Rapid Osseointegration Bestowed by Carbonate Apatite Coating of Rough Titanium

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Titanium (Ti) implants that realize rapid osseointegration are required for favorable outcomes. Rough implant surfaces favor osseointegration, hence, coating implants with natural bone mineral, i.e., carbonate apatite (CO$_3$Ap), may be effective for osseointegration. To achieve rapid osseointegration, rough-Ti substrates are coated with CO$_3$Ap (CO$_3$Ap-Ti) and the effects are evaluated in vitro and in vivo. For comparison, rough-Ti without coating (rough-Ti) and calcite-coated rough-Ti (calcite-Ti) substrates are fabricated. The adhesive strengths of calcite and CO$_3$Ap to the substrates are ≈76.6 and ≈76.8 MPa, respectively, being significantly higher than the strength defined in ISO13779-2 (15 MPa). Calcite and CO$_3$Ap coatings significantly promote preosteoblastic MC3T3-E1 cell proliferation. Additionally, the CO$_3$Ap coating promotes higher osteogenic differentiation activity than the calcite coating. Implantation of CO$_3$Ap-Ti into rabbit tibia defects prompts bone maturation, compared to calcite-Ti or rough-Ti implantation. The bone-implant contact percentage with CO$_3$Ap-Ti and calcite-Ti is higher than that with rough-Ti. Consequently, CO$_3$Ap-Ti acquires a robust bond with the host bone at an early stage (4 weeks postimplantation), compared to calcite-Ti or rough-Ti: the CO$_3$Ap-Ti–bone bonding strength is ≈1.9- and ≈5.5-fold higher than that of calcite-Ti and rough-Ti, respectively. Thus, CO$_3$Ap coating of Ti implants effectively achieve rapid osseointegration.

1. Introduction

Titanium (Ti) is one of the most widely used metals in implant devices and has been extensively used for dental and orthopedic surgery owing to its high mechanical strength and biocompatibility.[1,2] However, initial loosening of nontreated Ti implants often occurs if early loading is applied within 12–16 weeks due to the lack of rapid osseointegration of the implants with the bone.[3] Surface roughening of Ti implants is widely recognized as an effective method to obtain higher success rates for implantation.[4,5] Another effective method is the coating of Ti implants with bioactive materials.[6]

Hydroxyapatite [HAp, Ca$_{10}$(PO$_4$)$_6$(OH)$_2$] is a representative bioactive material, with a composition similar to that of human bone mineral.[7,8] Therefore, HAp coating of Ti implants has been studied for decades.[9–13] However, recent studies have revealed that the effectiveness of HAp coating is limited since poor adhesion and low resorbability of the HAp coating cause delamination upon prostheses and consequently generate osteolysis and aseptic loosening.[14–16] Furthermore, the debris and particles generated via of wear of HAp coatings have been described to induce cytotoxicity and immunogenicity.[17,18] Hence, these issues are associated with a high risk of implant failure.

The coating of Ti implants with natural bone mineral, i.e., carbonate apatite [CO$_3$Ap, Ca$_{10-a}$(PO$_4$)$_6$(OH)$_2$], may resolve the abovementioned challenges faced with HAp coatings as CO$_3$Ap has higher osteoconductivity than HAp.[19–21] Furthermore, CO$_3$Ap coatings can be replaced by bone in coordination with bone remodeling, whereas HAp is poorly resorbed and remains at the implantation site for more than 10 years.[22–24] Additionally, a proportionate amount of calcium and phosphorus ions released from CO$_3$Ap by osteoclastic resorption may promote osteoblastic differentiation.[20,22,25–27] Thus, CO$_3$Ap coating is expected to be effective for rapid osseointegration.

However, the effectiveness of CO$_3$Ap coating has not yet been demonstrated as the coating of Ti implants with CO$_3$Ap cannot be achieved by conventional methods, such as spray coating and spin coating, since CO$_3$Ap is decomposed by heat treatment.[28] Nevertheless, a fundamental technique has been developed herein to fabricate CO$_3$Ap by dissolution-precipitation reactions using calcium carbonate (e.g., calcite) blocks and granules as precursors, while maintaining the precursor shape.[19,21,22,25] Thus, calcite coating of Ti implants is considered to be foundational for effective CO$_3$Ap coating.

The present research group previously succeeded in robust coating of a rough-Ti substrate with calcite.[11,32] Hence, exploiting the calcite coating as a precursor for dissolution-precipitation reactions may be effective for achieving robust CO$_3$Ap coating of rough-Ti. Herein, CO$_3$Ap-coated rough-Ti (CO$_3$Ap-Ti) substrates, with strong adhesive strength between
CO3Ap coating and Ti surface, were fabricated by dissolution-precipitation reactions of calcite-coated rough-Ti (calcite-Ti). Furthermore, the effectiveness of coating Ti substrates with CO3Ap was evaluated in vitro and in vivo by comparison to calcite-Ti and rough-Ti coating methods.

2. Results and Discussion

2.1. Characteristics

The surface and interface morphologies of the samples were evaluated by scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) element mapping (Figure 1 and Figure S1, Supporting Information). The SEM images (Figure 1b) showed that the surface of rough-Ti was rough and uneven with numerous microholes, whereas that of calcite-Ti was fully coated with cubic or needle-like particles, with visible aggregation of crystals and no uncoated regions. The CO3Ap-Ti surface was also coated with a uniform layer composed of spherical particles. Cross-section EDX element mapping (Figure 1c) showed that both calcium and phosphorus ions were detected on the CO3Ap-Ti surface, whereas only calcium ions were present on the calcite-Ti surface. These results suggest that CO3Ap-Ti and calcite-Ti were coated with apatite and calcium carbonate, respectively. Furthermore, the thicknesses of calcite coating and CO3Ap coating were ≈5 and ≈9 µm, respectively.

The surface roughness and topography of the sample surfaces were measured by 3D-laser scanning. The 3D profiles captured for evaluation of the topography (Figure 2a) of each sample revealed that the surface of the CO3Ap-Ti was vastly different in height (peak-to-valley view) compared to calcite-Ti and rough-Ti surfaces. The Rₐ values for arithmetical mean deviation were calculated as a parameter describing the difference in roughness of the sample surfaces (Figure 2b). The Rₐ values of CO3Ap-Ti, calcite-Ti, and rough-Ti were 2.9 ± 0.5, 2.2 ± 0.6, and 2.0 ± 0.5 µm, respectively. Thus, the Rₐ value of CO3Ap-Ti was significantly higher than that of calcite-Ti and rough-Ti (p < 0.05), whereas no significant difference was observed between calcite-Ti and rough-Ti. The appropriate increase in surface roughness of the Ti implant was reported to be conducive to both cell movement and cell growth. For example, the Ti with rough surface (Rₐ ≈ 2.2 µm) had a positive effect on the proliferation and differentiation of MC3T3-E1 cells, compared to Ti with smooth surface (Rₐ ≈ 0.7 µm). In addition, Albrektsson and Wennerberg suggested that an Rₐ of ≈2 µm in a Ti-based implant surface could provide an optimal degree of roughness to promote osseointegration. These findings suggest that the Rₐ values of CO3Ap-Ti, calcite-Ti, and rough-Ti were favorable for reaching potential bone cell differentiation and osseointegration.

The contact angles of rough-Ti, calcite-Ti, and CO3Ap-Ti were 84.3°, 66.6°, and 1.9°, respectively (Figure 2c and Figure S2, Supporting Information). Thus, calcite coating significantly improved the water wettability, i.e., hydrophilicity, of rough-Ti; meanwhile, CO3Ap coating further improved the hydrophilicity of calcite-Ti. The improvement of surface hydrophilicity has been reported to enhance cell attachment, proliferation, and differentiation. Hence, CO3Ap-coating of Ti is considered effective for improving these cellular behaviors.

The X-ray diffractometry (XRD) pattern showed that the rutile phase of Titania was formed on Ti surfaces after annealing (Figure 3a). In the XRD pattern of calcite-Ti, diffraction peaks derived from calcite, as well as Ti and rutile were detected,
indicating that calcite coating was formed on the Ti surface by decomposition of Ca(NO$_3$)$_2$, and subsequent reaction with CO$_2$ at 550 °C. In the XRD pattern of the CO$_3$Ap-Ti, the diffraction peaks for calcite disappeared and typical apatite peaks appeared. In the attenuated total reflection (ATR)-Fourier transform infrared spectroscopy (FTIR) spectrum of calcite-Ti (Figure 3b), carbonated bands were present at 1390 cm$^{-1}$ ($\nu_3$ region), 875 cm$^{-1}$ ($\nu_2$ region), and 720 cm$^{-1}$ ($\nu_4$ region). In the spectrum of HAp, the phosphate bands were observed at 560–600 and 960–1100 cm$^{-1}$, while the hydroxyl band was observed at 630 cm$^{-1}$.[39] In the spectrum of CO$_3$Ap-Ti, the bands assigned to phosphate in apatite were present at 552, 600, 958, and 1010 cm$^{-1}$, whereas the hydroxyl band was not detected. Furthermore, the spectrum of CO$_3$Ap-Ti showed carbonate bands at 870 cm$^{-1}$ in the $\nu_2$ region, and 1408 and 1458 cm$^{-1}$ in the $\nu_3$ region. The doublet carbonate bands at 630 and 900–1200 cm$^{-1}$ in the $\nu_3$ region indicated that carbonate substituted hydroxyl and phosphate, respectively, in the apatite crystal. The above results (Figures 1–3) demonstrate that Ti substrates were coated with calcite, which was then successfully converted into AB-type CO$_3$Ap.[40] The mechanism of formation for CO$_3$Ap coating from calcite coating is described in Equations (1) and (2).

\[
\text{CaCO}_3 \rightarrow \text{Ca}^{2+} + \text{CO}_3^{2-} \quad (1)
\]

\[
\text{Ca}^{2+} + \text{PO}_4^{3-} + \text{CO}_3^{2-} \rightarrow \text{Ca}_{10-a} \left( \text{PO}_4 \right)_{a-b} \left( \text{CO}_3 \right)_{b} \quad (2)
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Calcite contains both Ca$^{2+}$ and CO$_3^{2-}$ and also possesses moderate solubility.[41] When calcite-Ti was immersed in the Na$_3$HPO$_4$ solution, the calcite coating dissolved, thereby supplying Ca$^{2+}$ and CO$_3^{2-}$, as shown in Equation (1). Meanwhile, the Na$_3$HPO$_4$ solution contained PO$_4^{3-}$, allowing for calcite to be transformed into CO$_3$Ap based on the dissolution-precipitation reactions due to the chemical equilibrium, as shown in Equation (2). Subsequently, the CO$_3$Ap coating, with a lower soluble phase than calcite, crossed the saturation limit causing CO$_3$Ap to precipitate at the surface of the calcite coating. The type of CO$_3$Ap was determined by considering where the CO$_3$ site occupied two different positions in the apatite structure.[22,42,43]

The tensile adhesion strengths between coatings and Ti substrates in CO$_3$Ap-Ti and calcite-Ti were measured by pull-off test. The photographs of coatings after this test (Figure 4a) revealed that both CO$_3$Ap-Ti and calcite-Ti showed a primary adhesion failure mode, as illustrated in Figure 4b, ensuring that the value of the adhesive strength between the coating and Ti substrate were accurately measured. The coating adhesive strengths of CO$_3$Ap-Ti and calcite-Ti were significantly different at 76.8 ± 2.7 and 56.6 ± 16.1 MPa, respectively ($p < 0.05$; Figure 4c). To date, the International Organization for Standardization (ISO13779-2, 2018)[44] defined that the mean tensile coating adhesion strength should not be less than 15 MPa, and no individual result should be less than 10 MPa. Both the tensile coating adhesion strengths of CO$_3$Ap-Ti and calcite-Ti were significantly higher than that defined by ISO, suggesting that CO$_3$Ap-Ti and calcite-Ti had sufficient tensile coating adhesion strength for clinical use.

The corrosion resistance of samples was then evaluated via immersion in Ringer solution (isotonic solution relative to the body fluids, pH = 6.7) at 37 °C to mimic corrosion under physiological conditions.[45–47] Samples, i.e., untreated pure Ti (smooth-Ti), rough-Ti, calcite-Ti, and CO$_3$Ap-Ti, were immersed in 10 mL test solution and stored at 37 °C for 7 d. The amount of Ti element dissolved was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES). Results show that the Ti released from samples was below the detection
sensitivity limit, which satisfied the ISO standard for testing metallic materials used in fixed prostheses (ISO 22674). Therefore, CO3Ap-Ti exhibited sufficient corrosion resistance and was considered safe for clinical use.

To more thoroughly demonstrate the advantages of this coating strategy, it will be useful to perform a comparison with an apatite coating generated by biomimetic strategies. However, the current manufacturing process for the biomimetic coating is controlled by many parameters, including the concentration and pH of the solution, as well as the pretreatment strategy of the substrate. These parameters have a significant influence on the phase, composition, crystallinity, and growth rate of apatite coatings, which pose considerable difficulties in actual applications. Hence, the bionic growth process requires further exploration; while the innovation of the current strategy lies in the establishment of a method that readily facilitates the strong coating of implant materials with CO3Ap, which improves the actual application.

2.2. In Vitro Study

To evaluate the effects elicited by the various coatings of Ti substrates on cell proliferation and differentiation, MC3T3-E1 cells were incubated on CO3Ap-Ti, calcite-Ti, and rough-Ti for a maximum of 21 d. After 12 h of culture, the MC3T3-E1 cells adhered to the surface of CO3Ap-Ti and calcite-Ti and exhibited a multilateral spindle shape with pseudopodia (Figure 5a and Figure S3, Supporting Information). In contrast, on the rough-Ti surface, most cells did not spread and remained round or polygonal in shape with few pseudopodia. Fluorescence microscopy images (Figure 5b) also showed that MC3T3-E1 cells on the rough-Ti were polygonal with few pseudopodia at 3 h of culture, whereas cells on calcite-Ti and CO3Ap-Ti were multilateral spindle shape with extended pseudopodia. At 24 h, cells on the rough-Ti became multilateral spindle shape and extended their pseudopodia. Tate et al. revealed that osteoblasts extend their pseudopodia toward their mineralized side and gradually become dendritic during osteogenesis. This finding suggests that calcite coating and CO3Ap-coating are effective for promoting osteogenesis. The fluorescence images also showed that the cell density on the rough-Ti was significantly lower than that on the calcite-Ti and the CO3Ap-Ti (Figure 5b,c).

To further confirm whether the coatings affect the growth of MC3T3-E1 cells, the number of cells in each group was examined by a cell proliferation assay (Figure 5d). At 3 and 7 d, the number of cells on calcite-Ti and CO3Ap-Ti was significantly higher than that on rough-Ti (p < 0.05). Cell viability was also evaluated by live/dead staining, where live and dead cells are stained green and red, respectively (Figure 5e). The fluorescence images showed the following trends in cell viability: calcite-Ti > CO3Ap-Ti > rough-Ti at day 1; and CO3Ap-Ti > calcite-Ti > rough-Ti at day 3 (Figure 5e). However, the quantitative analysis exhibited no significant difference in cell viability on day 1 or 3 between the rough-Ti, calcite-Ti, and CO3Ap-Ti (Figure 5f). Therefore, none of the coatings were found to impair or improve MC3T3-E1 cell viability compared to rough-Ti. The above results demonstrate that calcite coating and CO3Ap-coating of Ti were effective for improving initial cell adhesion and proliferation. Initial cell adhesion is reportedly critical for cell growth, as it triggers the onset of the cell cycle, including proliferation and differentiation, once the cells are attached to a substrate. Thus, the proliferation on the calcite-Ti and CO3Ap-Ti was likely improved owing to the improved initial cell adhesion by calcite coating and CO3Ap-coating of Ti.

The CO3Ap-Ti coating also promoted expression of the osteoblast-related gene that encodes runt-related transcription factor 2 (Runx2) at day 7 (p < 0.05) compared to calcite-Ti and rough-Ti (Figure 6a and Figure S4, Supporting Information). Furthermore, alkaline phosphatase (ALP) activity, a marker of early osteoblast differentiation, in the calcite-Ti and CO3Ap-Ti groups was significantly higher than that in the rough-Ti group (Figure 6b) at day 7. Meanwhile, at day 14, the ALP activity in the CO3Ap-Ti group was significantly higher than that in the calcite-Ti and rough-Ti groups, whereas no significant
difference was observed between the rough-Ti and calcite-Ti groups. Runx2 is required for the expression of multiple osteogenic genes and functions by binding to regulatory sites in osteogenic gene promoters to activate transcription.\[53,57\] Once Runx2 becomes activated, preosteoblasts undergo a multi-stage differentiation with each stage characterized by expression of particular molecular markers (e.g., collagen I, ALP, bone sialoprotein, osteocalcin).\[58–60\] Thus, the earlier expression of high Runx2 expression indicates earlier osteogenic differentiation, e.g., ALP activity or bone nodule formation. Hence, the difference in ALP activity observed on day 14 between CO3Ap-Ti and calcite-Ti was likely associated with the earlier and higher Runx2 expression in the CO3Ap-Ti group. Moreover, Alizarin Red staining revealed the presence of calcific deposition on the rough-Ti, calcite-Ti, and CO3Ap-Ti at day 21 (Figure 6c). The calcified deposition areas (Figure 6d) of rough-Ti, calcite-Ti, and CO3Ap-Ti were 25.3 ± 2.1%, 29.1 ± 2.4%, and 42.9 ± 4.5%, respectively. Thus, CO3Ap-Ti significantly promoted the differentiation of osteoblasts compared to rough-Ti and calcite-Ti (p < 0.05).

### 2.3. In Vivo Study

To evaluate early osseointegration, CO3Ap-Ti, calcite-Ti, and rough-Ti were implanted into rabbit tibia defects (Figure 7a,b). At 4 weeks after implantation, the bone-implant contact (BIC) percentages, an index of osseointegration, were histologically determined by the Villanueva–Goldner staining of tissue sections prepared as shown in Figure 7c. In this process, the mineralized bone (MB) becomes stained green, and osteoid pink (Figure 7d). The histological images at the interface between the implant (i.e., CO3Ap-Ti, calcite-Ti, or rough-Ti) and the host bone (HB) revealed that abundant MB was formed on the CO3Ap-Ti surface, whereas osteoid rather than MB was primarily present on the rough-Ti surface (Figure 7e). The BIC percentages in rough-Ti, calcite-Ti, and CO3Ap-Ti were 48.2% ± 7.8%, 59.9% ± 15.4%, and 72.9% ± 6.4%, respectively (Figure 7f). Thus, the BIC in CO3Ap-Ti was significantly higher than that in rough-Ti and calcite-Ti. These results demonstrate that the CO3Ap coating on Ti implants promoted osteogenesis and bone maturation.

Furthermore, the adhesion strength between the implant and HB was evaluated as another index of osseointegration by the detaching test (Figure 8a). The adhesion strengths of rough-Ti, calcite-Ti, and CO3Ap-Ti to bone were 8.7 ± 4.3, 24.0 ± 8.9, and 42.3 ± 14.7 N, respectively (Figure 8b). Thus, the adhesion strength of CO3Ap-Ti to bone was significantly higher than that of rough-Ti and calcite-Ti. The high adhesion strength of CO3Ap-Ti corresponded to its high BIC. Thus, owing to the high BIC of the CO3Ap-Ti, the connectivity of bone formed on the CO3Ap-Ti surface with HB was ensured, resulting in high adhesion strength between CO3Ap-Ti and HB. The SEM images of rough-Ti, calcite-Ti, and CO3Ap-Ti surfaces after the detaching test showed that bone tissues were formed on these surfaces (Figure 9). Notably, abundant bone tissue was observed on the CO3Ap-Ti surface (Figure 9c), compared to rough-Ti and calcite-Ti surfaces (Figure 9a,b). Importantly, a portion of CO3Ap coating remained (Figure 9c), whereas the calcite coating completely disappeared (Figure 9b). These differences in resorption speed were derived from the fact that CO3Ap was primarily resorbed by osteoclasts, while calcite was spontaneously dissolved. Based on the above in vitro and in vivo results, the exceedingly rapid loss of calcite coating caused only extremely short-term positive effects on cell proliferation and differentiation within 1 week; however, these positive effects were lost within 2 weeks. In contrast, the gradual resorption of CO3Ap coating produced improvements in cell proliferation and differentiation for a longer period of time. Even within 1 week, the effects of CO3Ap coating were nearly equal to those of calcite coating, according to in vitro results. Therefore, the resorption speed of CO3Ap coating was considered suitable within 4 weeks. Compared to the other coating, the adequate resorption speed of CO3Ap led to superior osseointegration and subsequent replacement of the coating by bone in coordination with bone remodeling, resulting in high BIC and high adhesion strength to bone.

### 3. Conclusion

Robust coating of Ti substrates with CO3Ap was achieved. The CO3Ap-Ti promoted cell proliferation and differentiation, compared to rough-Ti and calcite-Ti. Furthermore, the BIC and
adhesion strength to bone achieved with CO3Ap-Ti was significantly higher than those with rough-Ti and calcite-Ti. Thus, CO3Ap coating of Ti implants was effective for enhancing osseointegration. This study demonstrates the coating effects within a 4 week period. In a future study, the longer-term effects of coating will be investigated.

4. Experimental Section

Materials: Unless otherwise stated, all chemicals used in this study were purchased from Wako Pure Inc., Co. (Osaka, Japan). Grade II commercial pure Ti (smooth-Ti) was purchased from T&I Co (Saitama, Japan). Two specifications of pure Ti, disks and plates, were prepared for in vitro and in vivo evaluations, respectively. The Ti disks were 14.5 mm

Figure 5. a) SEM images revealing the morphology of MC3T3-E1 cells on the surface of rough-Ti, calcite-Ti, and CO3Ap-Ti at 12 h after seeding (magnification × 500). b) Fluorescence images of attachment of MC3T3-E1 cells on rough-Ti, calcite-Ti, and CO3Ap-Ti for 3 and 24 h. F-actin were stained with Phalloidin (red) and the nuclei were stained with Hoechst (blue) c) The cell density of MC3T3-E1 on samples after 3 and 24 h of culture (n = 4). d) Cell number determined by the cell counting kit-8 (CCK-8) proliferation assay at 3, and 7 d (n = 4). e) Live–dead cell assay after 1 and 3 d of culture in rough-Ti, calcite-Ti, and CO3Ap-Ti; Viable cells are green and dead cells are red (images magnification: ×10). f) Quantitative analysis of live–dead cells (n = 4). *p < 0.05 ***p < 0.001 for comparisons between the indicated groups.
in diameter and 1 mm in height, and the Ti plates were 10 mm in length and 1 mm in height. The mouse preosteoblastic cell line MC3T3-E1 was obtained from Cell Bank (Riken Biosource, Saitama, Japan), aged 19–20 weeks and weighing between 2.9 and 3.1 kg, were used in this study. The animals were housed and cared in the official animal center at Kyushu University and fed standard diet and water ad libitum.

Sample Preparation: The sample preparation was modified from a previous study. In brief, the pure Ti was roughened in a dual-acidic solution of 50 vol% H2SO4 and 7 vol% HCl for 30 min at 70 °C. The rough-Ti was then removed from the acid solution and washed in an ultrasonic cleaner for 15 min with ethanol and distilled water. The roughening procedure was concluded, and the samples were allowed to dry at room temperature overnight. Afterward, Ca(NO3)2 ethanol solution (0.5 µ) was prepared as the starting material. The Ca(NO3)2 solution (8.25 µL) was added dropwise to both surfaces of Ti substrate. When the droplet covered the whole Ti surface, the samples were placed in an electric furnace for heat treatment. The heating program was as follows: the furnace was set to 550 °C at a heating rate of 3 °C min−1, kept at constant temperature for 5 h, and then cooled in the furnace to room temperature naturally. The process was performed under 100 mL min−1 CO2 gas flow. In addition, the rough-Ti without Ca(NO3)2 was treated following the above-explained heat protocol to generate control samples. For composition conversion of the coating from calcite to CO3Ap, each calcite-Ti was soaked in Na2HPO4 aqueous solution (30 mL, 0.2 µ) at 80 °C for 7 d. As a result, CO3Ap-Ti was obtained. The CO3Ap-Ti was immersed in distilled water (30 mL) at the same temperature for 24 h to remove the residual Na2HPO4 solution from the Ti surface. Finally, the CO3Ap-Ti was rinsed with distilled water three times and dried at room temperature. Although the ultrasonic process caused the calcite coating to partially peel from the substrate, CO3Ap coating seems to resist the shedding due to its high coating strength (Figure S5, Supporting Information). Therefore, CO3Ap coating may prove to be washable by ultrasonic treatment. For cell and animal experiments, all samples were dry heat-sterilized at 170 °C for 1 h.

Analysis of Surface Characteristics: The surface and cross-sectional morphologies of the sample were acquired by SEM. X-ray intensities of calcium (Ca, purple), phosphorus (P, yellow), and titanium (Ti, cyan) were analyzed across the interface to evaluate the thickness of the coating. The coating polymorphism on the Ti surface was identified by XRD. The chemical structure of the coating was analyzed by FTIR with ATR accessories (FT-IR-6200; Japan Spectroscopic Company (JASCO) Co., Tokyo, Japan); the background spectrum was obtained by scanning the noncoated rough-Ti substrate. Additionally, HAp powders (HAp-200, Taihei Chemical Industrial Co., Osaka, Japan) were analyzed by ATR-FTIR and served as a control. The arithmetic mean deviation surface roughness (Rq) and topography were measured by laser scanning confocal microscopy (VK-9710, Keyence, Japan). Contact angles were measured with a contact angle meter (DM500, Kyowa Interface Science, Saitama, Japan); measurements were made after carefully adding water dropwise (1 µL per droplet) onto the material surfaces for 60 s (n = 4). The adhesive tensile strength of the coating on Ti was determined by pull-off test, using a universal mechanical strength tester (Romulus, Quad Group Inc., NY, USA). The surface of coated Ti was bound to the stud with prefabricated thermosetting resin (diameter of 2.7 mm). The stud was then pulled off at a rate of 2 mm s−1, and the load was recorded until the sample detached from the Ti surface. The maximum load was recorded and converted to pressure as adhesive strength of the coating.

Ti Release from Samples: ICP-AES (Optima 7300DV, PerkinElmer, MA, USA) was applied to analyze the amount of Ti ion released from
samples. One plate of smooth-Ti, rough-Ti, calcite-Ti, and CO₃Ap-Ti were immersed in 10 mL of Ringer solution (Otsuka, Osaka, Japan) containing 86 mg NaCl, 3 mg KCl, and 3.3 mg CaCl₂·2H₂O (pH 6.7) at 37 °C. The total number of Ti ions released from samples into the solution were measured over a period of 7 d.

**Cell Culture:** The cells were cultured in growth medium consisting of alpha-minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 U mL⁻¹), streptomycin (100 µg mL⁻¹), and amphotericin B (0.25 µg mL⁻¹; Thermo Fisher K.K., Tokyo, Japan). The cells were cultured at 37 °C in a humidified air atmosphere containing 5% CO₂. The growth medium was replaced every 3 d until the cells approached confluence. Then, the confluent cells were harvested using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Gibco, Thermo Fisher K.K., Grand Island, NY, USA), and seeded into new culture dishes after centrifugation. The above operations are repeated until sufficient amounts of cells were obtained for each test.

**Cell Attachment:** The overall condition of MC3T3-E1 cells attached to the surface of Ti was observed by SEM after a 12 h culturing period. In brief, MC3T3-E1 cells were seeded on each Ti sample for observation of attachment (as mentioned above) in 24-well plates at 1 × 10⁴ cells per well (n = 4). The growth medium was aspirated from each well, and cells were washed twice with phosphate buffered saline

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Figure 7. Illustration of the in vivo evaluation including a) work plan and schematics of the animal model, b) surgical photographs of the defect created in the tibia (10 mm in length × 1.6 mm in width) and of the bone harvested at 4 weeks postimplantation and c) illustration of the sectioning and staining method (Villanueva–Goldner stain). Representative Villanueva–Goldner-stained histological sections: d) general view of a representative histological section and e) high-magnification view of the cortical bone of sections in rough-Ti, calcite-Ti, and CO₃Ap-Ti after 4 weeks of healing showing bone-implant contact (BIC) and osteoid (Os). Yellow arrows indicate BIC areas. Black arrows highlight unmineralized bone areas. f) Histomorphometric analysis of newly regenerated bone at 4 weeks after implantation: BIC in rough-Ti, calcite-Ti, and CO₃Ap-Ti (n = 3). *p < 0.05 **p < 0.01 for comparisons between the indicated groups.
(PBS). The washed cells were then fixed with 1 mL of 3% glutaraldehyde in sodium phosphate buffer at 4 °C for 30 min. Subsequently, the cells were dehydrated in a graded series of ethanol from 50% to 99.5% and evaporated using hexamethyldisilazane. Before SEM observation, the Ti samples with fixed cells were sputter-coated with gold-palladium.

Immunofluorescence Staining: MC3T3-E1 cells were seeded on samples in 24-well plates for 3 and 24 h with an initial density of $5 \times 10^4$ cells per well ($n = 4$), rinsed thrice with PBS, and fixed with 4% paraformaldehyde for 10 min. The fixed cells were then permeated by 0.5% Triton X-100 for 5 min and blocked with PBS with Tween-20 (PBST) containing 1% bovine serum albumin for 30 min at ~20 °C. The cells were stained for actin using Acti-stain 555 phalloidin (PHDH1-A; Cytoskeleton Inc., Denver, USA) with Hoechst 33342 (Dojindo) as a nuclear stain. Between each step, the samples were washed with PBS or PBST. Finally, the samples were fixed with mounting medium and stored at 4 °C in dark. Fluorescence images were captured using a fluorescence microscope (BZ-X710; Keyence CO., Tokyo, Japan) and cell density was quantified microscopically and expressed as cells cm$^{-2}$.

Cell Proliferation: MC3T3-E1 cells were cultured at a density of $2 \times 10^4$ cells per well ($n = 4$) under the conditions explained above for proliferation assessment. The culture medium was changed every 2 d. At 3, and 7 d, the proliferation of cells was determined using cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan). After washing with PBS three times, the samples were incubated in growth medium supplemented with CCK-8 solution at 37 °C for 1 h, and 100 µL of the resulting medium was then transferred to a 96-well plate. The absorbance was measured at 450 nm with a microplate reader (M200; Tecan Group Ltd., Victoria, Australia).

Live–Dead Cell Test: Live–dead cell viability was analyzed to compare cellular growth on each Ti group. MC3T3-E1 cells were seeded on samples in 24-well plates for 1 and 3 d with an initial density of $5 \times 10^4$ cells per well ($n = 4$). Next, a live–dead viability assay kit for mammalian cells (Kit II, Takara, Shiga, Japan) was used to determine the percentage of live cells to dead cells for both platforms. Calcein-AM ($2 \times 10^{-6}$ M) and ethidium homodimer-1 ($4 \times 10^{-6}$ M) were added to PBS and incubated with the cells at room temperature for 40 min. Subsequently, fluorescence images were acquired as previously described, and image analyses for cell counts were performed via fluorescence microscopy.

ALP Activity: To examine the ALP activity, MC3T3-E1 cells were seeded in 24-well plates at an initial density of $6 \times 10^4$ cells per well ($n = 4$). The cells were cultured in differentiation medium prepared by supplementing the growth medium with β-glycerol phosphate ($10 \times 10^{-3}$ M) and ascorbic acid (50 µg mL$^{-1}$). The culture medium was changed every 2 d. After 7 and 14 d, the cells were washed in PBS three times and lysed using cell lysis buffer M, and the ALP activity was then assessed using a LabAssay ALP kit. The relative activity was normalized against the total

Figure 8. Illustration of the a) detaching test in bone harvested at 4 weeks after implantation of Ti plates in the tibia defects of rabbits and b) adhesion strength of bone-to-implant in rough-Ti, calcite-Ti, and CO$_3$Ap-Ti ($n = 8$). * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ for comparisons between the indicated groups.

Figure 9. SEM images of the surface of rough-Ti, calcite-Ti, and CO$_3$Ap-Ti at 4 weeks after implantation in the tibia defects of rabbits (magnification ×1000).
protein concentration of each sample, which was determined using the Protein Assay Rapid Kit.

Osteogenic Gene Expression: The samples and MC3T3-E1 cells (5 × 10⁴ cells per well) were incubated in the above differentiation medium for 7 or 14 d. Next, the total RNA was isolated using a Total RNA Kit for Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) (CellAmp Direct RNA Prep Kit, Takara, Shiga, Japan) and reverse transcribed to cDNA using a One Step TB Green PrimeScript qRT-PCR Kit (Takara, Shiga, Japan). qRT-PCR analysis was performed in triplicate with a Bio-Rad CFX Connect Real-Time PCR Detection System. Runx2 gene expression was measured, and glyceraldehyde-3-phosphatedehydrogenase (Gapdh) was selected as the housekeeping gene for normalization. The real-time PCR cycling conditions were 95 °C for 10 s and 60 °C for 30 s at 40 cycles. The primer sequences for the abovementioned gene are listed in Table S1 in the Supporting Information.

Alizarin Red Stain: Osteogenic differentiation was induced by culturing cells for 21 d in differentiation medium. Differentiation was examined by staining extracellular matrix calcification with Alizarin Red S (Calcified Nodule Staining kit; Cosmo Bio Co., Ltd., Tokyo, Japan). The calcified nodule was stained red-purple, and the stained area (%) was analyzed with software of ImageJ (n = 4).

Surgical Procedure: Animal experiments were conducted following the protocols approved (Approval ID: A19-144-0) by the Animal Care and Use Committee of Kyushu University. For surgery, the rabbits were anesthetized with intramuscular injection of ketamine (30 mg kg⁻¹) and xylazine (5 mg kg⁻¹). After shaving and disinfecting with iodine, the proximal tibia was exposed. A hole (1.6 mm in diameter) was made 2 cm away from the knee joint using a straight drill (φ1.6 mm, K-501, HOZAN TOOL Inc., Osaka, Japan), and then a straight defect (1.6 mm × 10 mm) was created by sawing straightly from that hole with a diamond file (φ1.6 mm, SB-178D, TAIYO SEIKO Inc., Osaka, Japan) under saline rinsing. Ti implants were placed into the defects, and the incisions were closed with silk sutures. Four weeks later, the rabbits were euthanized by an overdose of anesthesia, and 24 epiphyses of the tibia block with Ti were harvested.

Bone Bonding Test and Osseointegration Observation: Bone was fixed in 10% formalin and dehydrated in a graded alcohol solution, and then embedded in methacrylate. The embedded tissue was then sectioned into 1 mm thick slices and ground to the final thickness of 50 μm. In order to quantify the osseointegration around the Ti implant, the sections were subjected to Villanueva–Goldner staining and observed using a microscope (BX2710, Keyence, Osaka, Japan), and the obtained photographs were analyzed to calculate the BIC. BIC is defined as direct contact between bone and implant without fibrous tissue; the chosen area of the implant surface, only at the parts that contained compact bone, was 2 mm wide (n = 3). The details for the BIC calculation are shown in Figure S6 in the Supporting Information. The harvested bone block with Ti was carefully trimmed to isolate the bone tissue on both sides and at the edges of the plate. A load test device was set to apply alterable traction to the bone. The detaching failure load was recorded when the implant separated from the bone (n = 8). After testing, the surface of implants was studied using SEM.

Statistical Analysis: Statistical and graphical analysis was performed using Prism 8.2.1 (GraphPad Software, Inc., La Jolla, CA, USA). The data gathered from each experiment were used to calculate the mean with standard deviation. Base on the normal distribution of data and group numbers in each experiment, Student t-test and one-way Analysis of Variance (ANOVA) (Fisher’s Least Significant Difference test and Kruskal–Wallis test) were chosen correspondingly to analyze the statistical difference. A probability level less than 0.05 (p < 0.05) was considered a significant difference between two groups.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Keywords
calcite, carbonate apatite, coatings, osseointegration, titanium

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