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Original Article

Characteristics and biological responses of selective laser melted Ti6Al4V modified by micro-arc oxidation

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Abstract *Background/purpose:* Additive manufacturing (AM) technology, such as selective laser melting (SLM), has been used to fabricate medical devices of Ti-6wt.% Al-4wt.%V (Ti6Al4V) alloys in dentistry. Strontium (Sr) has been shown to have the potential to treat osteoporosis. The aim of this study was to investigate the physicochemical and biological properties of strontium-containing coatings on selective laser melted Ti6Al4V (SLM-Ti6Al4V) substrate.
Materials and methods: The disk of Ti6Al4V was prepared by SLM method. The strontium-containing coatings were prepared by micro-arc oxidation (MAO) in aqueous electrolytes. The surface topography, chemical composition, and phase of strontium-containing MAO (SrMAO) coatings were performed by scanning electron microscope (SEM), energy dispersive X-ray spectrometer (EDS), and thin film X-ray diffraction (TF-XRD), respectively. The apatite-forming ability of the MAO coatings was conducted in simulating body fluid (SBF), and the cell proliferation was determined by methylthiazoletetrazolium (MTT) assay.
Results: The microstructure of SLM-Ti6Al4V displays acicular α-phase organization. The TF-XRD results indicated that the phase of SrMAO coating was anatase, rutile, and titanium. The calcium, phosphorus, and strontium were detected in the coatings by EDS. Using the SEM, the surface morphology of SrMAO coatings exhibited a uniform 3D porous structure. The SrMAO coatings could induce a bone-like apatite layer after immersion in SBF, and presented significantly higher cell proliferation than untreated specimens in *in-vitro* experiments.
Conclusion: All findings in this study indicate that SrMAO coatings formed on SLM-Ti6Al4V surfaces exhibit a benefit on biological responses and thereby are suitable for biomedical

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applications.

Introduction

Dental implants are an important treatment plan option for replacing missing natural teeth. The implant site should remain undisturbed for a minimum of 3—8 months to promote proper healing and osseointegration between the implant and bone.[1] Titanium and titanium-based alloys have been widely used as dental implants because of their mechanical properties and bioinert. It has been pointed out to have problems such as Young's modulus being significantly higher than that of bones[2] and being extremely difficult to machine.[3] It is an important issue for developing alternative manufacturing processes of titanium alloys to solve this problem. The three-dimensional (3D) printing technology, also known as additive manufacturing (AM), provides new approaches for addressing these hurdles. Selective laser melting (SLM) is one kind of AM process used to produce medical devices.

SLM uses a fiber laser as a source of heat for melting the metal powder in the process. This method provides better intricate geometries, optimum material usage, and tooling time than conventional manufacturing methods for avoiding some of the challenges associated with Ti-6wt.%Al-4wt.%V (Ti6Al4V) conventional fabrication. Furthermore, SLM technology offers various advantages, especially for complex designs like dental implants. It eliminates the need for molds and welding, and reduces production time and cost.[4] Even though Ti6Al4V alloys have excellent mechanical properties, they do not chemically bond to bone or actively induce bone ingrowth as compared to the Ca/P bioceramic coated implants. Osseointegration is a prerequisite for successful dental implants. Calcium phosphate ceramics, which is an important bioceramic material, is usually deposited on implant surfaces to achieve an early and functional bone apposition.[5] The micro-arc oxidation (MAO) technology is an effective technique for forming bioceramic coating to improve alloy surface properties such as osseointegration.

The strontium, which is the one of main elements comprising human bone, enhances osteoblast activity and differentiation, whereas it inhibits osteoclast production and proliferation *in vitro*.[6] Owing to the potentiality of strontium for bone healing applications, it was seldom reported about the coating containing strontium on the surface of elective laser melted Ti6Al4V (SLM-Ti6Al4V) substrates. Furthermore, the titanium alloys coated with ceramic material can reduce Young's modulus.[7] In the present study, the MAO process was used to modify the SLM-Ti6Al4V surface in aqueous electrolytes, allowing strontium, calcium, and phosphorus to be incorporated into a coating matrix. The thickness, phase, composition, and surface morphology of the coatings were evaluated. The biological properties were evaluated by *in vitro* tests, in terms of cell proliferation and bioactivity.

Materials and methods

SLM-Ti6Al4V were manufactured by SLM Machine (Renishaw AM250, Renishaw, Taichung, Taiwan), and the process parameters include power (780 W), powder size (10—40 μm), scan speeding (10^4 mm/s), and layer thickness (50—100 μm). They were selected as substrates, which were 12.7 mm in diameter and 2 mm in thickness. The substrates were ground by sequences of silicon carbide papers, then ultrasonically cleaned in acetone, ethanol and de-ionized water prior to the MAO process. The MAO electrolyte was prepared by dissolving 0.06 M $NaH_2PO_4 \cdot H_2O$, 0.117 M $Ca(CH_3COO)_2 \cdot H_2O$, and 0.013 M $Sr(OH)_2 \cdot 8H_2O$ in de-ionized water.

MAO treatments were carried out for 600 s using AC power supply (GPR-60H30, Gitek Electronics Co., Ltd, New Taipei, Taiwan) in a 1 L double-walled glass cell, and the electrolyte temperature was maintained at 20 °C by a circulating water system. A square waveform voltage signal was applied with a positive-to-negative pulse ratio of 350 V/-150 V at 100 Hz frequency and 90% duty cycle. A stainless steel plate was used as a cathode in the electrochemical cell. After MAO treatment, the samples were rinsed with ethanol and de-ionized water, sequentially, and dried in a 60 °C oven.

The phase structure of the substrates and strontium-containing MAO (SrMAO) coatings was identified by thin film X-ray diffraction (TF-XRD, Rigaku D/max III. V, Tokyo, Japan). A metallurgical microscope observed the microstructures. Surface morphology and the cross-sections of the SrMAO coatings were observed using a scanning electron microscope (SEM, JSM-6390LV, JEOL, Tokyo, Japan) and chemical analysis was performed using an energy dispersive X-ray spectrometer (EDS, INCA 350 Oxford, Oxfordshire, UK). Surface roughness was measured by surfcorder (SE 1200, Kosaka Laboratory Ltd., Tokyo, Japan). The static water contact angle of the SrMAO coatings at ambient temperature was measured by an optical contact angle meter (Model 100 SB, Sindatek, Taipei, Taiwan) equipped with a controlled liquid dispensing system. A 2 μL droplet of deionized water was dropped onto the surface and recorded after 30 s since the drops fell onto the surface. The contact angle plug-in for ImageJ software (version 1.53K, NIH, Bethesda, ML, USA) assisted the measurements.

The apatite-forming ability of the SrMAO coatings was conducted following ISO 23317 in the solution simulating body fluid (SBF).[8] The SBF was kept at pH 7.4 and maintained at 37 °C for SrMAO coatings immersion. The characteristics of SrMAO coatings were evaluated after 1, 3, and 7 days, then the surface morphology was observed using an SEM with an EDS for chemical analysis. Besides, a culture of human osteoblastic cells (MG63 cells, ATCC number: CRL-

1427™, Manassas, VA, USA) were grown in an incubator supplied with 5% CO_2 at 37 °C. The cell suspensions were seeded on all specimens in 24-well plates at a cell density of 5 × 10^3 cells/cm^2. The seeded specimens were cultured in the CO_2 incubator (5% CO_2) and the complete medium was changed every other day. Following 1, 3, and 7 days of culturing, the cell proliferation was determined by a methylthiazoletetrazolium (MTT, Sigma, St. Louis, MO, USA) assay. A total solution of 100 μL from each well was aspirated and poured into a 96-well plate for measurement by ELISA reader (Sunrise-Basic, Tecan, Austria) at 570 nm.

The mean ± standard deviation (SD) values were used for the expression of data. The one-way analysis of variance (ANOVA) was employed for cell proliferation. A *P*-value of less than 0.01 was regarded as statistically significant.

Results

Substrate characterization

The microstructure of the SLM-Ti6Al4V specimen was observed by a metallographic microscope after grinding and polishing (Fig. 1). The structures printed using SLM technology display acicular α-phase organization. The phase compositions of the SLM-Ti6Al4V specimen were mainly composed of the α-Ti phase and some α′-Ti phase in the alloy (Fig. 2). The α′-Ti phase appearance is supersaturated in vanadium relative to the equilibrium α because of the very fast cooling rates during the SLM process hindering vanadium diffusion.

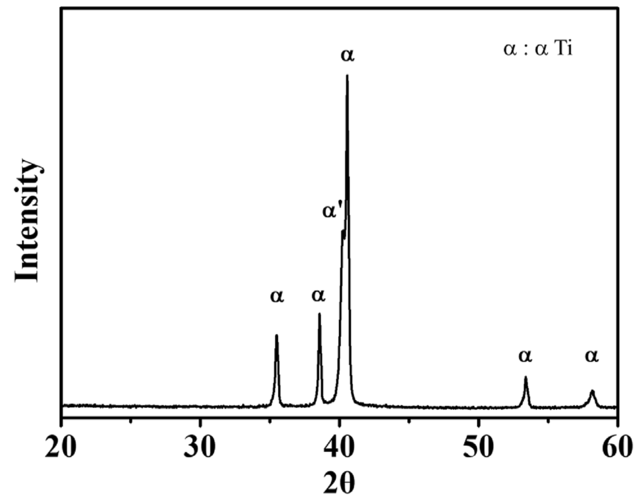

Figure 2 X-ray diffraction (XRD) pattern of selective laser melted Ti6Al4V (SLM-Ti6Al4V) substrate.

SrMAO coatings structural analysis

The surface morphology of SrMAO coatings showed rough and three-dimensional structures with open pores (Fig. 3(b)). The substrate surface was smooth before the MAO process (Fig. 3(a)), but became rough and porous when the spark discharge occurred. The plentiful open pores were characteristically produced by the MAO process. The elemental compositions of SLM substrate and SrMAO coatings were measured by EDS analysis (Table 1). The SrMAO coatings are mainly composed of titanium, aluminum, vanadium, strontium, calcium, phosphorus, and oxygen elements, which come from the used electrolytes and the substrate.

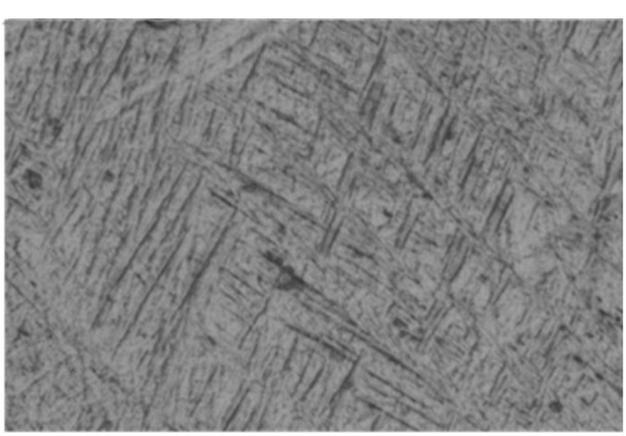

Figure 1 Metallurgical microscope images of selective laser melted Ti6Al4V (SLM-Ti6Al4V) microstructures (magnification: 200x).

The TF-XRD patterns of the SLM specimen after MAO treatment are shown in Fig. 4, and presented features of a crystal structure within the oxide films, indicating the presence of crystallized TiO_2. Three kinds of crystalline phases, anatase (TiO_2) (JCPD #00-002-0406), rutile (TiO_2) (JCPD #01-088-1172), and titanium (JCPD #01-089-5009), were observed in the diffraction patterns. The appearance of titanium peaks should come from the oxide layers of X-ray penetration. The main peak of these peaks is at 25.41° and 27.77° for specimens, indicating that SrMAO coatings are mainly composed of rutile structures containing anatase. However, SrMAO coating was composed of titanium, aluminum, vanadium, strontium, calcium, phosphorus, and oxygen by EDS analysis (Table 1). It suggested that the SrMAO coatings are composed of titanium oxide containing Sr—Ca—P—O amorphous phase.

The specimen average roughness (Ra) of untreated SLM-Ti6Al4V and the SrMAO coatings are 0.52 ± 0.05 μm and 0.64 ± 0.07 μm, respectively, which indicated that the surface roughness was submicron meter level. The method of contact angle could evaluate material surface hydrophilicity, and materials with better hydrophilicity present lower contact angles. The deionized water droplets could be spread preferably on the SrMAO coating surface (Fig. 5(b)), and the contact angle was less than that of uncoated SLM-Ti6Al4V specimens (Fig. 5(a)). Smaller contact angles correspond to excellent hydrophilicity, which is the required feature for materials to be used in biomedical applications. The cross-sectional morphology of SrMAO coatings was observed (Fig. 6), and presented a structure comprising two layers. The coating was uniformly and continuously formed on the SLM-Ti6Al4V substrate surface, and the thickness was 4.2 ± 0.4 μm. Furthermore, compared with the continuous and dense structure of the inner layer, the outer layer had a thicker porous structure.

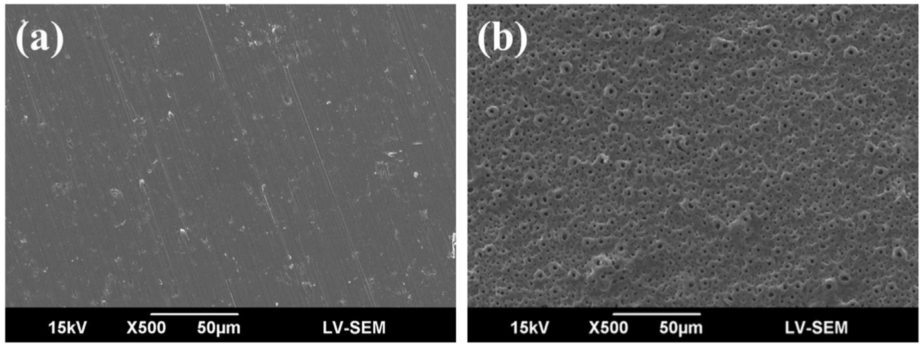

Figure 3 Surface morphology of (a) as-polished SLM-Ti6Al4V substrate, and (b) the strontium-containing MAO (SrMAO) coatings formed on the SLM-Ti6Al4V substrate by scanning electron microscopy (SEM).

Table 1 Elemental compositions (at%) of selective laser melted Ti6Al4V (SLM-Ti6Al4V) substrate and strontium-containing coatings MAO (SrMAO) coatings.

	Ti	Al	V	Sr	Ca	P	O
SLM-Ti6Al4V	87.91	8.65	3.44	nil	nil	nil	nil
SrMAO coatings	15.88	1.82	0.63	0.91	6.16	5.00	69.60

Unit: at%.

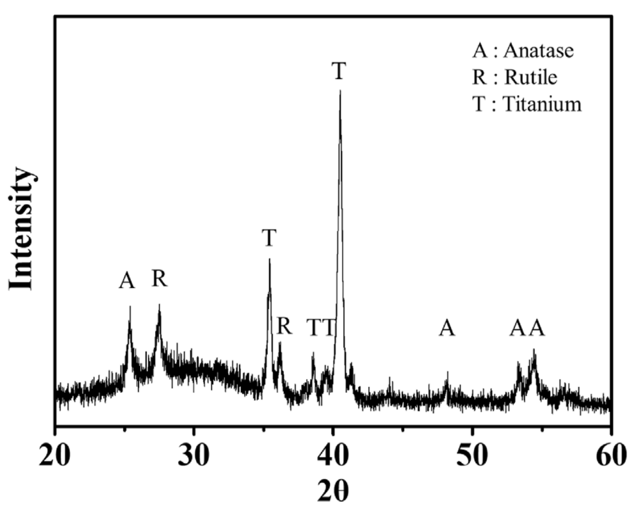

Figure 4 Thin film X-ray diffraction (TF-XRD) pattern of the strontium-containing MAO (SrMAO) coating.

Bioactivity and biological properties

Bioactivity has suggested that an apatite layer may promote bonding between bone and an artificial implant. SBF immersion is a well-known method for the preliminary determination of the material bioactivity, *in vitro*. When a material is soaked in SBF or implanted in a body environment, the bone-like apatite layer formed on its surface indicates that it is biocompatible and bond to bone. Fig. 7 shows the morphologies of SrMAO coatings after being soaked in SBF for 0, 1, 3, and 7 days. Few precipitates formed on the surfaces of SrMAO coatings after immersion for 1 day, and a porous structure was observed. The surfaces of SrMAO coatings were completely covered with precipitates for 7 days. However, the surfaces of the SLM-Ti6Al4V substrate were not covered with precipitates after immersion for 0—7 days. The surface compositions of SrMAO coatings were measured by EDS analysis (Table 2). The content of calcium and phosphorus showed a significant increase from day 3 to day 7. The strontium content increased from day 0 to day 3 and there were no signals of strontium, titanium, aluminum, and vanadium for soaking 7 days. These results suggest that an apatite layer was formed on the surface of SrMAO coatings when exposed to SBF. The precipitate of the apatite layer, mainly composed of calcium and phosphorus, increased the signal of calcium and phosphorus but decreased the signal of strontium.

The MTT assay was used to determine the rate of cell proliferation on SrMAO coatings and control substrates. The growth of MG63 cells on the surface of each specimen was

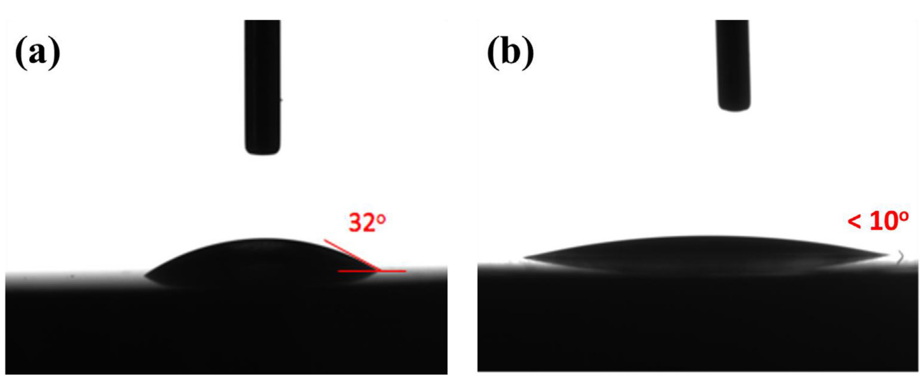

Figure 5 Surface static contact angle of (a) uncoated SLM-Ti6Al4V and (b) the strontium-containing MAO (SrMAO) coating.

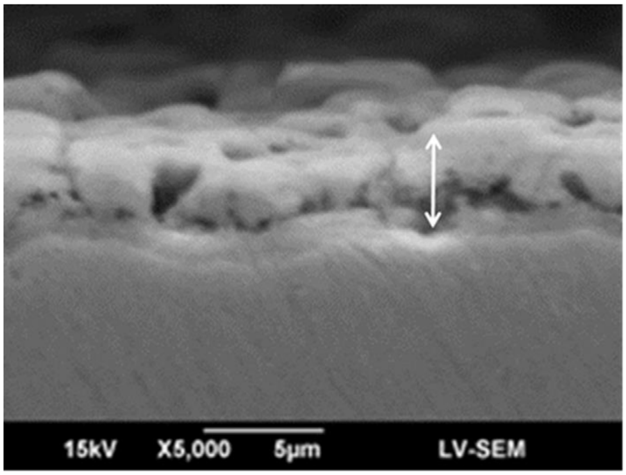

Figure 6 Cross-sectional images of the strontium-containing MAO (SrMAO) coating.

evaluated after 1, 3, and 7 days of cell culturing (Fig. 8). The significant increase in cell numbers as a function of time was observed in SrMAO coatings throughout the 1–7 day culture period. The cell numbers on SrMAO coatings are higher than as-polished substrates after 1-day, 3-day, and 7-day cultures, and there is a significant difference found between the SrMAO coatings and SLM-Ti6Al4V substrates ($P < 0.01$). Therefore, the SrMAO group exhibited a higher level of cell proliferation than that of the untreated group.

Discussion

Ti6Al4V is one of the widely used titanium alloys for engineering and biomedical applications. However, the traditional casting of Ti6Al4V has some disadvantages such as poor machinability with the complex geometries of a dental screw. AM can provide a more suitable method of desired structures with intricate geometries for fabricating biomedical materials. Ti6Al4V is known as an ($\alpha + \beta$) phase alloy, which presents roughly 94% vol. for the hexagonal compact (hcp) α phase and 6% vol. for the body centered cubic (bcc) β phase.[9] In the SLM process, the subsequent rapid solidification of the molten pool will result in the non-equilibrium phase for the formation of acicular α' martensite. This α' phase shows a hcp structure (similar to the pure α phase), and it exists the alloying elements of the SLM-Ti6Al4V alloy to form a very fine entangled needles microstructure.[10] The results were consistent with microstructural and XRD analysis (Figs. 1 and 2).

To improve the biocompatibility of Ti6Al4V alloys, there are many methods to modify the surface composition and surface morphology. The MAO technique is a simple, controllable, and cost-effective method, which combines chemical and morphological modification in a single step. Furthermore, the MAO coating properties are easily varied by controlling process parameters, such as voltage, current, processing duration, and electrolyte constituents. Because of the plasma-like state of the surface during the MAO process, the erupted gases oxygen and hydrogen can form a three-dimensional microstructure with a large number of crater-like pores (Fig. 3).[11] The enlarged surface area and rough microstructure could provide physical support for physiological responses such as cell proliferation and attachment. Besides, the crystallization of TiO_2 matrix appeared to occur simultaneously at random points on the substrate surface where the spark discharge occurs (Fig. 4). The application of higher voltages led to the formation of mixed phases of anatase and rutile in the MAO coatings. The MAO coating only consisted of anatase phase at low voltage, but a mixture of rutile and anatase phases appeared at high voltage.[12]

Many electrolytes have been used to anodize titanium and titanium alloys and the composition of oxide coatings is generally determined by the electrolyte type which are incorporated during the process. It is known that calcium and phosphorus are beneficial to bond with bone tissue. Various elements are responsible for a variety of biochemical functions, which are important for the different steps of bone regeneration between osteoblasts, osteoclasts, and osteocytes such as strontium and zinc.[13] In particular, strontium can have the dual effects of increasing bone cell proliferation and inhibiting bone resorption, which is helpful for patients with osteoporosis.[14] From the EDS results, it was indicated that strontium, calcium, and phosphorus existed in SrMAO coatings (Table 1). During the MAO process, the coatings were infused with electrolytes that contained strontium, calcium, and phosphorus. Although these elements are undetectable by XRD, it can be inferred that the titanium oxide composition includes a Sr–Ca–P–O amorphous phase, which is based on previous studies.[15]

It is generally accepted that surface properties of MAO coatings heavily influence materials biocompatibility. The hydrophilicity is directly related to the water contact angle and the increase in the hydrophilicity of the implant surface affects the interaction between the implant and the surrounding biological environment. The water contact angle of SrMAO coatings is below 10° (Fig. 5), indicating that the coatings show excellent hydrophilicity. Chu et al.[16] reported that the contact angle of the MAO samples decreases dramatically with increasing the applied voltage. Due to smaller pores and roughness formed at lower applied voltages, water cannot wet the smaller pores of the MAO coating surface. Other studies indicated that the contact angle value is related to the surface roughness. For hydrophilic materials, the surface becomes more hydrophilic as it becomes rougher compared with smooth surfaces, such as the TiO_2 coating produced by the MAO process.[17] Furthermore, the surface morphology of MAO coatings is also a crucial factor in the nucleation of apatite. The concave regions of three-dimensional structures act as nucleation sites for bone-like apatite anchors. This phenomenon explains that the three-dimensional structure combines compositions of coatings to accelerate apatite nucleation by increasing the ionic activity product of apatite.

SBF is a metastable buffer solution similar to human plasma. The biomimetic system has been reported to induce the formation of a bone-like apatite layer on the surface. The apatite completely covers the surfaces of SrMAO coatings after being soaked in SBF for 7 days (Fig. 7) which suggests that it possesses a good bioactivity. The incorporation of phosphorus and calcium into the oxide

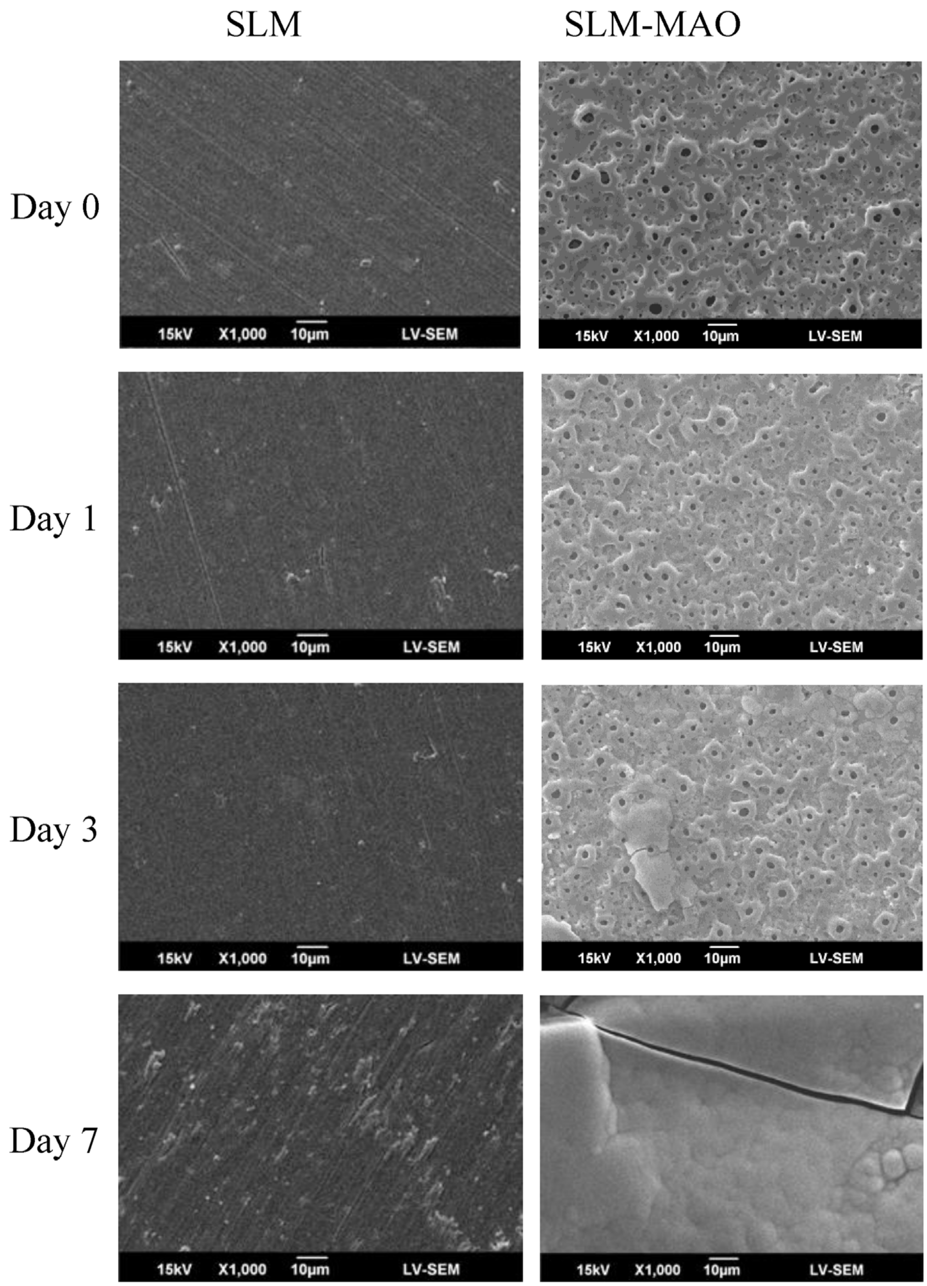

Figure 7 Scanning electron microscopy (SEM) images of the strontium-containing MAO (SrMAO) coatings soaked in simulating body fluid (SBF) for 1—7 days.

Table 2 Elemental compositions (at%) of the selective laser melting (SLM) substrate and strontium-containing coatings MAO (SrMAO) coatings after soaking in simulated body fluid (SBF).

	Soaking Time	Ti	Al	V	Sr	Ca	P	O
SLM-Ti6Al4V	Day 0	87.93	6.03	6.04	nil	nil	nil	nil
	Day 1	90.09	5.75	4.15	nil	nil	nil	nil
	Day 3	89.44	6.48	4.08	nil	nil	nil	nil
	Day 7	90.07	5.9	4.03	nil	nil	nil	nil
SrMAO coatings	Day 0	15.88	1.82	0.63	0.91	6.16	5.00	69.60
	Day 1	16.58	1.06	0.55	1.23	6.89	7.34	66.34
	Day 3	16.55	1.81	1.51	1.14	10.10	5.18	63.70
	Day 7	nil	nil	nil	nil	30.72	17.06	52.22

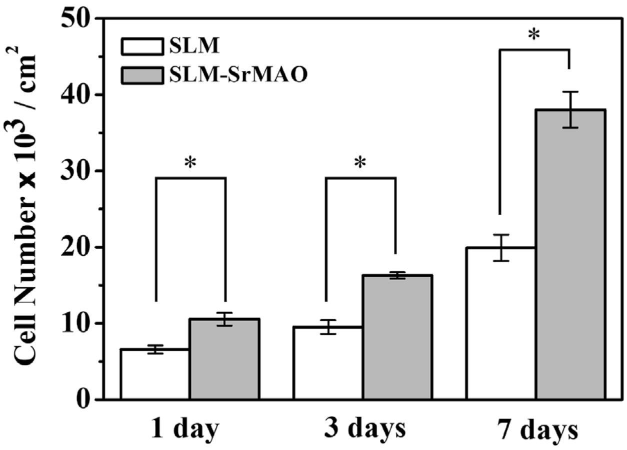

Figure 8 The methylthiazoletetrazolium (MTT) cell proliferation assay results of MG63 cells after culturing for 1, 3, and 7 days. * indicates a significant difference (P-value < 0.01).

coatings enhanced the formation of the apatite layer. A previous study[18] showed that SrMAO coatings containing strontium soaked in SBF, the surface was covered with a layer of the apatite phase after 7 days of immersion. Besides, the strontium ion concentrations increased in SBF solution, which indicated that the strontium ions would be released from MAO coatings. The released strontium ions have been shown to affect the early formation of biomimetic coatings on single crystalline rutile substrates and have a critical impact on cellular growth and gene expression in the human body.[19,20]

The biocompatibility of a material depends not only on its physical and chemical surface properties, but also on the initial reaction of cells upon the material surface. The porous structure of MAO coatings provides a rough surface that mechanically interlocks with bone cells to enhance their growth and increase the fixation and stability of the implant. The surface roughness of titanium implants has an impact on the rate of osteointegration and biomechanical fixation.[21] The average roughness of SrMAO coatings is 0.64 μm, which belongs to the submicron level in this study (Fig. 3). Im et al.[22] indicated that submicron features on titanium were found to improve osteoblast adhesion and proliferation. The research demonstrated that submicron structures showed cytocompatibility properties for endothelial and bone cells.

A study suggested that a surface modification method of anodic oxidation for 3D printed implants induced a better promotion effect on osteoblast proliferation and differentiation compared with untreated surface or polished surfaces.[23] Culturing cells on SrMAO coatings of specimens led to a significant increase in the number of cells after 1–7 day culture, compared to culturing on untreated specimens (Fig. 8). The MAO technology can create calcium phosphate coatings on implants for enhancing long-term corrosion resistance and biocompatibility.[24] Furthermore, SrMAO coatings containing strontium contents provided a preferential surface for cell proliferation compared with coating without strontium content, and the optimal content of strontium could improve biological performance.[15] Landi et al.[25] found that low concentrations of strontium can promote to stimulate bone formation, but high concentrations have deleterious effects on bone mineralization.

In conclusion, the surface of Ti6Al4V alloys fabricated by the SLM method was modified through the MAO process. The phase compositions of SLM-Ti6Al4V substrates were $\alpha + \alpha'$ phases. The SrMAO coating surface produced open pores and three-dimensional structures, and the average thickness and roughness were 4.2 μm and 0.64 μm, respectively. The anatase and rutile phases were identified in SrMAO coatings, and the electrolyte containing strontium, calcium, and phosphorus has been applied to produce coatings with Sr–Ca–P–O phase embedded in the TiO_2 matrix. The SrMAO coatings showed excellent hydrophilicity, and induced the apatite layer after being soaked in SBF. Furthermore, SrMAO coatings significantly enhance cell proliferation in *in vitro* tests. All findings indicate that the proposed SrMAO coatings containing strontium formed on SLM-Ti6Al4V substrates show considerable promise as a biomaterial for implants.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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