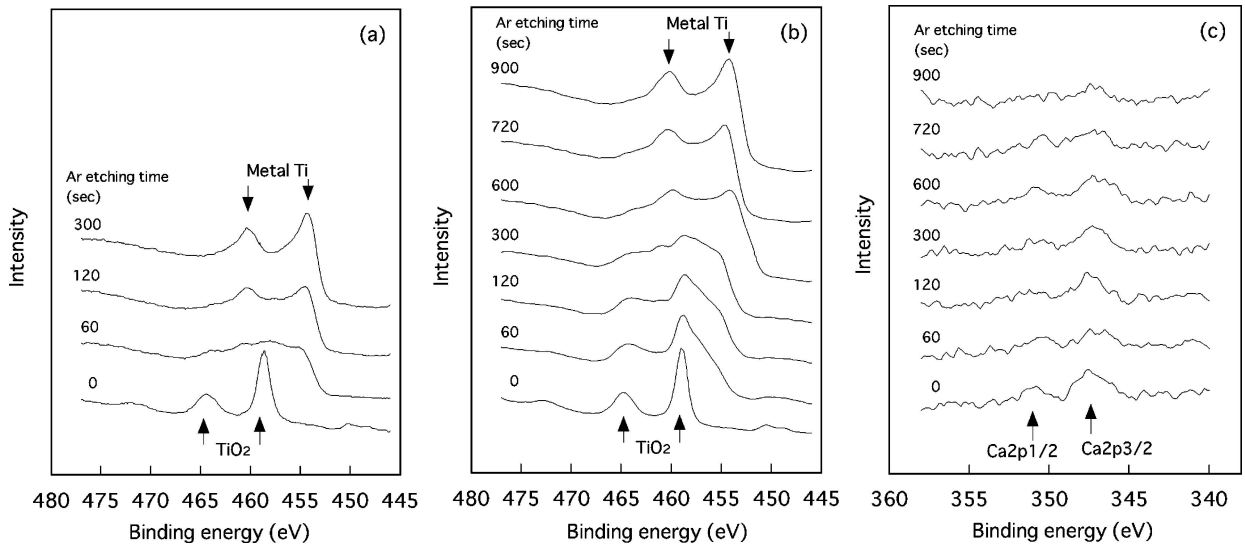

Figure 5 XPS spectra of (a) Ti2p of non-treated specimen, (b) Ti2p and (c) Ca2p after 10 mmol/L $CaCl_2$ hydrothermal treatment.

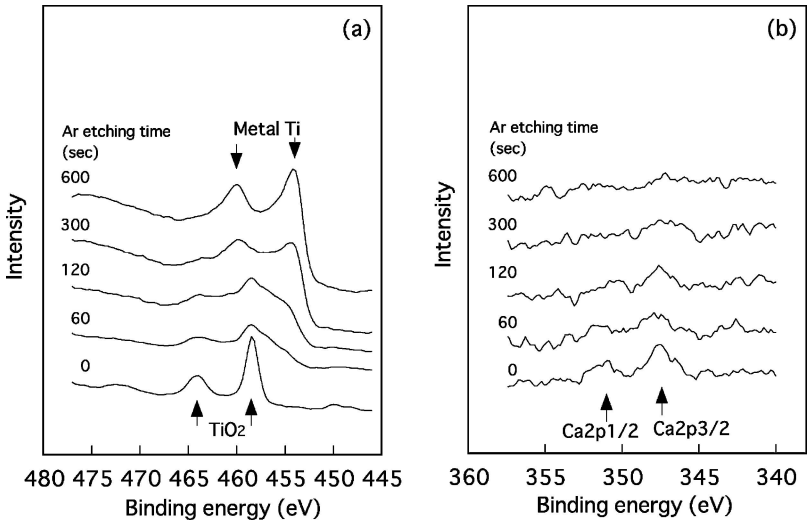

Figure 6 XPS spectra of (a) Ti2p and (b) Ca2p after 100 mmol/L $CaCl_2$ hydrothermal treatment.

etching time indicating that calcium content in the surface of specimen gradually decreased from the surface to the interior of the oxide layer. The result of XPS analysis on the surface of the hydrothermally treated with 100 mmol/L $CaCl_2$ solution is shown in Fig. 6. It was seen that the TiO_2 peaks disappeared by Ar^+ ion etching more quickly than with 10 mmol/L $CaCl_2$ solution. This fact suggests that TiO_2 layer was thinner when hydrothermally treated with 100 mmol/L $CaCl_2$ solution. Ca peaks also disappeared simultaneously with disappearance of TiO_2 layer.

3.4. Initial cell attachment and proliferation

Fig. 7 shows the initial attachment and proliferation of MC3T3-E1 cells. The amount of initial attached cells

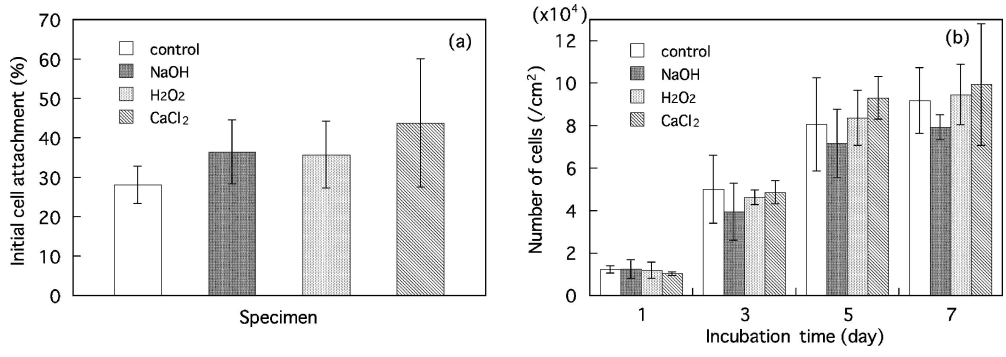

Figure 7 Initial cell attachment and proliferation of osteoblast-like cell MC3T3-E1. (a) initial cell attachment after 5 h incubation and (b) number of cells after 1, 3, 5 and 7 days incubation.

on non-treated control specimen and NaOH, H_2O_2, $CaCl_2$-HT treated specimens were almost the same value (Fig. 7(a)) $p < 0.05$). This result indicated that these chemical treatments did not affect the initial attachment of MC3T3-E1 cells to the titanium surface.

The growth rate of MC3T3-E1 cells on each plate is shown in Fig. 7(b). After 5 days incubation, the growth rate of MC3T3-E1 cells on $CaCl_2$-HT treated specimen was significantly higher than that on other chemical treated specimens ($p < 0.05$). The increase in growth rate was slowing down at 7 days incubation because of the confluent growth.

4. Discussion

The results obtained in the present study clearly demonstrated that hydrothermal treatment of titanium with 10 and 20 mmol/L $CaCl_2$ solution is effective for the formation of so-called a bone-like apatite on its surface when the titanium was immersed in SBF. The enhanced formation of bone-like apatite is probably due to the existence of calcium on the surface of titanium. It has been reported that Ti–O–Ca bonding was formed when NaOH treated titanium was immersed in SBF [20, 21]. As shown in Figs. 5 and 6, calcium peaks decreased synchronously with the TiO_2 oxide layer by Ar^+ etching. This means calcium exists not only on the superficial, but also in the whole surface oxide layer, indicating the formation of Ti–O–Ca bonding. Therefore, it is reasonable that titanium treated hydrothermally in the presence of $CaCl_2$ showed faster deposition of bone-like apatite than titanium treated with NaOH since $CaCl_2$ treated titanium does not require period for the formation of Ti–O–Ca bonding in the SBF. The existence of Ti–O–Ca bonding probably made a nucleation of apatite easy. XPS analysis also revealed that surface TiO_2 layer became thicker. However, the TiO_2 layer in the case of titanium hydrothermally treated with 100 mmol/L $CaCl_2$ solution became rather thinner than with 10 mmol/L $CaCl_2$ (Fig. 6). Hamada *et al*. reported that the precipitation of apatite was inhibited by hydrothermal treatment in 5.4 mol/L $CaCl_2$ solution at 121 °C for 2 h [24]. In the present study, the apatite formation was also less with higher $CaCl_2$ concentration. This inhibition was caused by the decrease in thickness of the surface TiO_2 oxide layer and lack of calcium ions contained in the layer. Therefore, it was thought that the high concentration $CaCl_2$ solution decreased the thickness of a surface oxide layer because of corrosive nature of chloride ion [24]. Since TiO_2 layer is known to be closely related to the formation of bone-like apatite, thickness of TiO_2 layer may also be one of the factors for the faster bone-like apatite formation [27, 28]. It was suggested that the thickness of the oxide film on the titanium surface and the presence of calcium would effectively contribute to the deposition of apatite. Even if the Ti–O–Ca bonding was formed, when the thickness of a TiO_2 oxide layer was too thin, the deposition of the apatite could not be promoted. The above results suggested that the hydrothermal treatment of titanium in the presence of 10–20 mmol/L $CaCl_2$ solutions at 200 °C result in the existence of calcium on the surface of titanium and formation of thicker TiO_2 layer.

The amount of initial attached cells on non-treated control titanium and NaOH, H_2O_2, $CaCl_2$-HT treated specimen were almost the same values. After 5 days incubation, the growth rate of MC3T3-E1 cells on $CaCl_2$-HT treated specimen was significantly higher than that on other chemical treated specimens. These results indicated that the surface TiO_2 oxide layer including Ca formed via $CaCl_2$-HT treatment did not affect the attachment and accelerated the proliferation of MC3T3-E1 cells on the titanium implant. Therefore, the hydrothermal treatment with 10–20 mmol/L $CaCl_2$ solution at 200 °C was expected to be an excellent method for the fabrication of titanium implant with good bioactivity and osteoconductivity.

5. Conclusion

The calcium containing TiO_2 oxide layer was formed on the titanium surface by hydrothermal treatment with 10–20 mmol/L $CaCl_2$ solution at 200 °C for 24 h. The thickness of surface TiO_2 oxide layer increased by the treatment and became approximately three times thicker than non-treated titanium. The thickness of the TiO_2 oxide layer and presence of calcium in the layer on the titanium surface effectively contributed to the precipitation of bone-like apatite on the titanium during immersion in SBF. The $CaCl_2$ hydrothermal treatment did not affect the initial attachment and accelerated the growth rate of MC3T3-E1 cells to the titanium surface. The hydrothermal treatment with 10–20 mmol/L $CaCl_2$ solution at 200 °C was expected to be a excellent method for the fabrication of titanium implant with good bioactivity and osteoconductivity.

Acknowledgments

This study was supported by a Grant-in Aid for Scientific Research (165919562) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1. M. J. FILAGGI, N. A. COOMBS and R. M. PILLIAR, *J. Biomed. Mater. Res.* **25** (1991) 1211.
2. K. HAYASHI, T. MASHIMA and K. UENOYAMA, *Biomaterials* **20** (1999) 111.
3. W. R. LACEFIELD, *Ann. Biomed. Mater. Res.* **523** (1988) 73.
4. L. M. BOULTON, P. J. GREGSON, M. TUKE and T. BALDWIN, *Mate. Lett.* **12** (1991) 1.
5. M. WEI, A. J. RUYS, B. K. MILTHORPE and C. C. SORRELL, *J. Biomed. Mater. Res.* **45** (1999) 11.
6. H. MONMA, *J. Mater. Sci.* **29** (1994) 949.
7. M. OGISO, Y. YAMASHITA and T. MATSUMOTO, *J. Dent. Res.* **77** (1997) 1426.
8. M. WEI, A. J. RUYS, M. V. SWAIN, S. H. KIM, B. K. MILTHORPE and C. C. SORRELL, *J. Mater. Sci. Mater. Med.* **10** (1999) 401.
9. T. KOKUBO, F. MIYAJI, H. M. KIM and T. NAKMURA, *J. Am. Ceram. Soc.* **79** (1996) 1127.
10. H. M. KIM, F. MIYAJI, T. KOKUBO and T. NAKAMURA, *J. Biomed. Mater. Res.* **32** (1996) 409.
11. *Idem.*, *J. Ceram. Soc. Japan* **105** (1997) 111.

12. *Idem., J. Mater. Sci. Mater. Med.* **8** (1997) 341.
13. H. B. WEN, Q. LIU, J. R. DE WIJIN and K. DE GROOT, *Biomaterials* **19** (1998) 215.
14. S. W. HA, K. L. ECKERT, E. WINTERMANTEL, K. GRUNER, M. GUECHEVA and H. VONMONT, *J. Mater. Sci.: Mater. Med.* **8** (1997) 881.
15. H. M. KIM, H. TAKADAMA, T. KOKUBO, S. NISHIGUCHI and T. NAKAMURA, *Biomaterials* **21** (2000) 353.
16. C. OHTSUKI, H. IIDA, S. HAYAKAWA and A. OSAKA, *J. Biome. Mater. Res.* **35** (1997) 39.
17. M. C. DE ANDRE, M. R. T. FILGUEIRAS and T. OGASAWARA, *ibid.* **46** (1999) 441.
18. S. NISHIGUCHI, H. KATO, H. FUJITA, M. OKA, H. M. KIM, T. KOKUBO and T. NAKAMURA, *Biomaterials* **22** (2001) 2525.
19. X. X. WANG, S. HAYAKAWA, K. TSURU and A. OSAKA, *J. Biomed. Mater. Res.* **54** (2001) 172.
20. H. TAKADAMA, H. M. KIM, T. KOKUBO and T. NAKAMURA, *ibid.* **57** (2001) 441.
21. H. TAKADAMA, H. M. KIM, T. KOKUBO and T. NAKAMURA, *ibid.* **55** (2001) 185.
22. T. HANAWA, M. KON, H. UKAI, K. MURAKAMI, Y. MIYAMOTO and K. ASAOKA, *ibid.* **34** (1997) 273.
23. T. KOKUBO, H. KUSHITANI, S. SAKKA, T. KITSUGI and T. YAMAMURO, *ibid.* **24** (1990) 721.
24. K. HAMADA, M. KON, T. HANAWA, K. YOKOYAMA, Y. MIYAMOTO and K. ASAOKA, *Biomaterials* **23** (2002) 2265.
25. H. KODAMA, Y. AMAGI, S. KASAI and S. YAMAMOTO, *Jpn. J. Oral Biol.* **23** (1981) 899.
26. T. MOSSMAN, *J Immunol Meth* **65** (1983) 55.
27. B. FENG, J. Y. CHEN, S. K. QI, L. HE, J. Z. ZHAO and X. D. ZHANG, *J. Mater. Sci. Mater. Med.* **13** (2002) 457.
28. K. S. HWANG, Y. H. LEE, B. A. KANG, S. B. KIM and J. S. OH, *ibid.* **14** (2003) 521.

Received 22 March 2005
and accepted 25 May 2005