

Bioactive effects of strontium loading on micro/nano surface Ti6Al4V components fabricated by selective laser melting

Yu Shimizu^{a,*}, Shunsuke Fujibayashi^a, Seiji Yamaguchi^b, Shigeo Mori^c, Hisashi Kitagaki^c, Takayoshi Shimizu^a, Yaichiro Okuzu^a, Kazutaka Masamoto^a, Koji Goto^a, Bungo Otsuki^a, Toshiyuki Kawai^a, Kazuaki Morizane^a, Tomotoshi Kawata^a, Shuichi Matsuda^a

^a Department of Orthopaedic Surgery, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan

^b Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan

^c Osaka Yakin Kogyo Co., Ltd., 4-4-28 Zuiko, Higashiyodogawa-ku, Osaka 533-0005, Japan

ARTICLE INFO

Keywords:

Selective laser melting
Ti6Al4V
Strontium
Solution treatment
Nanoscale network
Osseointegration

ABSTRACT

Selective laser melting (SLM) titanium alloys require surface modification to achieve early bone-bonding. This study investigated the effects of solution and heat treatment to induce the sustained release of strontium (Sr) ions from SLM Ti6Al4V implants (Sr-S64). The results were compared with a control group comprising an untreated surface [SLM pure titanium (STi) and SLM Ti6Al4V (S64)] and a treated surface to induce the release of calcium (Ca) ions from SLM Ti6Al4V (Ca-S64). The surface-treated materials showed homogenous nanoscale network formation on the original micro-topographical surface and formed bone-like apatite on the surface in a simulated body fluid within 3 days. *In vitro* evaluation using MC3T3-E1 cells showed that the cells were viable on Sr-S64 surface, and Sr-S64 enhanced cell adhesion-related and osteogenic differentiation-related genes expression. *In vivo* rabbit tibia model, Sr-S64 provided significantly greater bone-bonding strength and bone-implant contact area than those in controls (STi and S64) in the early phase (2–4 weeks) after implantation; however, there was no statistical difference between Ca-S64 and controls. In conclusion, Sr solution and heat treatment was a safe and effective method to enhance early bone-bonding ability of S-64 by improving the surface characteristics and sustained delivery for Sr.

1. Introduction

Titanium (Ti) alloys, particularly the Ti6Al4V metals, are widely used in the orthopedic and dental fields because of their excellent biocompatibility and mechanical properties. Ti6Al4V metals have two major problems; complicated structures are difficult to be fabricated by traditional manufacturing methods, and smooth Ti6Al4V metals have limited bone-bonding ability [1].

Applying additive manufacturing (AM) to the field of orthopedic and dental implants is one of the solutions to the first problem. Selective laser melting (SLM) and selective electron beam melting (SEBM) are two representative metallic AM technologies, which have enabled the direct fabrication of predefined three-dimensional porous metals with complicated internal structures derived from metal powders. The primary difference between SLM and SEBM is the energy

source used to melt the powder material. SLM has the advantages of constructing scaffolds with detailed features because the beam energy and diameter used in SLM is smaller than that used in SEBM. AM technologies have been successfully utilized to produce titanium alloy implants, and this field will attract more attention in the future. AM-produced Ti6Al4V implants are already in clinical use [2], and AM-produced β -type titanium alloys such as Ti-35Nb-2Ta-3Zr, which is an alloy of low modulus, have been recently developed [3].

Tailoring the implant surface properties to regulate the surrounding bone response is one of the solutions to the second problem. SLM-Ti6Al4V alloys as fabricated characteristically have rough surfaces with Ra values of $\sim 20 \mu\text{m}$, which is considered to be beneficial for increasing surface area and initial interference stability [4]. However, some concerns have been reported regarding poor osseointegration and subsequent loosening even in highly porous AM implants [5]. Tsukanaka

Abbreviations: SLM, selective laser melting; AM, additive manufacturing; WCA, water contact angle; BIC, bone implant contact; STi, commercial pure titanium fabricated by selective laser melting; S64, Ti6Al4V fabricated by selective laser melting; Ca-S64, Ca solution and heat-treated Ti6Al4V fabricated by selective laser melting; Sr-S64, Sr solution and heat-treated Ti6Al4V fabricated by selective laser melting

* Corresponding author.

E-mail address: ys12687@kuhp.kyoto-u.ac.jp (Y. Shimizu).

<https://doi.org/10.1016/j.msec.2019.110519>

Received 22 August 2019; Received in revised form 27 November 2019; Accepted 2 December 2019

Available online 05 December 2019

0928-4931/ © 2019 Elsevier B.V. All rights reserved.

et al. demonstrated that bioactive surface treatment was necessary to improve bone-bonding ability of SLM-Ti because the original surface of SLM Ti did not have a positive effect on osteoblast differentiation [6]. SLM implants are often applied in challenging situations, such as surgery in patients with critical bone defects or osteoporosis [2,7]. Therefore, early chemical integration on the bone-implant interface should be warranted and many recent studies have investigated the surface modification of SLM implants [8,9].

The incorporation of bioactive trace elements, such as calcium (Ca), magnesium (Mg), zinc (Zn), and strontium (Sr), are typical surface modifications. Strontium ranelate, which contains two cations of Sr, is a proven therapeutic drug for postmenopausal osteoporosis [10]. Sr is commonly thought to regulate bone metabolism as a dual-action bone agent that promotes the proliferation of pre-osteoblasts, the differentiation of osteoblasts and the inhibition of osteoclast differentiation and activation [11,12]. This modulation of bone turnover through the promotion of bone formation and the simultaneous inhibition of bone resorption is considered effective even in the peri-implant regeneration process [13]. Recently, the local administration of Sr from functionalized implant surfaces has been reported to allow the higher local dose and avoid systemic side effects [14], as well as enhance implant osseointegration [15]. We have recently developed a method to induce strontium to pure Ti plates by devising alkali and heat treatment and confirmed its superiority over conventional alkali and heat treatment *in vitro* and *in vivo* [16,17].

To our knowledge, few studies have investigated the effects of Sr incorporation on SLM Ti6Al4V surfaces. The purpose of this study was to elucidate the effects of the solution and heat treatment to induce the release of Sr ions on SLM-Ti6Al4V by conducting surface analysis, *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Sample preparations

The SLM materials were fabricated as previously reported [17]. Dense cylindrical (diameter 18 mm, thickness 2 mm) implants for *in vitro* study and dense cuboid (15 × 10 × 2 mm) implants for *in vivo* study were designed using a computer aided-design program (Magics®, Materialise, Leuven, Belgium) and manufactured by SLM technology. SLM pure Ti implants (STi), as a negative control without alloy components were fabricated using commercially pure Ti powder (> 99.5% pure) with particle diameters < 45 µm (Osaka Titanium Technologies, Amagasaki, Hyogo, Japan) by an EOSINT M270 SLM machine (Electro Optical Systems, Krailling, Germany). SLM Ti6Al4V implants (S64) were fabricated using commercial Ti6Al4V alloy powder with particle diameters < 51.4 µm by an EOS M290 machine (Electro Optical Systems, Krailling, Germany). The implants were processed in an atmosphere of Ar with Yb fiber laser system. The process parameters for the EOSINT M270 SLM machine were as follows: laser power, 120 W; scanning speed, 225 mm/s; hatch spacing, 120 µm; hatch offset, 20 µm. The process parameters for the EOS M290 SLM machine were Electro Optical Systems recommended conditions (not disclosed). The powder layer thickness was set at 30 µm for both machines. Fabricated SLM materials were washed with acetone, 2-propanol, and ultrapure water for 30 min each in an ultrasonic cleaner and then dried.

2.2. Surface treatment

Among the samples prepared, S64 was subjected to two types of solution and heat treatment according to two previously reported procedures; Ca solution and heat treatment (Ca-S64) [18]; and Sr and Ca solution and heat treatment (Sr-S64) [17]. Briefly, S64 was initially treated with 5 M NaOH solution at 95 °C for 24 h and then gently rinsed with ultrapure water for 30 s. Subsequently, for the Ca treatment, S64 metals were soaked in 100 mM CaCl₂ solution at 40 °C for 24 h, heated

Table 1

Surface treatment of SLM samples used in this study. Abbreviations: STi (commercial pure titanium fabricated by SLM), S64 (Ti6Al4V fabricated by SLM), Ca-S64 (Ca solution and heat-treated Ti6Al4V fabricated by SLM), Sr-S64 (Sr solution and heat-treated Ti6Al4V fabricated by SLM), WCA (water contact angle).

Implants	1st step	2nd step	3rd step	4th step
STi	None	None	None	None
S64	None	None	None	None
Ca-S64	5 M NaOH; 95 °C; 24 h	100 mM CaCl ₂ ; 40 °C; 24 h	600 °C; 1 h	H ₂ O; 80 °C; 24 h
Sr-S64	5 M NaOH; 95 °C; 24 h	50 mM CaCl ₂ + 50 mM SrCl ₂ ; 40 °C; 24 h	600 °C; 1 h	1 M SrCl ₂ ; 80 °C; 24 h

to 600 °C for 1 h, and finally soaked in ultrapure water at 80 °C for 24 h. For the Sr treatment, S64 metals were soaked in a mixed solution of 50 mM CaCl₂ + 50 mM SrCl₂ solution at 40 °C for 24 h, heated to 600 °C for 1 h, and treated with 1 M SrCl₂ solution at 80 °C for 24 h (Table 1). Thus, we prepared STi and three types of S64 samples (S64, Ca-S64, Sr-S64) and sterilized them with ethylene oxide gas before *in vitro* and *in vivo* experiments (Fig. 1).

2.3. Sample surface analysis

2.3.1. Surface morphology and microstructure characterization

The surfaces of the three types of SLM Ti6Al4V samples (S64, Ca-S64, Sr-S64) were observed by field emission scanning electron microscopy (FE-SEM; S-4300, Hitachi Co., Tokyo, Japan) to investigate the changes in the surface morphology induced by chemical solution and heat treatment. For the microstructural observation, cross-sections of the samples perpendicular and parallel to the building direction were prepared, and were subjected to subsequent mechanical polishing and acid etching (HNO₃:H₂O (10:90 vol%)). The microstructures were examined using an inverted metallurgical microscope (Eclipse MA200, Nikon, Japan).

2.3.2. Apatite-forming ability in the simulated body fluid (SBF)

The three types of SLM Ti6Al4V samples (S64, Ca-S64, Sr-S64) were soaked in 40 mL of SBF (ISO 23317) with ion concentrations nearly equal to those of human blood plasma: Na⁺ 142.0, K⁺ 5.0, Mg²⁺ 1.5, Ca²⁺ 2.5, Cl⁻ 147.8, HCO₃⁻ 4.2, HPO₄²⁻ 1.0, and SO₄²⁻ 0.5 mM, at 36.5 °C for 3 days [19]. Subsequently, the samples were removed from the SBF, gently washed with ultrapure water and dried at 40 °C. Apatite formation on the surfaces of the samples was examined by FE-SEM.

2.3.3. Energy-dispersive X-ray analysis of the surface

The surfaces of the SLM Ti6Al4V samples (S64, Ca-S64, Sr-S64) were examined for elemental compositions with an energy-dispersive X-ray spectrometer (EDX; EMAX-7000, Horiba Ltd., Kyoto, Japan) for Ti, Al, V, O, Ca and Sr. Measurements were made at five locations and the average value was used for analysis.

2.3.4. X-ray photoelectron spectroscopy

The elemental distributions of the treated SLM Ti6Al4V samples (Ca-S64 and Sr-S64) were analyzed at a near-surface depth using X-ray photoelectron spectroscopy (XPS; PHI 5000 Versaprobe II, ULVAC-PHI, Inc., Kanagawa, Japan) under Ar sputtering at a rate of 4 nm/min (SiO₂ conversion). In this analysis, an Al Kα radiation line was used as the X-ray source. The XPS take-off angle was set at 45°, which enabled the system to detect photoelectrons to a depth of 1–5 nm from the surface.

2.3.5. Thin-film X-ray diffraction

The surface structures of the four types of samples were analyzed using thin-film X-ray diffractometry (TF-XRD; model RNT-2500, Rigaku Co., Japan). In TF-XRD, the measurement was performed using a CuK X-

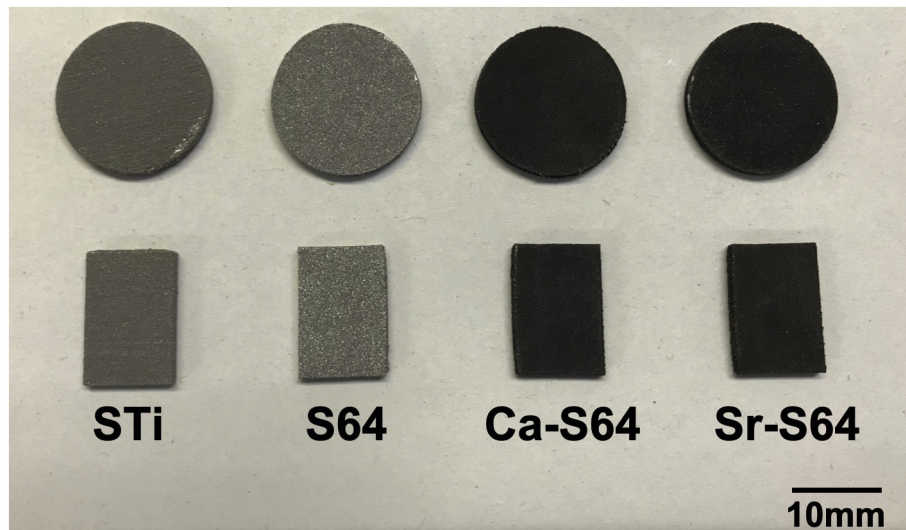


Fig. 1. Sample appearance of four groups of SLM-Ti, SLM-Ti6Al4V untreated and treated implants.

ray source at 50 kV and 200 mA. The glancing angle of the incident beam was set to 1° against the sample surface.

2.3.6. Ca and Sr ion release

To evaluate the release of Ca and Sr ions, the treated SLM Ti6Al4V samples (Ca-S64 and Sr-S64) were immersed in 3 mL of phosphate-buffered saline (PBS) at a pH of 7.4 at 36.5°C , then gently shaken for up to 14 days. Ca and Sr ion eluting concentrations in the PBS were detected by inductively coupled plasma emission spectroscopy (ICP; SPS3100, Seiko Instruments Inc., Japan) [17]. Three samples were severally immersed in PBS that was renewed at every time point, and the average values of their accumulative ion release were used for analysis.

2.3.7. Wettability

The wettability of the four types of samples was evaluated by water contact angle (WCA) as previously reported [20]. A 4 μL ultrapure water was dropped onto each surface, and the contact angle was measured. Three samples were examined independently for each group, and the mean values were used for analysis.

2.3.8. Surface micrometer-scale roughness

The topography of the surfaces was measured using a contact probe profilometer (Mitsutoyo SurfTest SJ-210) on a micrometer scale. Three implants in each group were measured and each implant was measured five times in the vertical and horizontal direction, and the mean Ra was calculated.

2.4. In vitro study

In all the *in vitro* experiments, osteoblast-like cell line MC3T3-E1 cells were cultured in α -MEM (Gibco, USA) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C with 5% CO_2 in a humidified atmosphere, and the medium was changed every 2 days. After incubation for the required period, each experiment was performed as described below. Four or five discs for each of the four types of STi and S64 groups (S64, Ca-S64, Sr-S64) were used per experiment, and the experiments were repeated at least three times to confirm the similar qualitative trends of these experiments.

2.4.1. Confirmation of cell adhesion by SEM and immunostaining

MC3T3-E1 cells were seeded onto STi, S64, Ca-S64 and Sr-S64 disc samples in 12-well plate at 5×10^4 cells/well and cultured for 5 h and 24 h. Each sample was washed with PBS and fixed in 2.5%

glutaraldehyde at 4°C for 2 h on ice, followed by dehydration in an ethanol series. The substrates were subsequently freeze-dried, coated with a thin Pt-Pd film and observed using FE-SEM (S-4700, Hitachi Co., Tokyo, Japan). For Immunofluorescence staining, MC3T3-E1 cells were seeded onto the four samples at 2×10^4 cells/well. After 3 h and 24 h of culturing, the cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After blocking with 4% FBS, the cells were stained with rhodamine-phalloidin for 20 min to detect actin, and with DAPI for 10 min in the dark to detect nucleus. Observations were performed using fluorescence microscopy (BZ-X710, Keyence, USA).

2.4.2. Cell proliferation

Cellular proliferation, viability, and cytotoxicity were quantified using XTT assays, which analyze the number of viable cells by measuring the cleavage of the yellow tetrazolium salts added to the culture medium. MC3T3-E1 cells were seeded onto the four samples at 5×10^4 cells/well. After 3 and 5 days of culturing, XTT working solution (Roche Applied Science, USA) was added onto all substrates, which were then incubated for 4 h at 37°C . Following incubation, 150 μL samples of the medium were transferred to 96-well plates, and the OD values (A405 nm–A620 nm) were measured on a microplate reader (iMark™ Microplate Absorbance Reader, BIO-RAD Laboratories, Hercules, California).

2.4.3. Alkaline phosphatase (ALP) activity

MC3T3-E1 cells were seeded onto the four samples at 5×10^4 cells/well and cultured for 7 and 14 days. After incubation at 37°C with 6.7 mM *p*-nitrophenyl phosphate (pNPP) (Wako Pure Chemical Industries, Japan) for 30 min, the absorbance of *p*-nitrophenol liberated in the reactive solution was measured on a microplate reader and ALP activity was calculated. The ALP levels were normalized against the total protein level in the cell lysates, and the values relative to control (S64) were calculated.

2.4.4. Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis

MC3T3-E1 cells were seeded onto the four samples at 5×10^4 cells/well and cultured for 7 days. The total RNA of the cells attached to each sample was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed using ReverTra Ace qPCR Master Mix (Toyobo, Japan). RT-qPCR was performed in triplicate on a Step One Plus system (Applied Biosystems, Foster City, CA, USA) using Thunderbird SYBR qPCR Mix (Toyobo, Japan). The primer sequences are described in

Table 2
Primer sequences of each target gene used for RT-PCR in the current study.

Gene	Primer sequence (F, forward; R, reverse; 5'-3')
<i>Gapdh</i>	F: TGTCCGTCGTGGATCTGAC R: CCTGCTTCACCACTTCTTG
<i>Integrin-β1</i>	F: TTGGGATGATGTCGGGAC R: AATGTTTCAGTGCAGAGCC
<i>Alp</i>	F: ACTCAGGGCAATGAGGTCAC R: CACCCGAGTGGTAGTCACAA
<i>Ocn</i>	F: AGACTCCGGCGCTACCTT R: CTCGTCACAAGCAGGGTTAAG
<i>Opn</i>	F: GGAGGAAACCAGCCAAGG R: TGCCAGAATCAGTCACATTCAC

Table 2. mRNA expression levels were normalized to those of *Gapdh*, and the values relative to control (S64) were calculated.

2.5. In vivo study

2.5.1. Animals

Thirty-six adult male Japanese white rabbits (weight 2.8–3.5 kg) were used in this study with approval from the Animal Research Committee, Graduate School of Medicine, Kyoto University, Japan (approval number: Med Kyo 17561 and 18262). All rabbits were individually housed in standard cages and fed standard rabbit food and water.

2.5.2. Surgical procedure

The surgical procedures used have been described previously [21,22]. Rabbits were anesthetized by intravenous injection slowly of pentobarbital sodium (40 mg/kg), inhaled isoflurane, and local administration of 1% lidocaine solution. After disinfection, 3-cm longitudinal skin incision was made on the medial side of the knee. The fascia and the periosteum were split and retracted to expose the tibial cortex. A 16 × 2 mm hole was made using a dental burr from the medial to the lateral cortex running parallel to the longitudinal axis of the tibia. After the hole was irrigated with normal saline, each sample was implanted in the frontal direction, perforating the tibia (Fig. 2(a)). After saline irrigation again, the fascia and the skin were sutured layer

by layer. Similar surgical procedure was performed on bilateral tibia. 20 rabbits were used for mechanical testing and the remaining 16 were used for histological examination. 18 rabbits were sacrificed at 2 weeks after surgery, and 18 at 4 weeks after surgery. At each time point, 5 legs from each sample were subjected to biomechanical testing and 4 legs to histological evaluation. Following euthanasia, segments of the proximal tibia bearing the implanted plates were cut, and all the specimens were wrapped in wetted gauze soaked in saline to keep them moist after harvesting.

2.5.3. Biomechanical testing

The detaching test was conducted within a few hours at the latest after explantation to measure the bone-bonding strength. The detaching test protocol was based on previously described procedures [21]. Traction force was applied vertically to the implant surface at a cross-head speed of 35 mm/min using an Instron-type autograph (Model 1101; Aikoh Engineering Co. Ltd., Nagoya, Japan). In this system, specially-designed hooks held the bone-plate-bone construct (Fig. 2(b)). The detaching failure load was measured when the implant detached from the bone.

2.5.4. Histology

The harvested specimens were fixed in phosphate-buffered 10% formalin (pH 7.25) for 10 days and dehydrated in ascending concentrations of ethanol, defatted in xylene and embedded in polyester resin. The specimens were cut into ~500-μm thick slices using a band saw (BS-300CP, EXACT Cutting Systems, Norderstedt, Germany) perpendicular to the axis of the implant, and the slices were ground to a thickness of 50–60 μm using a grinding-sliding machine (Microgrinding MG-400, EXACT Cutting Systems). Each section was then stained with Stevenel's blue and Van Gieson's picrofuchsin to stain calcified bone bright red and soft tissue blue. The sections were observed using a transmitted light microscope (model Eclipse 80i, Nikon Co., Tokyo, Japan) combined with a digital camera (model DS-55 M-L1, Nikon Co., Japan). The stained sections were evaluated by quantitative histomorphometry for the amount of bone in direct contact with the implant surface, bone-implant contact (BIC) ratio, using 2-dimensional imaging software (Image J; National Institutes of Health, Bethesda, MA, USA). The BIC ratio was measured from three sections per sample and the

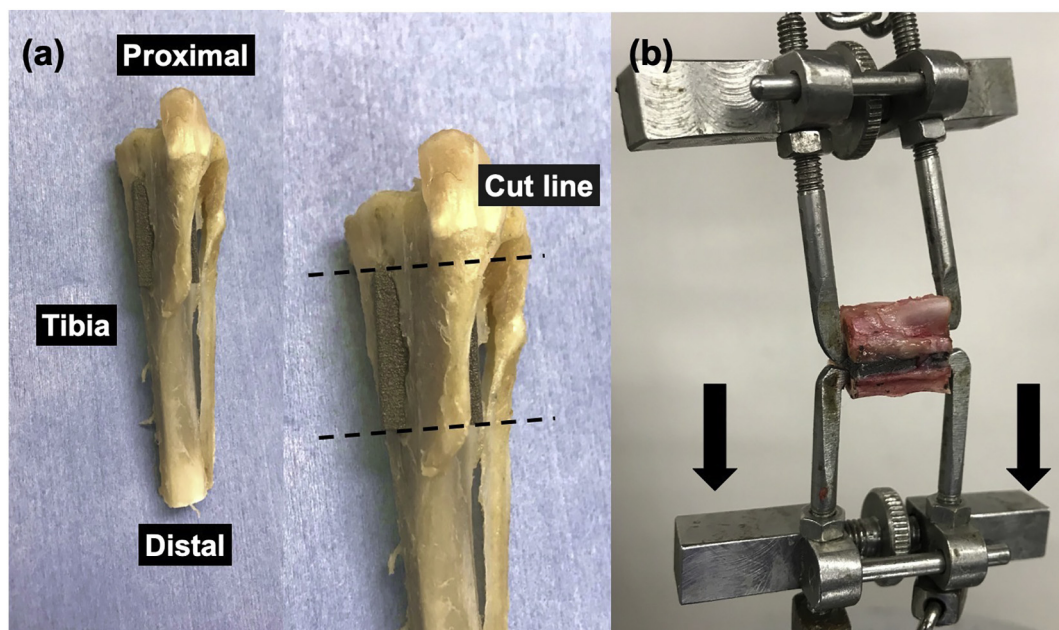


Fig. 2. (a) Representative sample images of *in vivo* SLM implant insertion into the rabbit tibia. (b) The biomechanical test used in *in vivo*; the anterior and posterior cortical bone were held, and tensile load at the time detachment at bone-implant interface occurred was recorded as failure load.

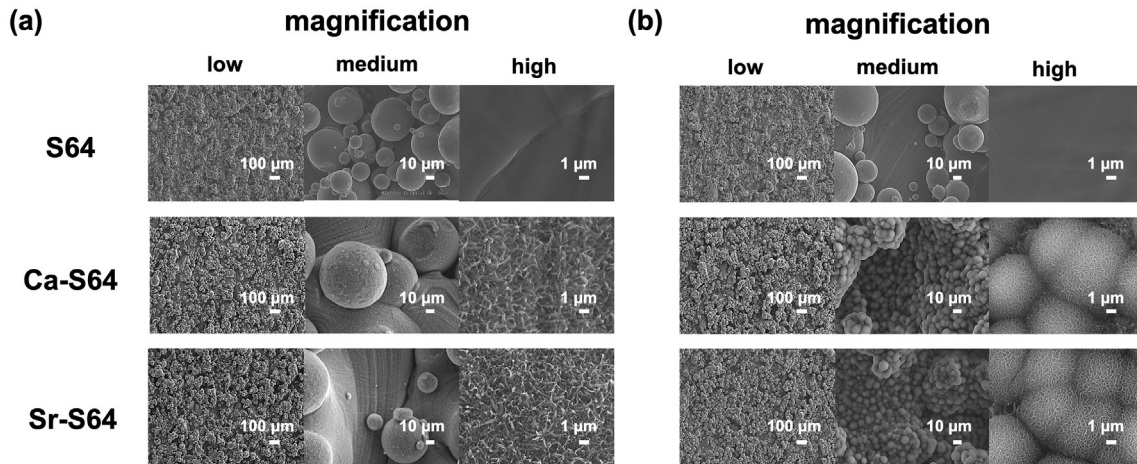


Fig. 3. (a) FE-SEM photographs of material surfaces of S64, Ca-S64 and Sr-S64. (b) FE-SEM photographs of the surface of S64, Ca-S64 and Sr-S64 soaked in SBF for 3 days.

mean value was calculated. Other thick sections were cut with a band saw, polished with diamond paper, and a thin layer of carbon applied for observation by using SEM and EDX.

2.6. Statistical analysis

All data are shown as means $\pm$ standard deviations. Significant differences among sample groups were determined using one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. A p value of < 0.05 was considered to be statistically significant. All analyses were performed using JMP Pro 13.0.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Surface characteristics

Fig. 3(a) shows the FE-SEM photographs of the surfaces of the surfaces of the SLM Ti6Al4V samples (S64, Ca-S64 and Sr-S64). Partially-melted and spherical sintered Ti6Al4V particles were bonded to the surface of the S64 materials at low and medium magnification. Both bioactive treatments resulted in the formation of fine nanoscale networks over the entire surface at high magnification. **Fig. 3(b)** shows the FE-SEM photographs of the surfaces of the SLM Ti6Al4V samples (S64, Ca-S64 and Sr-S64) soaked in SBF for 3 days. No precipitates were observed on S64. Conversely, the walls of Ca-S64 and Sr-S64 were completely covered with spherical bone-like apatite.

Fig. 4 shows the microstructures of the four types of samples. There were no unmelted regions, residual pores, and cracks in all the groups. S64 exhibited a fine acicular martensitic microstructure, which was different from that of STi. Because the heat-treatment temperature in this study was approximately 600 °C, which is lower than the temperature at the β -phase field of titanium alloys, the microstructures of Ca-S64 and Sr-S64 showed almost no difference from that of S64.

Table 3 shows the results of the EDX analysis of the SLM Ti6Al4V metal surfaces (S64, Ca-S64 and Sr-S64). Both bioactive treatments (Ca-S64 and Sr-S64) reduced alloy elements on the surface. 2.7% of the Ca was incorporated into the surface layer of Ca-S64, and 1.5% of the Sr was incorporated while keeping 2.6% Ca into the surface layer of Sr-S64.

Fig. 5 shows the depth profiles of the XPS spectra of Ca-S64 and Sr-S64. Ti and O were the main elements on the surfaces of both samples, which is consistent with the EDX results (**Table 3**). Larger amounts of Ca and Sr ions were observed near the top surface. They were gradually decreased with increasing depth up to approximately 2 μ m.

Fig. 6 shows the XRD spectra of the materials. Broad diffraction peaks around 25° and 48° were observed in Ca-S64 and Sr-S64, indicating that Ca may exist in the calcium titanate (CT) in the form of CaTi_4O_9 , CaTi_2O_5 , and CaTi_4O_5 in Ca-S64 and Sr may exist in the strontium-containing calcium titanate (SrCT) in the form of $\text{Sr}_x\text{Ca}_{1-x}\text{Ti}_2\text{O}_5$, $\text{Sr}_x\text{Ca}_{1-x}\text{Ti}_4\text{O}_9$, and $\text{Sr}_x\text{Ca}_{1-x}\text{Ti}_4\text{O}_5$ in Sr-S64, as reported previously [17,18]. There should be rutile forms of titanium dioxide after chemical and heat treatments, but they could not be detected owing to the roughness of the material surface.

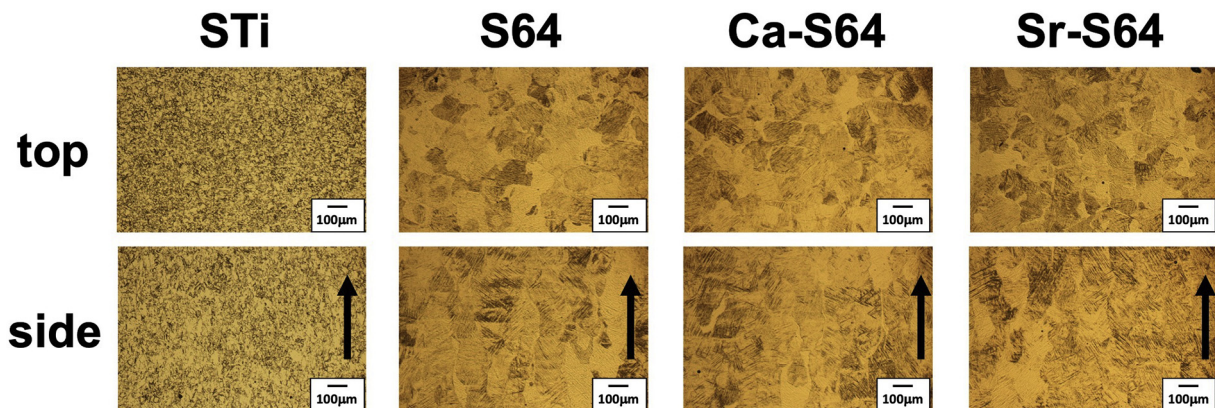


Fig. 4. Microstructures of cross-sections of the samples perpendicular (top) and parallel (side) to the building direction. The black arrows in the side views indicate the building direction.

Table 3
Chemical composition of the surface layers of S64, Ca-S64 and Sr-S64 analyzed by EDX.

Treatment	Element/at. %					
	O	Ti	Al	V	Ca	Sr
S64	0	88.4	7.2	4.8	0	0
Ca-S64	66.2	29.1	1.2	0.9	2.7	0
Sr-S64	62.5	31.7	0.7	1	2.6	1.5

Fig. 7 shows the concentration of the Ca and Sr ions released from the samples subjected to both bioactive treatments, as a function of the square root of the soaking time in PBS. Sr-S64 released 7.6 ppm of Sr ions within 1 h, and slowly released another 5.2 ppm over 14 days (Fig. 7(a)). Ca-S64 showed a release of Ca ions that reached 1.65 ppm after 14 days (Fig. 7(b)). Sr-S64, however, released Ca ions only reached levels as low as 0.07 ppm even after 14 days.

Table 4 shows the WCA of the four groups. STi was the most hydrophobic and its WCA was $75 \pm 9^\circ$. S64 became slightly hydrophilic compared with STi and its WCA was $34 \pm 6^\circ$. Both Ca-S64 and Sr-S64 showed super hydrophilicity. Table 4 also shows the roughness parameter (Ra) of the four groups. The surface roughness values of the four groups were almost the same. Both bioactive treatments (Ca-S64 and Sr-S64) did not affect the original microscale roughness of the native S64 material.

3.2. In vitro cell responses

3.2.1. Cell adhesion

SEM images showed the cells were well spread among the spherical particles in all groups (Fig. 8(a)). Immunofluorescence imaging also showed that cells of all groups were well spread although the concentration of actin filaments (red) varied because of the surface roughness (Fig. 8(b)). After 5 h of seeding, the shape of cells was found to be polygonal and sharp filopodia were observed in STi and S64. Lamellipodia were seen to protrude from cells in Ca-S64 and Sr-S64. Subsequently, after 24 h of seeding, more extended lamellipodia and filopodia connecting the cells were observed in the cells cultured on Ca-S64 and Sr-S64 than in the untreated groups (STi and S64). The cell morphology observed by fluorescent immunostaining was compatible with the SEM observations.

3.2.2. Cell proliferation

After 3 days of cultivation, Ca-S64 and Sr-S64 showed higher cell viability than untreated groups (STi and S64). After 5 days of

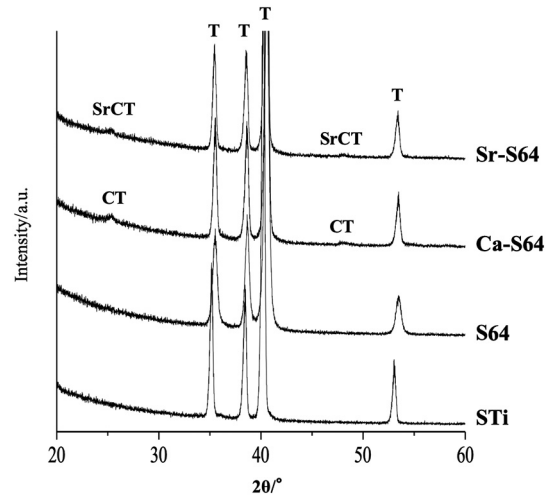


Fig. 6. XRD spectra of the material surfaces. T: α -Ti; CT: CaTi_4O_9 , CaTi_2O_5 , CaTi_4O_5 ; SrCT: $\text{Sr}_x\text{Ca}_{1-x}\text{Ti}_2\text{O}_5$, $\text{Sr}_x\text{Ca}_{1-x}\text{Ti}_4\text{O}_9$, $\text{Sr}_x\text{Ca}_{1-x}\text{Ti}_4\text{O}_5$.

cultivation, there was no significant difference in cell proliferation ability between all groups (Fig. 9(a)). S64, Ca-S64 and Sr-S64 had no cytotoxic effects in spite of their alloy elements such as V and Al.

3.2.3. ALP assay

The average of the ALP activity at the protein level in group with bioactive treatments (Ca-S64 and Sr-S64) tended to be higher than that of untreated group (STi and S64), but there was no statistically significant difference (Fig. 9(b)).

3.2.4. RT-qPCR

Fig. 10 shows the relative gene expression level of *Alp*, *Ocn*, *Opn* and *Integrin- β 1* after 7 days of cultivation of MC3T3-E1 cells on each material. Sr-S64 significantly promoted the expression of osteogenesis related genes including the *Alp*, *Ocn*, *Opn* and *Integrin- β 1*, and the gene expression of Sr-S64 was statistically noninferior compared with all other groups. Ca-S64 significantly promoted the expression of *Alp* above that of STi, but *Ocn* and *Opn* genes, late markers of osteogenic differentiation, had significantly lower expressions in Ca-S64 than in all other groups.

3.3. In vivo study

All rabbits tolerated the surgical procedure uneventfully. No

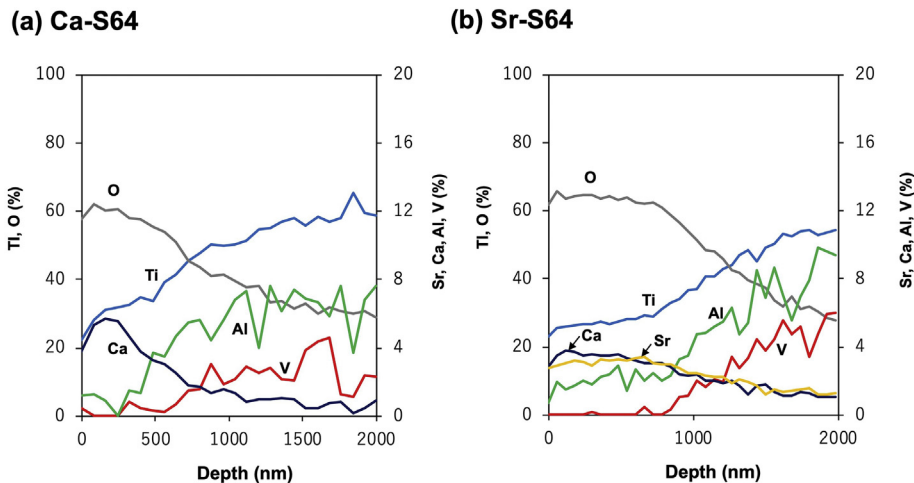


Fig. 5. XPS depth profiles of the surface of the SLM Ti6Al4V samples subjected to chemical and heat treatments (a) Ca-S64 (b) Sr-S64.

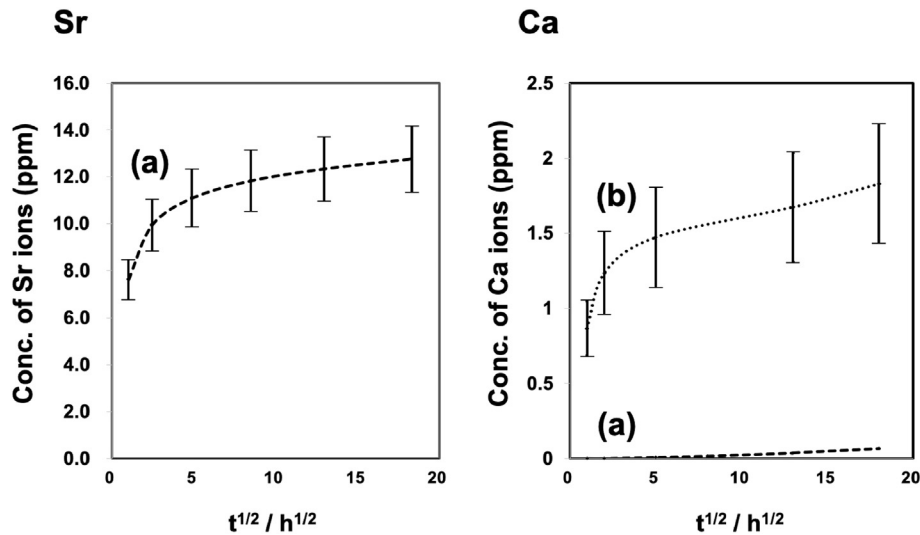


Fig. 7. Concentrations of Sr and Ca ions measured by ICP, which was released from (a) Sr-S64, (b) Ca-S64 as a function of square root of soaking time in PBS.

Table 4
Surface characteristics of SLM samples.

Implants	WCA (SD) (°)	Roughness (Ra) (μm)
STi	75 (6)	13.4
S64	34 (9)	16.6
Ca-S64	0	16.5
Sr-S64	0	17.2

infection of the operative site or implant dislocation was observed during necropsy. No apparent adverse tissue reactions were observed around the implants.

3.3.1. Results of biomechanical testing

Fig. 11 shows the bone-bonding strength between implant and bone at 2 and 4 weeks. At 2 weeks, Sr-S64 had a significantly higher average failure load (51.3 N) than S64 (16.0 N) and STi (11.7 N). The average failure load of Ca-S64 tended to be higher than STi, but the difference did not reach statistical significance ($p = 0.06$). At 4 weeks, Sr-S64 had a significantly higher average failure load (103.6 N) than S64 (60.4 N) and STi (54.4 N).

The symbol “*” indicates statistical difference between groups ($p < 0.05$) and the symbol “***” indicates $p < 0.01$.

3.3.2. Results of histological examination and histomorphometry

Fig. 12(a) shows the BIC ratio from the histological analysis. The bioactive treatment implants (Ca-S64 and Sr-S64) had greater BIC than STi at 2 weeks. At 4 weeks, Sr-S64 had a significantly greater BIC than STi, while no statistical difference existed between Ca-S64 group and untreated groups (S64 and STi). This trend was consistent with those regarding biomechanical testing. Fig. 12(b) shows the representative images of the histology. At 2 weeks, all samples showed immature bone formation near the implant, and especially Sr-S64 and Ca-S64 had high level of new bone formation around the implant surfaces. At 4 weeks, all samples showed bone maturation, while Sr-S64 appeared to maintain new bone near the implant.

Fig. 13 shows representative SEM and EDX mapping images of the interface between the Sr-S64 implants and rabbit tibial bone. From 2 weeks, the new bone was formed to wrap around the spherical sintered powder Ti6Al4V particles and achieve osseointegration.

4. Discussion

In this study, experiments were performed on four kinds of solid

SLM metals; STi, S64, Ca-S64 and Sr-S64. Our data indicate that Sr solution and heat treatment significantly improves the surface bioactivity of S-64. Sr-S64 showed good cell viability, higher osteogenic differentiation-related and cell adhesion-related gene expression *in vitro*. In *in vivo* study, significantly higher mechanical bone bonding strength, histological new bone formation and significantly higher BIC ratios were observed from second to the fourth week after surgery in Sr-S64. Thus, the present study has identified several advantages of Sr-S64.

First, the SEM images showed homogenous micro/nano topography formation on the surface of the chemical solution and heat treatment groups (Fig. 3). This nano-network layer on Sr-S64 was composed of Sr-containing calcium hydrogen titanate layer of around 1 μm thick, which had a high scratch resistance, of ~50 mN [17,18]. Recently, nano roughness combined with micro roughness on the implant surface has been reported to effectively increase the bioactivity of implants by mimicking the actual bone structure [23,24]. Cell responses to micro/nano surfaces with various nanostructures have been investigated in numerous studies, and several studies reported the positive effects of various nanostructures [25,26]. Nano topography can increase the contact spot area and hydrophilicity [27], and subsequently increase adsorption of extracellular matrix proteins, thereby accelerating the cell adhesion [28]. Consistent with previous reports, Ca-S64 and Sr-S64 showed super hydrophilicity (Table 4). SEM and immunostaining images (Fig. 8) showed that MC3T3-E1 cells cultured on Ca-S64 and Sr-S64 showed more cytoplasmic elongation on the surface than controls, suggesting that the micro/nanostructure is more advantageous for cell adhesion than the microstructure only. Activated cell adhesion promotes the up-regulation of integrin β1, which can stimulate integrin-dependent pathways and several downstream pathways such as MAPK/ERK and PI3K/Akt, resulting in osteogenic differentiation [29]. In addition, nano topography can directly enhance osteoblast proliferation and differentiation by increasing the osteogenic differentiation markers, such as ALP and OCN [30,31]. Chemical treatments initiated with NaOH produce nanometer-scale roughness on the surface of the materials [32]. Various types of nanostructures produced by alkali-heat treatment have been reported in the literature, e.g., nanonet [8], nanotuft, nanotrabeclae [23], and nanopolymorphic [33]. Ca-S64 and Sr-S64 showed a disorderly arranged nanonet-like structure on the surfaces (Fig. 3). However, in this study, the effect of nanostructure alone cannot be examined, which needs to be addressed in future studies.

Second, the results of the EDX and XPS analysis indicated that the chemical solution and heat treatment in this study reduced the surface

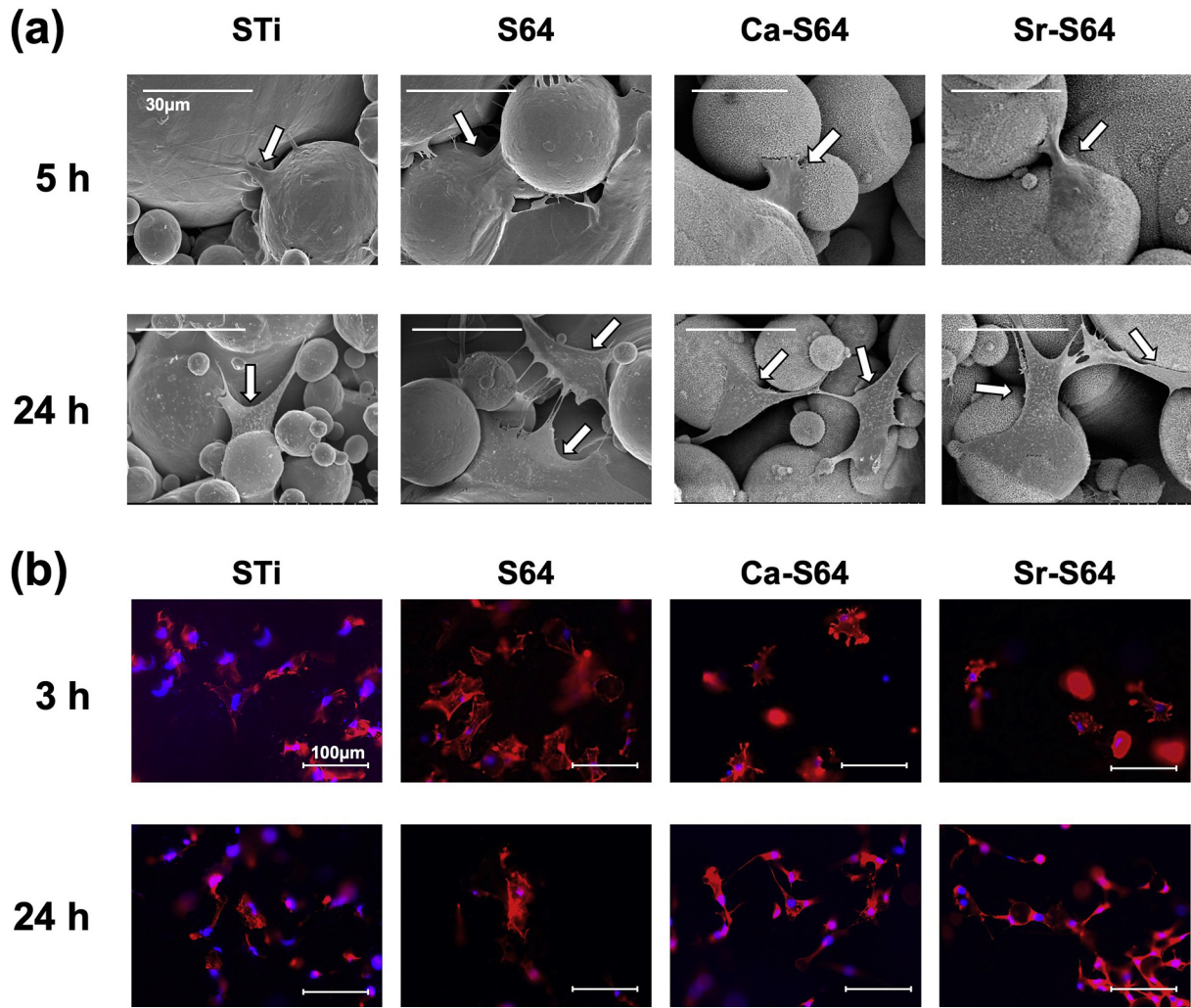


Fig. 8. Cell adhesion on each sample confirmed by SEM and fluorescent immunostaining images. (a) SEM images of the cultured osteoblasts on each material for 5 h and 24 h after seeding. The osteoblasts are indicated by white arrowheads. (b) Fluorescent immunostaining images of the cultured osteoblasts 3 h and 24 h after seeding. The actin filaments are indicated in red, and the nuclei are indicated in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

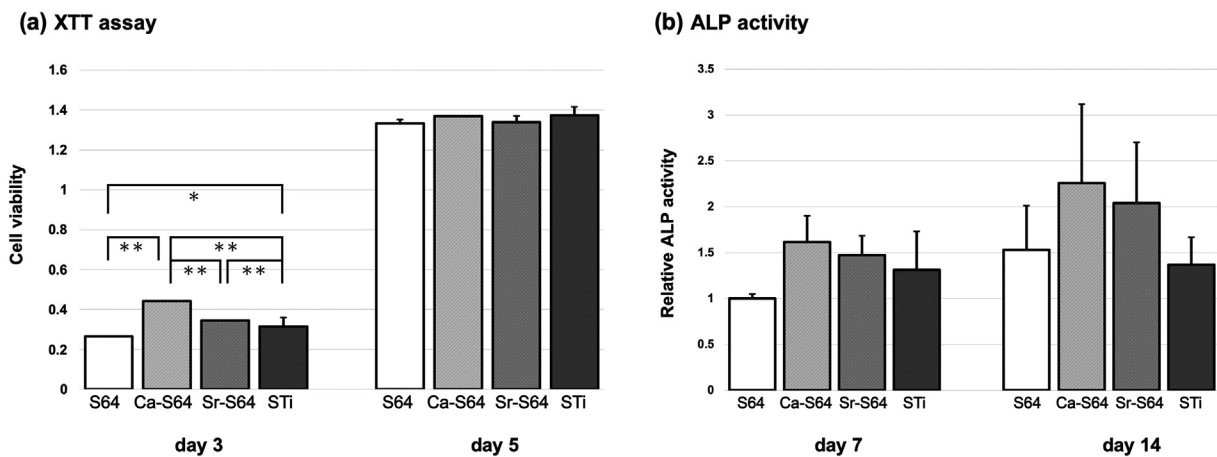


Fig. 9. Cell viability and ALP activity of MC3T3-E1 cells cultured on each sample type. (a) XTT assay (b) relative ALP activity.

composition of alloys (Table 3 and Fig. 5). Several studies have demonstrated that S64 as fabricated surface had no cytotoxicity [4,34]. Herein, XTT assay showed no cytotoxicity in Sr-S64 materials compared to STi (Fig. 9), which is considered to be sufficient evidence to show

that applying this chemical and heat treatment method to S64 is acceptable.

Third, enough Sr ions were released from Sr-S64 (Fig. 7). The concentration of Sr ion released from the Sr solution and heat-treated

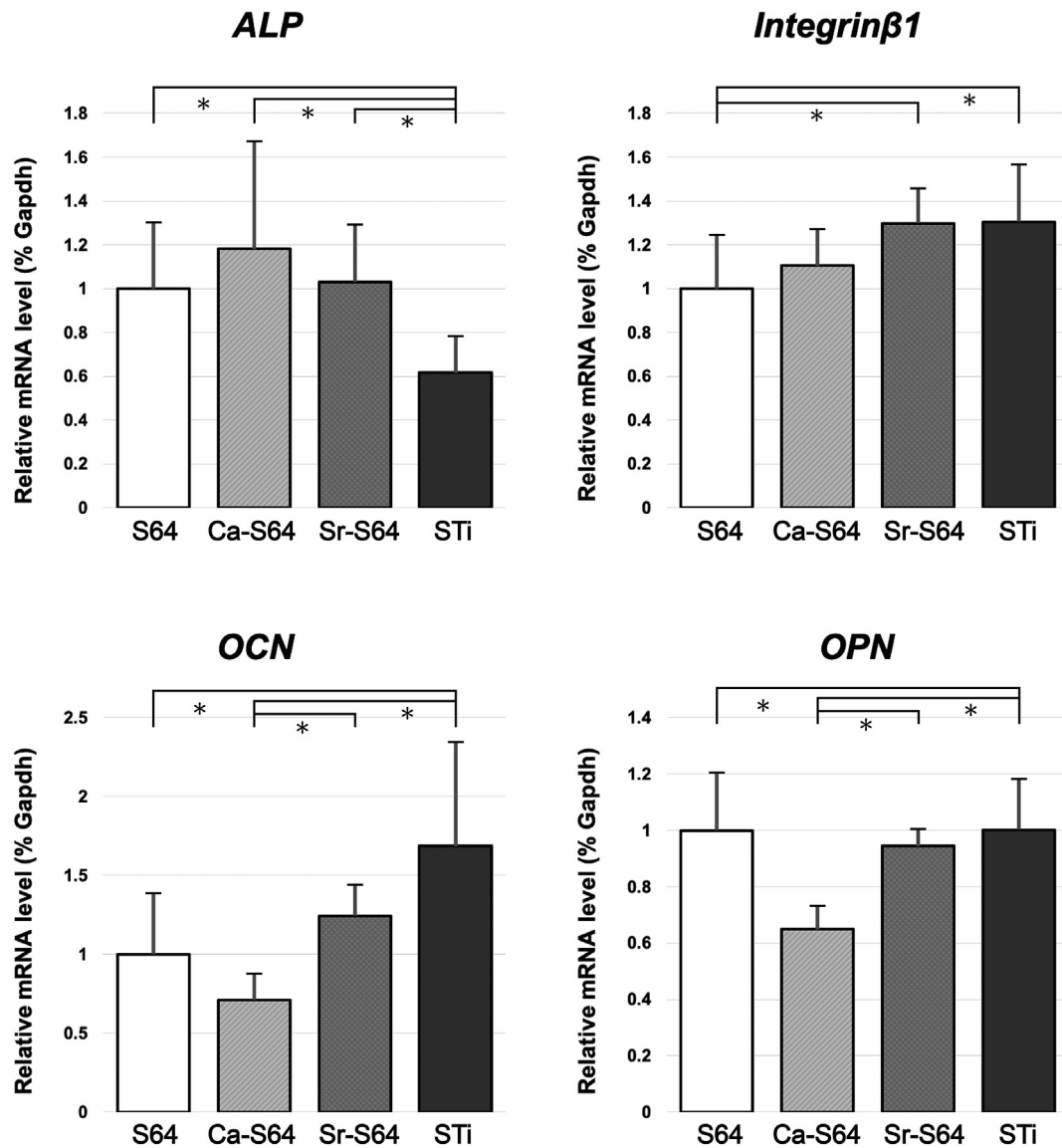


Fig. 10. Relative gene expression levels of MC3T3-E1 cells after 7-day culture using RT-qPCR. *Gapdh* was used as an internal control. The symbol “*” indicates statistical difference between groups ($p < 0.05$).

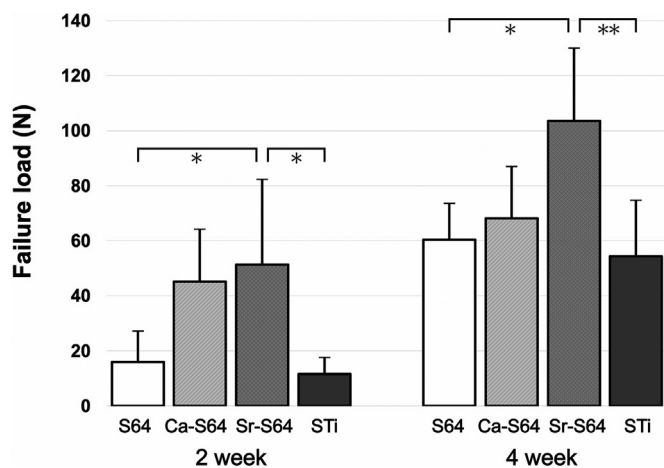


Fig. 11. Failure load (N) in the detaching test after implantation for 2 weeks and 4 weeks.

smooth Ti plates in our previous study was 0.92 ppm for 7 days [16], while the amount of Sr ions released from Sr-S64 in this study was 12.3 ppm over the same period, owing to the increased specific surface area. The optimal amount of local Sr administration remains unknown and the cell response to Sr at similar concentrations may vary depending on the cell lineage. In *in vitro* study, Brennan et al. reported that Sr ranelate (0.01–1 mM) promoted the replication, differentiation, and lifespan of human primary osteoblasts, in a dose-dependent manner [35]. Sila-Asna et al. reported that high dose (210.7 ppm) Sr inhibited osteoblastic differentiation in human MSCs [36]. The serum Sr ion concentration in the blood of osteoporosis patients receiving treatment with oral strontium ranelate (2 g/day) was approximately 8.7 ppm [37]. These ion concentrations help us to determine optimal concentration in the actual clinical settings. If the released amount of Sr ion from the material is well below this value, the efficacy of local Sr administration may be limited or less when applied to human. These findings agree with data from Guo et al. which suggest that between 0.25 and 0.5 mM Sr ranelate could be more appropriate for local application [38]. Accordingly, Sr ion concentration released from Sr-S64 would be in the optimal range and can also be adjusted by changing the concentration of SrCl_2 to be immersed during the chemical solution

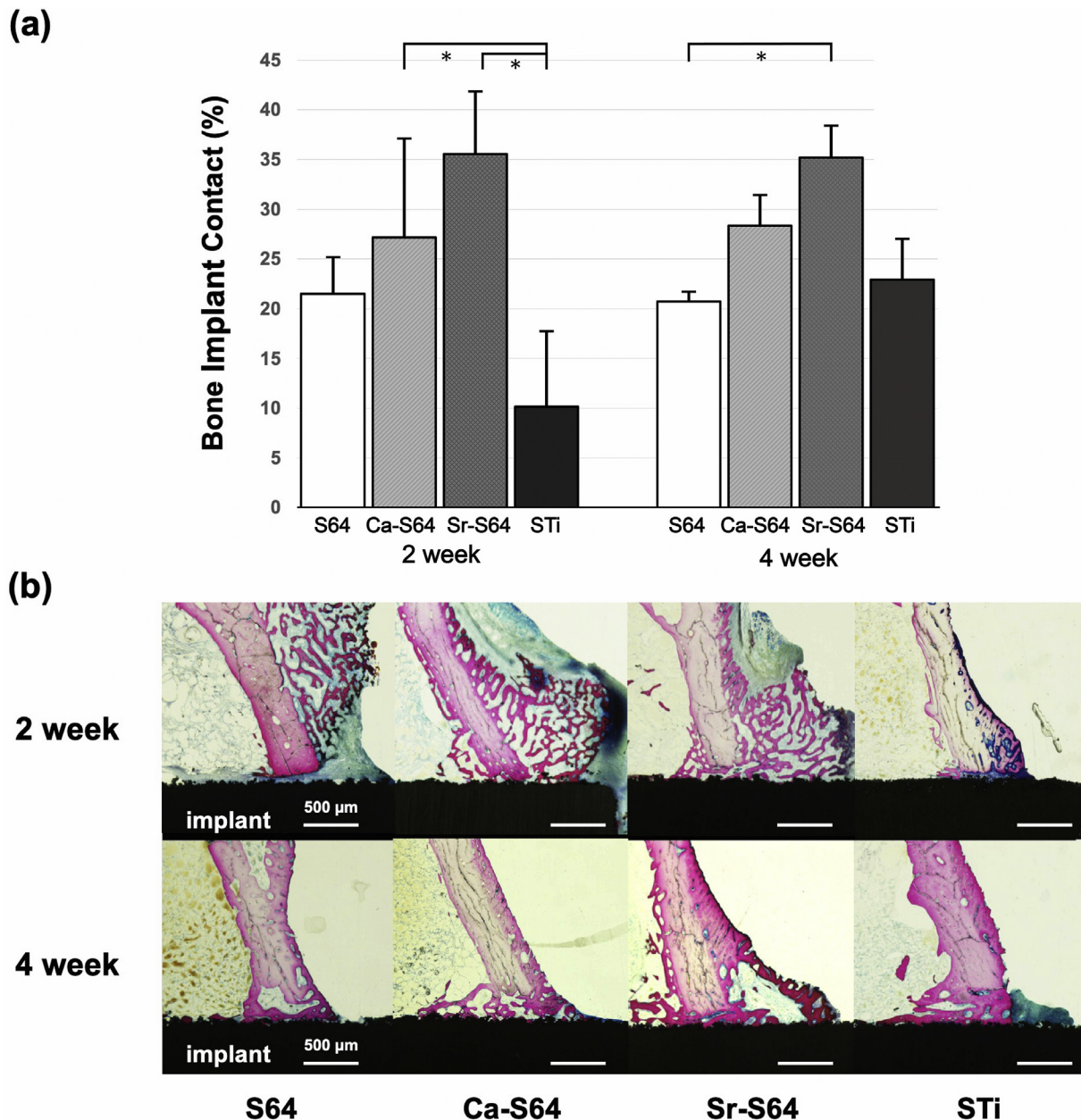


Fig. 12. (a) Bone-implant contact (BIC) ratios at 2 and 4 weeks after implantation. The symbol “*” indicates $p < 0.05$. (b) Representative histology samples of the implanted SLM metal samples stained with Stevenel's blue and Van Gieson's picrofuchsin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

process or by increasing the specific surface area by fabricating three-dimensional (3D) structure. In this study, to eliminate the effects of internal structure as possible, investigations *in vitro* and *in vivo* were conducted using metal plates with dense structure. Further studies are needed to explore the optimal balance between Sr ion concentration and SLM 3D structure in future.

In the *in vitro* study, our results indicated that Sr-S64 were comparable to the control, in terms of RT-qPCR results for some genes (Fig. 10). In the Ca-S64 group, middle and late osteogenic differentiation markers (OPN, OCN) were significantly lowered. Because Ca ion is a regulator of various cellular responses and can promote osteoblast proliferation and differentiation [39], some other potentially negative effects of the present surface treatments may have prevented the proliferation and differentiation of cells *in vitro*. With respect to Sr-S64, although micro/nano topography and hydrophilicity were the same as for Ca-64, *in vitro* results were either the same as or better than those of

other materials. These results suggest that Sr ions exert more positive effects on osteogenic cells than the potential drawbacks introduced by surface treatments. Previous studies have shown that Sr promotes gene expression of MC3T3-E1 cell lines *in vitro* [16,40,41]. *In vitro* assays, using only one cell type, can evaluate individual biological processes in a controlled manner, but cannot fully replicate the complex environments encountered *in vivo*, particularly when investigating the effects of multipotent drugs such as Sr. More detailed studies are needed to elucidate the underlying molecular mechanisms of *in vitro* results that may be affected by various factors, including surface roughness, hydrophilicity, and surface composition.

Our findings in the current *in vivo* study indicated that Sr-S64 exhibited significantly good bone-bonding ability compared to untreated controls (Fig. 11). Okuzu et al. evaluated the same Sr solution and heat treatment on smooth Ti metal and reported that its strength of failure load was ~10 N at 4 weeks [16]. In contrast, the strength of failure load

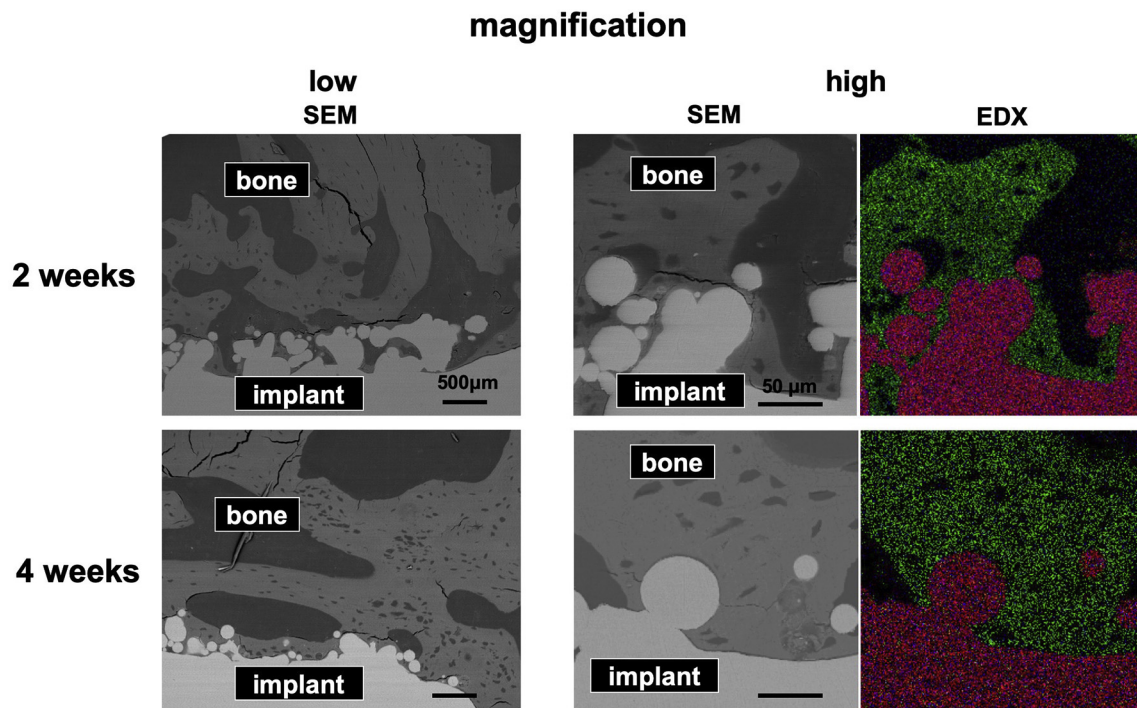


Fig. 13. Representative backscattered SEM images of the interface between Sr-S64 and rabbit tibial bone and EDX mapping analysis of composite (red indicates Ti and green indicates Ca). The specimens were partially cracked by the electron beam irradiation of SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of Sr-S64 herein was considerably higher (103.6 N), demonstrating the exceptional usefulness of this material. Histological images of Sr-S64 showed bone maturation had already begun at 4 weeks, with no increase in BIC compared with 2 weeks (Fig. 12). The increased failure load of Sr-S64 at 4 weeks suggests better peri-implant bone regeneration of the surrounding bone microarchitecture than controls, following the results of other studies [42]. Because Sr-S64 implants had sustained Sr ion release for 2 weeks and strontium is effective even at low concentrations [43], this strontium-rich implant is expected to have a favorable long-term course and thus requires long-term study.

Several limitations should be noted in this study. First, STi and S64 were manufactured by different machines, EOSINT M270 and EOS M290. Although comparing the SLM implants fabricated by different machines is not desirable, owing to the different manufacturing conditions, we used STi fabricated by EOSINT M270 as a control in this study because we have previously demonstrated its biocompatibility [6]. Second, we have not investigated the effect of Ca ions released from Sr-S64. Ca and Sr ions could exert synergistic or competitive effects [44]. Third, we have not investigated the peri implant bone quality. This could be better defined using a bone mineral density analysis. Fourth, with respect to the relative density of the fabricated specimens, our processing parameters were optimized previously [9]. However, in terms of biocompatibility, the optimal parameters for the surface roughness of SLM materials have not been fully elucidated. Metal powder particles adhering to the surface can increase the specific surface area, but may decrease the mechanical strength of the surface. Repetitive surface friction during the implantation process may damage the micro/nanostructures. Therefore, some studies adopted post-process treatments such as sandblasting, acid polishing, and heat treatments to remove or reduce the residual surface particles [8,9] [45]. Although the as fabricated SLM materials were adopted in this study to maximize the amount of Sr ions incorporated into the metal surfaces, further studies are required to investigate the effect of process parameters and post-process treatments on the bioactive treatment.

5. Conclusion

In this study, we have developed new surface modifications for S64 with sustained Sr ion release (Sr-S64). This simple chemical and heat treatment showed no adverse effects on surface composition and added a uniform nanoscale network structure to the S64 material surface, which formed bone-like apatite in SBF within 3 days. *In vitro* results using MC3T3-E1 cells on Sr-S64 exhibited good cell adhesion and differentiation. *In vivo* results using rabbits demonstrated that Sr-S64 showed significantly greater bone-bonding strength and BIC ratio than untreated controls from 2 to 4 weeks. This method enables focusing the design of SLM implants on mechanical properties rather than biological activity, thereby facilitating their fabrication and application to clinical use.

Funding

This work was supported by a Grant in Aid for Scientific Research from the Japan Society for the Promotion of Science (Kakenhi grant No. 15K06482).

CRediT authorship contribution statement

Yu Shimizu: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Investigation, Visualization, Writing - original draft. **Shunsuke Fujibayashi:** Conceptualization, Funding acquisition, Writing - review & editing, Project administration. **Seiji Yamaguchi:** Conceptualization, Data curation, Methodology, Investigation, Resources, Visualization, Writing - review & editing. **Shigeo Mori:** Data curation, Investigation, Resources, Visualization. **Hisashi Kitagaki:** Data curation, Investigation, Resources. **Takayoshi Shimizu:** Conceptualization, Formal analysis, Methodology, Writing - review & editing. **Yaichiro Okuzu:** Conceptualization, Methodology, Writing - review & editing. **Kazutaka Masamoto:** Conceptualization, Formal analysis, Methodology, Writing - review & editing. **Koji Goto:** Conceptualization, Methodology, Writing - review & editing. **Bungo**

Otsuki: Conceptualization, Methodology, Writing - review & editing.
Toshiyuki Kawai: Conceptualization, Formal analysis, Methodology, Writing - review & editing.
Kazuaki Morizane: Conceptualization, Methodology, Writing - review & editing.
Tomotoshi Kawata: Conceptualization, Methodology, Writing - review & editing.
Shuichi Matsuda: Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

None.

Acknowledgments

We greatly appreciate the technical support of Mr. Kohda and Ms. Furuta for the SEM studies.

References

- [1] W. Yan, T. Nakamura, M. Kobayashi, H. Kim, F. Miyaji, T. Kokubo, *Bonding of Chemically Treated Titanium Implants to Bone*, (1996).
- [2] T.L. Siu, J.M. Rogers, K. Lin, R. Thompson, M. Owbridge, Custom-made titanium 3-dimensional printed interbody cages for treatment of osteoporotic fracture-related spinal deformity, *World Neurosurg* 111 (2018) 1–5, <https://doi.org/10.1016/j.wneu.2017.11.160>.
- [3] N. Hafeez, S. Liu, E. Lu, L. Wang, R. Liu, W. Lu, L.C. Zhang, Mechanical behavior and phase transformation of β -type Ti-35Nb-2Ta-3Zr alloy fabricated by 3D-printing, *J. Alloys Compd.* 790 (2019) 117–126, <https://doi.org/10.1016/j.jallcom.2019.03.138>.
- [4] J. Vaithilingam, E. Prina, R.D. Goodridge, R.J.M. Hague, S. Edmondson, F.R.A.J. Rose, S.D.R. Christie, Surface chemistry of Ti6Al4V components fabricated using selective laser melting for biomedical applications, *Mater. Sci. Eng. C* 67 (2016) 294–303, <https://doi.org/10.1016/j.msec.2016.05.054>.
- [5] H. Imai, J. Miyawaki, T. Kamada, A. Maruishi, J. Takeba, H. Miura, Radiolucency around highly porous sockets and hydroxyapatite-coated porous sockets in total hip arthroplasty for hip dysplasia, *Eur. J. Orthop. Surg. Traumatol.* 29 (2019) 611–618, <https://doi.org/10.1007/s00590-018-2351-3>.
- [6] M. Tsukanaka, S. Fujibayashi, M. Takemoto, T. Matsushita, Bioactive treatment promotes osteoblast differentiation on titanium materials fabricated by selective laser melting technology, 35 (2016) 118–125, <https://doi.org/10.4012/dmj.2015-127>.
- [7] J. Van Der Stok, O.P. Van Der Jagt, S. Amin Yavari, M.F.P. De Haas, J.H. Waarsing, H. Jahr, E.M.M. Van Lieshout, P. Patka, J.A.N. Verhaar, A.A. Zadpoor, H. Weinans, Selective laser melting-produced porous titanium scaffolds regenerate bone in critical size cortical bone defects, *J. Orthop. Res.* 31 (2013) 792–799, <https://doi.org/10.1002/jor.22293>.
- [8] J. Yun Xu, X. shuai Chen, C. yu Zhang, Y. Liu, J. Wang, F. long Deng, Improved bioactivity of selective laser melting titanium: surface modification with micro-/nano-textured hierarchical topography and bone regeneration performance evaluation, *Mater. Sci. Eng. C* 68 (2016) 229–240, <https://doi.org/10.1016/j.msec.2016.05.096>.
- [9] D.K. Pattanayak, A. Fukuda, T. Matsushita, M. Takemoto, S. Fujibayashi, K. Sasaki, N. Nishida, T. Nakamura, T. Kokubo, Bioactive Ti metal analogous to human cancellous bone: fabrication by selective laser melting and chemical treatments, *Acta Biomater.* 7 (2011) 1398–1406, <https://doi.org/10.1016/j.actbio.2010.09.034>.
- [10] P.J. Meunier, C. Roux, S. Ortolani, M. Diaz-Curiel, J. Compston, P. Marquis, C. Cormier, G. Isaia, J. Badurski, J.D. Wark, J. Collette, J.Y. Reginster, Effects of long-term strontium ranelate treatment on vertebral fracture risk in postmenopausal women with osteoporosis, *Osteoporos. Int.* 20 (2009) 1663–1673, <https://doi.org/10.1007/s00198-008-0825-6>.
- [11] P.J. Marie, Strontium ranelate: new insights into its dual mode of action, *Bone* 40 (2007) 5–8, <https://doi.org/10.1016/j.bone.2007.02.003>.
- [12] Z. Saidak, P.J. Marie, Strontium signaling: molecular mechanisms and therapeutic implications in osteoporosis, *Pharmacol. Ther.* 136 (2012) 216–226, <https://doi.org/10.1016/j.pharmthera.2012.07.009>.
- [13] Y. Li, X. Li, G. Song, K. Chen, G. Yin, J. Hu, Effects of strontium ranelate on osseointegration of titanium implant in osteoporotic rats, *Clin. Oral Implants Res.* 23 (2012) 1038–1044, <https://doi.org/10.1111/j.1600-0501.2011.02252.x>.
- [14] A. Sabareeswaran, B. Basu, S.J. Shenoy, Z. Jaffer, N. Saha, A. Stamboulis, Early osseointegration of a strontium containing glass ceramic in rabbit model, *Biomaterials* 34 (2013) 9278–9286, <https://doi.org/10.1016/j.biomaterials.2013.08.070>.
- [15] W. Liu, M. Cheng, T. Wahafu, Y. Zhao, H. Qin, J. Wang, X. Zhang, L. Wang, The in vitro and in vivo performance of a strontium-containing coating on the low-modulus Ti35Nb2Ta3Zr alloy formed by micro-arc oxidation, *J. Mater. Sci. Mater. Med.* 26 (2015) 1–13, <https://doi.org/10.1007/s10856-015-5533-0>.
- [16] Y. Okuzu, S. Fujibayashi, S. Yamaguchi, K. Yamamoto, T. Shimizu, Strontium and magnesium ions released from bioactive titanium metal promote early bone bonding in a rabbit implant model, *Acta Biomaterialia* 63 (2017) 383–392.
- [17] S. Yamaguchi, S. Nath, T. Matsushita, T. Kokubo, Controlled release of strontium ions from a bioactive Ti metal with a Ca-enriched surface layer, *Acta Biomater.* 10 (2014) 2282–2289, <https://doi.org/10.1016/j.actbio.2014.01.026>.
- [18] T. Kizuki, H. Takadama, T. Matsushita, T. Nakamura, T. Kokubo, Preparation of bioactive Ti metal surface enriched with calcium ions by chemical treatment, *Acta Biomater.* 6 (2010) 2836–2842, <https://doi.org/10.1016/j.actbio.2010.01.007>.
- [19] T. Kokubo, H. Takadama, How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* 27 (2006) 2907–2915, <https://doi.org/10.1016/j.biomaterials.2006.01.017>.
- [20] T. Kizuki, T. Matsushita, T. Kokubo, Apatite-forming PEEK with TiO₂ surface layer coating, *J. Mater. Sci. Mater. Med.* 26 (2015) 1–9, <https://doi.org/10.1007/s10856-014-5359-1>.
- [21] T. Nakamura, T. Yamamuro, S. Higashi, T. Kokubo, S. Ito, A new glass-ceramic for bone replacement: evaluation of its bonding to bone tissue, *J. Biomed. Mater. Res.* 19 (1985) 685–698, <https://doi.org/10.1002/jbm.b.820190608>.
- [22] S. Fujibayashi, T. Nakamura, S. Nishiguchi, J. Tamura, M. Uchida, H.M. Kim, T. Kokubo, Bioactive titanium: effect of sodium removal on the bone-bonding ability of bioactive titanium prepared by alkali and heat treatment, *J. Biomed. Mater. Res.* 56 (2001) 562–570, [https://doi.org/10.1002/1097-4636\(20010915\)56:4<562::AID-JBM1128>3.0.CO;2-M](https://doi.org/10.1002/1097-4636(20010915)56:4<562::AID-JBM1128>3.0.CO;2-M).
- [23] N. Tsukimura, T. Ueno, F. Iwasa, H. Minamikawa, Y. Sugita, K. Ishizaki, T. Ikeda, K. Nakagawa, M. Yamada, T. Ogawa, Bone integration capability of alkali- and heat-treated nanobimorphic Ti-15Mo-5Zr-3Al, *Acta Biomater.* 7 (2011) 4267–4277, <https://doi.org/10.1016/j.actbio.2011.08.016>.
- [24] Q. Huang, T.A. Elkhoory, X. Liu, R. Zhang, X. Yang, Z. Shen, Q. Feng, Effects of hierarchical micro/nano-topographies on the morphology, proliferation and differentiation of osteoblast-like cells, *Colloids Surfaces B Biointerfaces* 145 (2016) 37–45, <https://doi.org/10.1016/j.colsurfb.2016.04.031>.
- [25] R. Brånemark, L. Emanuelsson, A. Palmquist, P. Thomsen, Bone response to laser-induced micro- and nano-size titanium surface features, *Nanomedicine Nanotechnology, Biol. Med.* 7 (2011) 220–227, <https://doi.org/10.1016/j.nano.2010.10.006>.
- [26] F. Liu, Y. Li, J. Liang, W. Sui, A. Bellare, L. Kong, Effects of micro/nano strontium-loaded surface implants on osseointegration in ovariectomized sheep, *Clin. Implant. Dent. Relat. Res.* 21 (2019) 377–385, <https://doi.org/10.1111/cid.12719>.
- [27] S.S.A. An, Rajangam, Fibrinogen and fibrin based micro and nano scaffolds incorporated with drugs, proteins, cells and genes for therapeutic biomedical applications, *Int. J. Nanomedicine* 3641 (2013), <https://doi.org/10.2147/ijn.s43945>.
- [28] L. Guida, A. Oliva, M.A. Basile, M. Giordano, L. Nastro, M. Annunziata, Human gingival fibroblast functions are stimulated by oxidized nano-structured titanium surfaces, *J. Dent.* 41 (2013) 900–907, <https://doi.org/10.1016/j.jdent.2013.07.009>.
- [29] S. Chen, Y. Guo, R. Liu, S. Wu, J. Fang, B. Huang, Z. Li, Z. Chen, Z. Chen, Tuning surface properties of bone biomaterials to manipulate osteoblastic cell adhesion and the signaling pathways for the enhancement of early osseointegration, *Colloids Surfaces B Biointerfaces* 164 (2018) 58–69, <https://doi.org/10.1016/j.colsurfb.2018.01.022>.
- [30] X. Liu, X. Song, P. Zhang, Z. Zhu, X. Xu, Effects of nano tantalum implants on inducing osteoblast proliferation and differentiation, *Exp. Ther. Med.* 12 (2016) 3541–3544, <https://doi.org/10.3892/etm.2016.3801>.
- [31] J. Wang, Y. An, F. Li, D. Li, D. Jing, T. Guo, E. Luo, C. Ma, The effects of pulsed electromagnetic field on the functions of osteoblasts on implant surfaces with different topographies, *Acta Biomater.* 10 (2014) 975–985, <https://doi.org/10.1016/j.actbio.2013.10.008>.
- [32] T. Kokubo, S. Yamaguchi, Novel bioactive materials developed by simulated body fluid evaluation: surface-modified Ti metal and its alloys, *Acta Biomater.* 44 (2016) 16–30, <https://doi.org/10.1016/j.actbio.2016.08.013>.
- [33] T. Ueno, N. Tsukimura, M. Yamada, T. Ogawa, Enhanced bone-integration capability of alkali- and heat-treated nanopolymorphic titanium in micro-to-nanoscale hierarchy, *Biomaterials* 32 (2011) 7297–7308, <https://doi.org/10.1016/j.biomaterials.2011.06.033>.
- [34] H. Wang, B. Zhao, C. Liu, C. Wang, X. Tan, M. Hu, A comparison of biocompatibility of a titanium alloy fabricated by electron beam melting and selective laser melting, (2016) 1–19, <https://doi.org/10.1371/journal.pone.0158513>.
- [35] T.C. Brennan, M.S. Rybchyn, W. Green, S. Atwa, A.D. Conigrave, R.S. Mason, Osteoblasts play key roles in the mechanisms of action of strontium ranelate, *Br. J. Pharmacol.* 157 (2009) 1291–1300, <https://doi.org/10.1111/j.1476-5381.2009.00305.x>.
- [36] M. Sila-Asna, A. Bunyaratvej, S. Maeda, H. Kitaguchi, N. Bunyaratavej, Osteoblast differentiation and bone formation gene expression in strontium-inducing bone marrow mesenchymal stem cell, *Kobe J. Med. Sci.* 53 (2007) 25–35.
- [37] P.J. Meunier, C. Roux, E. Seeman, S. Ortolani, J.E. Badurski, T.D. Spector, J. Cannata, A. Balogh, E.-M. Lemmel, S. Pors-Nielsen, R. Rizzoli, H.K. Genant, J.-Y. Reginster, The effects of strontium ranelate on the risk of vertebral fracture in women with postmenopausal osteoporosis, *Obstet. Gynecol. Surv.* 59 (2004) 526–527, <https://doi.org/10.1097/00006254-200407000-00021>.
- [38] X. Guo, S. Wei, M. Lu, Z. Shao, J. Lu, L. Xia, K. Lin, D. Zou, Dose-dependent effects of strontium ranelate on ovariectomy rat bone marrow mesenchymal stem cells and human umbilical vein endothelial cells, *Int. J. Biol. Sci.* 12 (2016) 1511–1522, <https://doi.org/10.7150/ijbs.16499>.
- [39] P. Habibovic, J.E. Barralet, Bioinorganics and biomaterials: bone repair, *Acta Biomater.* 7 (2011) 3013–3026, <https://doi.org/10.1016/j.actbio.2011.03.027>.
- [40] S. Peng, X.S. Liu, S. Huang, Z. Li, H. Pan, W. Zhen, K.D.K. Luk, X.E. Guo, W.W. Lu, The cross-talk between osteoclasts and osteoblasts in response to strontium treatment: involvement of osteoprotegerin, *Bone* 49 (2011) 1290–1298, <https://doi.org/10.1016/j.bone.2011.08.031>.
- [41] A. Barbara, P. Delannoy, B.G. Denis, P.J. Marie, Normal matrix mineralization induced by strontium ranelate in MC3T3-E1 osteogenic cells, *Metabolism* 53 (2004)

- 532–537, <https://doi.org/10.1016/j.metabol.2003.10.022>.
- [42] W. Querido, A.L. Rossi, M. Farina, The effects of strontium on bone mineral: a review on current knowledge and microanalytical approaches, *Micron* 80 (2016) 122–134, <https://doi.org/10.1016/j.micron.2015.10.006>.
- [43] A. Aimaiti, A. Maimaitiyiming, X. Boyong, K. Aji, C. Li, L. Cui, Low-dose strontium stimulates osteogenesis but high-dose doses cause apoptosis in human adipose-derived stem cells via regulation of the ERK1/2 signaling pathway, *Stem Cell Res Ther* 8 (2017) 1–12, <https://doi.org/10.1186/s13287-017-0726-8>.
- [44] X. Yuan, H. Cao, J. Wang, K. Tang, B. Li, Y. Zhao, M. Cheng, H. Qin, X. Liu, X. Zhang, Immunomodulatory effects of calcium and strontium co-doped titanium oxides on osteogenesis, *Front. Immunol.* 8 (2017) 6–9, <https://doi.org/10.3389/fimmu.2017.01196>.
- [45] W. Peng, L. Xu, J. You, L. Fang, Q. Zhang, Selective laser melting of titanium alloy enables osseointegration of porous multi-rooted implants in a rabbit model, *Biomed. Eng. Online* (2016) 1–13, <https://doi.org/10.1186/s12938-016-0207-9>.