Trabecular Bone Response to Titanium Implants with a Thin Carbonate-Containing Apatite Coating Applied Using the Molecular Precursor Method

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Purpose: *The influence of thin carbonate-containing apatite (CA) coating on the trabecular bone response to titanium implants was investigated.* ***Materials and Methods:*** *Thin CA coatings were deposited by a new method known as the molecular precursor method. Using a precursor solution composed of an EDTA-calcium complex, a CA film was deposited on the titanium surfaces. Uncoated and CA-coated titanium were placed in the trabecular bone of the left and right femoral condyles of 16 rabbits. After implantation periods of 2, 4, 8, and 12 weeks, the bone-implant interface was evaluated histologically and histomorphometrically.* ***Results:*** *Histologic evaluation revealed new bone formation around the uncoated and CA-coated implant surfaces after only 4 weeks of implantation. After 12 weeks, mature trabecular bone surrounded all implants. At 4 and 8 weeks of implantation, no difference existed in bone contact between uncoated and CA-coated implants. After 12 weeks of implantation, the CA-coated implant group showed a significantly higher percentage of bone contact than the uncoated implant group.* ***Discussion and Conclusion:*** *The present study demonstrated that thin CA coatings applied using the molecular precursor method showed greater bone-to-implant contact during the healing phase than uncoated controls. The results were similar to those observed with implants with calcium phosphate coatings deposited with a physical vapor deposition technique.* Int J Oral Maxillofac Implants 2006;21:851–858

Key words: *bone-implant contact, calcium phosphate coating, carbonate-containing apatite, dental implants, molecular precursor method*

It is well known that, compared with as-received titanium implants, implants coated with calcium phosphate induce faster bone adaptation and improve the implant integration.[1] The plasma-spray technique is now the most widely used method for the deposition of calcium phosphate coatings. Nevertheless, plasma-spray apatite coatings may have some shortcomings, such as degradation and fatigue behavior of the coating; the long-term clinical safety and prognosis have also been questioned.[2–4]

To overcome these problems, physical vapor deposition (PVD) techniques such as ion plating, magnetron sputtering, and ion beam dynamic mixing have been introduced to deposit thin calcium phosphate coatings on medical implants, especially oral implants.[5–7] PVD–deposited calcium phosphate coatings are more adherent to the underlying titanium surface and less prone to form cracks than plasma-sprayed coatings.[6,7] Frequently, these coatings have an amorphous structure directly after deposition, which can easily be improved by rapid heat treatment with, for example, infrared radiation.[8] The main component of the crystalline structure of these heat-treated films is hydroxyapatite. Previous cell culture and animal experiments have demonstrated the biological efficacy of this type of coating.[9–11]

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Furthermore, it has been demonstrated previously that the bone reaction to an oral implant is also influenced by local implantation site conditions, such as the presence of cortical or trabecular bone.[12] The deposition of carbonate-containing apatite (CA) film on a titanium substrate is interesting because of its chemical resemblance to bone mineral. Recently, Leeuwenburgh and coworkers[13,14] reported a new coating technology referred to as electrostatic spray deposition (ESD), which was originally developed to synthesize thick ceramic films for solid electrolytes.[15] They found that crystalline CA coatings were formed after heat treatment of as-deposited ESD coating. Siebers and colleagues[16] assayed the cell proliferation, alkaline phosphatase activity, and osteocalcin concentration of osteoblastlike cells on such CA coatings deposited by ESD. Despite favorable biological properties, a disadvantage of the ESD technique is that it is a line-of-sight technique, which makes it difficult to apply uniform coatings on complex implant surface geometries or inside porous scaffold materials, such as titanium fiber mesh.[17]

Sato and coworkers[18–20] also developed a novel method for coating ceramic or metallic materials with a metal oxide film. They call this technique the molecular precursor method. The principle of the method is the application of an alcoholic precursor solution of an ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA)-metal complex on the substrate, followed by firing of the material at a temperature of approximately 500°C to 700°C. Film formation can be obtained by a combination of anionic species involving metal ions and adequate alkylammonium cations without a polymerization process. Thin films of titanium oxide (TiO_2) and cobalt oxide (Co_3O_4) were applied to a glass substrate using a Ti-EDTA or Co-EDTA complex by the molecular precursor method. The molecular precursor method was also used to produce thin strontium titanate ($SrTiO_3$) films using a mixture of Ti-EDTA and Sr-EDTA complex.

Recently, Sato and colleagues[21] found that CA can be deposited on titanium by using a precursor solution of a Ca-EDTA complex. They found that the Ca-EDTA precursor solution without water is stable during storage. Thin crystalline CA films were applied to titanium substrates using the molecular precursor method, with firing at 600°C to 700°C.[22] The film thickness was less than 1 mm, and the tensile bond strength measurement and scratch test showed an excellent degree of adhesion of the coated film on the titanium after immersion in phosphate-buffered saline (PBS, pH = 7.4) solution. Moreover, a greater amount of precipitation of calcium phosphate crystals was observed on titanium coated with a thin CA film after immersion in simulated body fluid (SBF) compared with an uncoated titanium surface.[23]

To learn more about the biological response of CA film as produced by the molecular precursor method, animal study was conducted. The objective of this study was to compare the interfacial bone response around titanium implants coated with a thin layer of CA versus unaltered control titanium implants after implantation into the trabecular bone of the femoral condyles of rabbits.

MATERIALS AND METHODS

Preparation of Molecular Precursor Solution

The molecular precursor solution for the CA coating was obtained by adding metaphosphate salt to a Ca-EDTA/amine ethanol solution. The general procedure for the preparation of the molecular precursor solution is shown in Fig 1. The preparation of molecular precursor solution was performed in 3 steps: *(1)* preparation of Ca-EDTA/amine ethanol solution, *(2)* preparation of metaphosphate salt ($((C_4H_9)_2NH_2)_2P_2O_6 \cdot 2H_2O$), and *(3)* the addition of metaphosphate salt into Ca-EDTA/amine ethanol solution by adjusting the Ca/P to 1.67. The procedures were described in detail in a previous paper.[22,23]

Implant Materials

Thirty-two cylindric commercially pure titanium implants with a length of 6 mm were used. The diameter of all implants was 2.8 mm. Sixteen implants were left uncoated and the other 16 implants were provided with a thin CA coating using the molecular precursor method.

The coating procedure was performed according to the previously described method.[22,23] Before coating, titanium implants were cleaned ultrasonically in propanol, then dried at 100°C. The implants were immersed into molecular precursor solution for 5 minutes. Afterwards, the precursor films that formed on the titanium cylinder were dried at 60°C for 20 minutes and then fired at 600°C for 2 hours using a furnace (MSFT-1520-P, Nikkato, Tokyo, Japan) under atmospheric conditions.

The surface roughnesses (Ra, Ry, and Rz) of the uncoated and Ca-coated titanium implants were measured with a Handy Surf E-30A (Tokyo Seimitsu, Tokyo, Japan) with a scan length of 4 mm and a cut-off value of 0.8. The stylus was moved on the cylinder surface along the long axis. Three runs for each sample were performed. Ra, Ry, and Rz of uncoated titanium were 0.40 ± 0.08 μm, 4.54 ± 0.08 μm, and 2.75 ± 0.62 μm, respectively, and the final Ra, Ry, and Rz of CA-coated titanium implants were 0.37 ± 0.08 μm, 4.44 ± 0.85 μm, and 2.58 ± 0.36 μm, respectively (means ± SDs for the 3 runs).

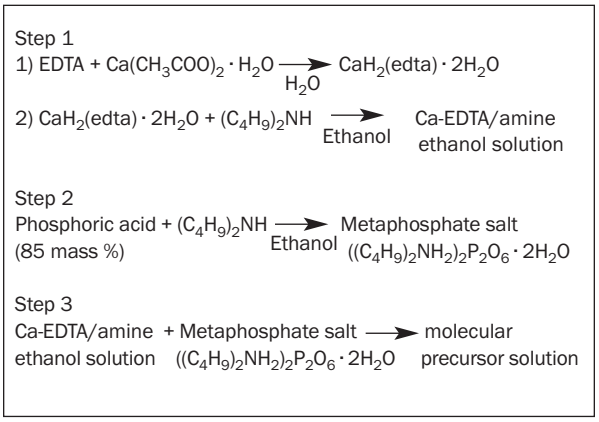

Fig 1 Schematic presentation of the preparation of the molecular precursor solution.

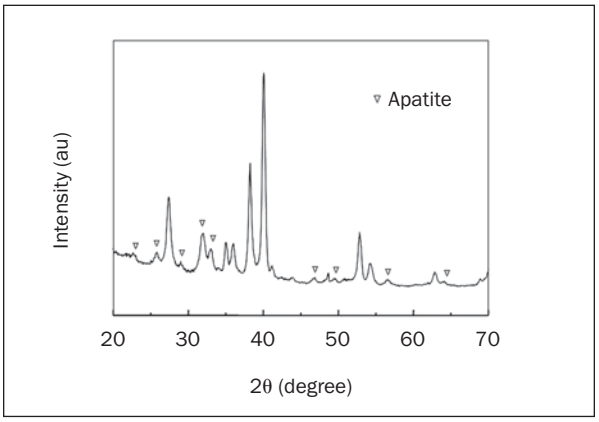

Fig 2 XRD patterns of CA coatings. The major reflections have been assigned to the peaks. au = arbitrary unit.

The deposited CA layer was characterized by x-ray diffraction (XRD; θ-2θ diffractometer, MXP-18 AHF22, Mac Science, Kanagawa, Japan) with a thin layer attachment (incident angle = 0.3 degree). The x-ray source was CuKα, and a power of 45 kV × 300 mA was used. The XRD pattern showed that the deposited coating had apatite structures (Fig 2). Fourier transform infrared reflection-adsorption spectroscopy (FT-IR-RAS; FT-210, Horiba, Kyoto, Japan) (Fig 3) revealed the presence of carbonate in the deposited films.

The thickness and Ca/P ratio of deposited film were determined by electron probe microanalysis (EPMA; X-8010, Hitachi, Tokyo, Japan) at an accelerating voltage of 25 kV by measuring the x-ray intensity of Ca-Kα, P-Kα, and Ti-Kα. The film thickness was approximately 0.16 ± 0.02 μm, and the Ca/P ratio was 1.49 ± 0.20.

After coating, the implants were cleaned ultrasonically in acetone, then boiled in ethanol and dried. Before surgery, all implants were sterilized in an autoclave.

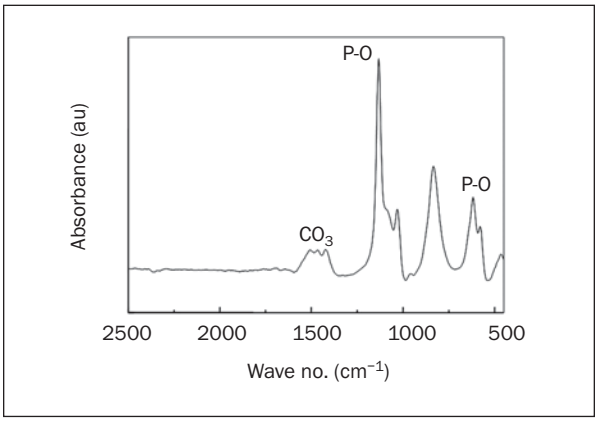

Fig 3 FT-IR spectrum of CA coatings. au = arbitrary unit.

Experimental Design and Implantation Procedure

The animal study was performed according to the animal experimental ethical guidelines of Nihon University School of Dentistry (certificate no. ECA-04-0019). Sixteen 3-month-old adult female Japanese white rabbits weighing about 3.5 to 4 kg were used.

The implants were placed into the trabecular bone of rabbits according to a previously used technique.[11,12] Each animal received 1 uncoated titanium and 1 CA-coated implant (1 in the left medial femoral condyle and 1 in the right). A total of 32 implants were placed, 16 uncoated and 16 CA-coated.

Pentobarbital sodium (25 mg/kg Nembutal) was injected intravenously; local anesthesia was induced by injection of xylocain. To reduce the perioperative infection risk, a prophylactic antibiotic (0.01 mg/kg Shiomalin, which is equivalent to latamoxef sodium) was administered postoperatively by a subcutaneous injection.

For the insertion of the implants, each animal was immobilized on its back. The hind legs of the rabbits were shaved, washed, and disinfected with iodine tincture.

A longitudinal incision was made on the medial surface of the femur, and the medial condyle was exposed. After exposure of the femoral condyle, a 0.6-mm pilot hole was drilled. The hole was gradually widened with a series of drills to the final diameter of the implant (2.8 mm). The bone preparation was performed with a very gentle surgical technique using a low rotational drilling speed (500 rpm) and continuous internal cooling. After press-fit insertion of the implants, the soft tissues were closed in separate layers using resorbable Vicryl 3-0 sutures (Ethicon, Somerville, NJ).

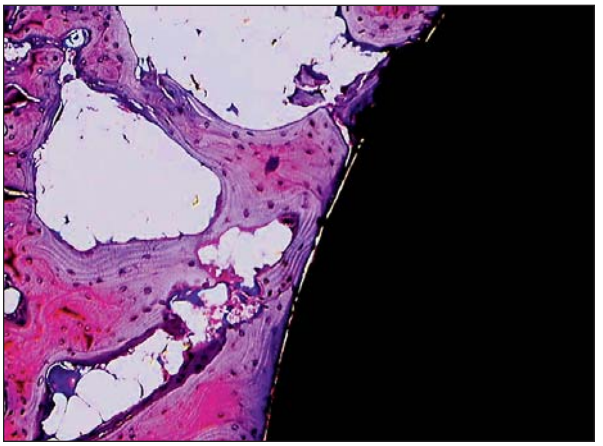

Fig 4 Histological section of an uncoated titanium implant after 2 weeks of implantation. The original drill hole can be recognized, and primary woven bone formation was observed on the implant surface (methylene blue and basic fuchsin; original magnification ×100).

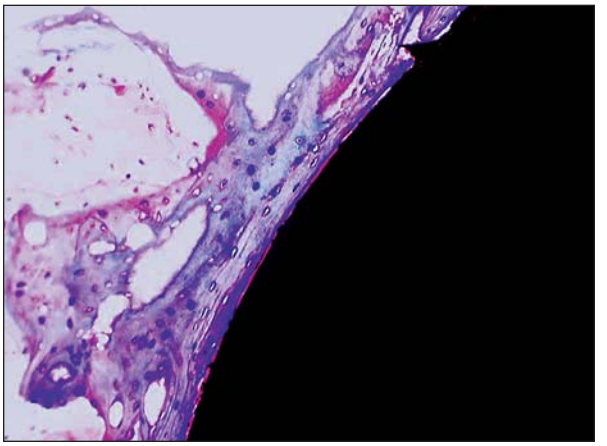

Fig 5 Histologic section of a CA-coated implant 2 weeks after implantation. Primary woven bone formation was observed (methylene blue and basic fuchsin; original magnification ×100).

Finally, the position and the fit of the implants were confirmed radiographically. The implants were placed transversely across the femoral condyle. Only at the cortical side were the implants surrounded by cortical bone. The rest of the implants were fully placed in trabecular bone.

Postoperatively, the animals were placed in a standard cage. They were fed water and rabbit food *ad libitum* and were allowed to move unrestricted at all times. The rabbits were sacrificed by injecting an overdose of pentobarbital sodium (Nembutal) peritoneally, according to the following schedule: 4 rabbits after 2 weeks of implantation, 4 after 4 weeks, 4 after 8 weeks, and 4 after 12 weeks (2 rabbits per coating group per time).

Histological Procedures and Evaluation

After the animals were sacrificed, the implants and surrounding bone were immediately excised. Excess tissue was removed from the excised femurs. Following the fixation in 10% buffered formalin solution, the specimens were prepared for histological evaluation. Therefore, the implant-containing tissue blocks were dehydrated through a graded series of ethanols and embedded in methylmethacrylate. After polymerization, nondecalcified thin sections were prepared using a cutting-grinding technique (EXAKT-Cutting Grinding System, BS-300CP band system & 400 CS microgrinding system, EXAKT Apparatebau, Norderstedt, Germany).[24] Sections with a final thickness of approximately 50 µm were made in a transverse direction perpendicular to the axis of the implants and were stained with methylene blue and basic fuchsin. The implant-bone interface was evaluated using a light microscope (Eclipse E800M; Nikon, Tokyo, Japan; magnification ×100).

Besides a descriptive evaluation, the percentage of bone contact was determined for the 4-, 8-, and 12-week specimens. The amount of bone contact was defined as the percentage of implant length at which there is direct bone-to-implant contact without intervening fibrous tissue. The measurements were performed along the total perimeter of the implant. Four or 5 sections were prepared from same implant and 1 or 2 sections were used for the histomorphometric evaluation. All measurements were performed at sections taken from the middle part of the implant. This part was completely surrounded by trabecular bone. The sections were selected at random. However, the sections were always taken at least 800 µm from each other. The measurements were made using a light microscope (Eclipse E800M; Nikon, magnification 12.5× to 125×) connected to a computer equipped with a video (KY-F55B, Victor, Yokohama, Japan) and an image analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD).

All measurement were statistically evaluated using a 1-way analysis of variance (ANOVA) and Student-Newman-Keuls test for multiple comparisons among the means at $P = .05$. The Barlett method was used to test the assumption that standard deviations of the group were equal.

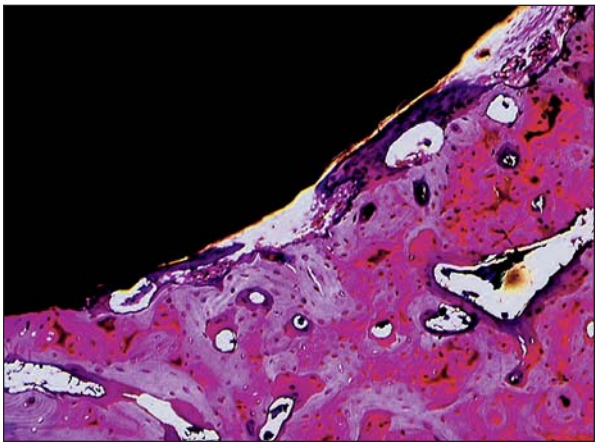

Fig 6 Histologic appearance of an uncoated titanium implant after 4 weeks of implantation. The original drill hole could still be recognized, and the formation of new bone was observed (methylene blue and basic fuchsin; original magnification ×100).

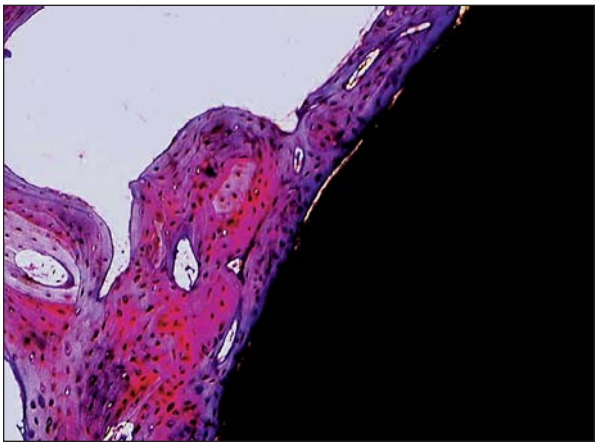

Fig 7 Histologic appearance of a CA-coated implant after 8 weeks of implantation. New bone had formed on the implant surface (methylene blue and basic fuchsin; original magnification ×85).

RESULTS

During the test period, the experimental animals remained in good health. At sacrifice, no clinical signs of inflammation or adverse tissue reactions were seen. All implants were still in situ at sacrifice.

Histology

Implantation Time of 2 Weeks. The overall trabecular bone response to the 2 different implant surfaces was similar (Figs 4 and 5). The original drill hole could be recognized in all cases. Primary woven bone formation was observed. In addition, small bone fragments that had apparently been loosened during the drilling process were observed. The bone fragments were interspersed between the bone marrow spaces.

Implantation Time of 4 Weeks. In these specimens, the healing process had proceeded. In all sections the primary woven bone was reduced in size and had reoriented. The space in the lattice of the primary woven bone was filled with new bone (Fig 6). The new bone was in direct contact with the implant surface without intervening fibrous tissue layers. Despite extensive bone formation, the original drill hole could occasionally still be recognized.

Implantation Time of 8 Weeks. The healing process had further proceeded (Fig 7). The quality of bone was increased compared with that at 4 weeks. Mature bone was seen in direct contact with the implant surface. No signs of an inflammatory response were detected in the tissue surrounding the implants.

Implantation Time of 12 Weeks. At 12 weeks remodeling and compaction of the bone-implant interface were complete (Figs 8 and 9). No clear differences in bone response to the 2 different implants could be seen. The new bone had completely remodeled into mature trabecular bone. The original drill hole could no longer be recognized. All implant surfaces were partially covered with bone. Bone marrow tissue was present in between areas of bone contact. Only 1 implant was seen in direct contact with the epiphyseal growth plate.

Histomorphometric Evaluation

Table 1 shows the results of the measured percentage of bone-implant contact. In both uncoated and CA-coated groups, the percentage of direct bone-implant contact showed a statistically significant increase through the study period. After 12 weeks of implantation, the amount of bone contact was significantly higher ($P < .05$) for both implant surfaces compared with the 4- and 8-week measurements.

At 4 and 8 weeks, no significant difference existed in bone contact between uncoated and CA-coated implants ($P > .05$). However, at 12 weeks, the amount of bone contact for the CA-coated implants was significantly higher compared with the uncoated titanium implant ($P > .05$).

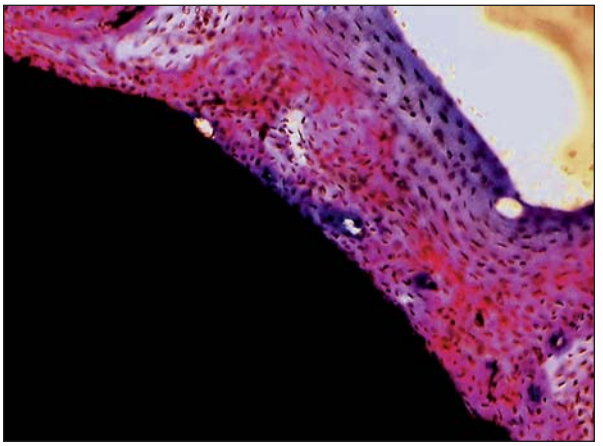

Fig 8 Histologic appearance of an uncoated titanium implant after 12 weeks of implantation. Mature trabecular bone was observed around the implant (methylene blue and basic fuchsin; original magnification ×100).

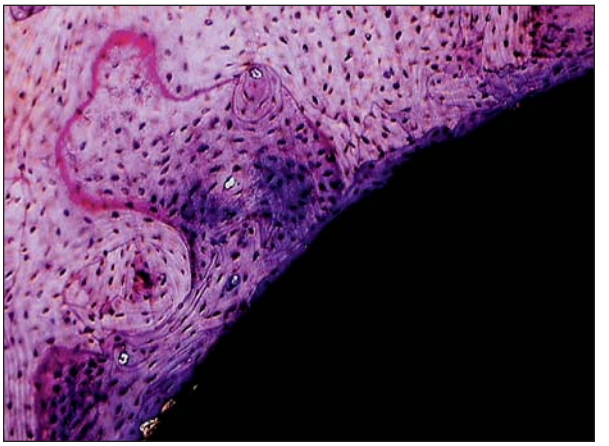

Fig 9 Histologic appearance of a CA implant after 12 weeks. The implant surface was almost completely covered with new bone (methylene blue and basic fuchsin; original magnification ×100).

Table 1 Percentage of the Measured Bone-Implant Contact

	Implantation period					
	4 weeks		8 weeks		12 weeks	
	Mean	SD	Mean	SD	Mean	SD
Uncoated	46.4	16.6	58.2	12.4	71.3	6.9
CA coated	47.7	14.9	61.8	10.8	* 80.7	5.6

*Means were significantly different at $P \leq .05$.

DISCUSSION

The aim of the present study was to evaluate the effect of a CA coating on the trabecular bone response. Based on the histomorphometric analysis, the greatest amount of bone contact was observed with CA-coated implants at 12 weeks.

At 2 weeks of implantation, primary woven bone formation hampered correct quantification of the bone reaction. Nevertheless, the observed effect of the thin CA coating agrees with previous in vitro and in vivo studies.[9–14,25–27] The present results also confirmed a previous SBF immersion experiment.[23] Various hypotheses have already been postulated to explain the effect and final biological mechanism of Ca-P ceramic coatings.[28,29] For example, it has been suggested that increased adsorption of cell adhesion proteins and/or growth factors on Ca-P ceramics evokes increased bone cell adhesion and differentiation. An alternative hypothesis is that dissolution of Ca^{2+} ions from the coating results in a supersaturated interface condition, followed by the deposition of a more bonelike mineral layer.

Previous studies found crystalline thin CA films produced by the molecular precursor method to be stable after 12 months of immersion in PBS solution.[22] A slight decrease of the Ca/P ratio of the coating was observed after PBS immersion.[22] It was assumed that ion exchange reaction occurred between the coated film and PBS solution via the dissolution/precipitation process. The in vivo dissolution behavior of the present CA films is still not clear. In general, adequate insight into the role of CA coatings in bone healing is still lacking.

Wolke and coworkers[30] suggested that 1-μm-thick heat-treated Ca-P sputter coatings on roughened titanium implants appear to be of sufficient thickness to show bioactive properties under in vivo conditions. They placed such implants subcutaneously into the backs of rabbits. Mohammadi and colleagues[31,32] examined the bone response toward calcium phosphate sputter-coated implants with different thicknesses and crystallinities, ie, 0.1-μm and 2.0-μm amorphous and 0.1-μm and 2.0-μm crystalline. They concluded that sputtered crystalline calcium phosphate–coated implants with an ultra-thin 0.1-μm thickness were associated with improved bone response compared with uncoated titanium implants after both early and long-term implantation. No further improvement in the bone response was observed with 2-μm coatings. The current animal study demonstrated that implants with a CA coating with a thickness of approximately 0.16 μm showed greater contact during the healing phase than an untreated control implants.

The advantage of the molecular precursor method is that CA coating can be deposited onto titanium implants of any shape. Moreover, the thin film produced by the molecular precursor method

already possesses a crystalline structure, which avoids the use of postdeposition annealing procedures. For example, PVD methods provide amorphous calcium phosphate coatings, but heat treatment procedures are needed to obtain a crystalline apatitic film.[8] Two steps, deposition of Ca-P film and heat treatment crystallization are needed for PVD methods, while using molecular precursor methods, crystalline Ca-P film can be deposited in 1 step. Film deposition and crystallization are performed at once. The application of CA coatings using the molecular precursor methods on implants with complex geometries or on porous scaffold materials such as titanium fiber mesh will be the topic of a future experiment.

The percentage of bone-implant contact obtained in the present study was comparable with that obtained for Ca-P sputter coated implants.[12] Thus, it can be suggested that thin CA coatings deposited with the molecular precursor method produce bone responses similar to those observed with calcium phosphate coatings deposited with a PVD technique, such as magnetron sputtering.

Finally, the percentage of bone-implant contact for both uncoated and coated implant surfaces was extremely high. Albrektsson and colleagues[33] suggested that osseointegration corresponds to approximately 60% bone contact. The bone contact in the present study was clearly above this limit with or without CA coating. The final efficacy of this increased bone contact has to be demonstrated in long-term studies in which the implants are subjected to clinical loading conditions.

In conclusion, the present study shows that thin CA coatings produced by the molecular precursor method showed greater bone-to-implant contact during the healing phase compared with uncoated controls.

ACKNOWLEDGMENTS

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