Improved bioactivity of selective laser melting titanium: Surface modification with micro-/nano-textured hierarchical topography and bone regeneration performance evaluation

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Abstract

Selective laser melting (SLM) titanium requires surface modification to improve its bioactivity. The microrough surface of it can be utilized as the micro primary substrate to create a micro-/nano-textured topography for improved bone regeneration. In this study, the microrough SLM titanium substrate was optimized by sandblasting, and nano-porous features of orderly arranged nanotubes and disorderly arranged nanonet were produced by anodization (SAN) and alkali-heat treatment (SAH), respectively. The results were compared with the control group of an untreated surface (native-SLM) and a microtopography only surface treated by acid etching (SLA).

The effects of the different topographies on cell functions and bone formation performance were evaluated in vitro and in vivo. It was found that micro-/nano-textured topographies of SAN and SAH showed enhanced cell behaviour relative to the microtopography of SLA with significantly higher proliferation on the 1st, 3rd, 5th and 7th day ($P < 0.05$) and higher total protein contents on the 14th day ($P < 0.05$). In vivo, SAN and SAH formed more successively regenerated bone, which resulted in higher bone-implant contact (BIC%) and bone-bonding force than native-SLM and SLA. In addition, the three-dimensional nanonet of SAH was expected to be more similar to native extracellular matrix (ECM) and thus led to better bone formation. The alkaline phosphatase activity of SAH was significantly higher than the other three groups at an earlier stage of the 7th day ($P < 0.05$) and the BIC% was nearly double that of native-SLM and SLA in the 8th week. In conclusion, the addition of nano-porous features on the microrough SLM titanium surface is effective in improving the bioactivity and bone regeneration performance, in which the ECM-like nanonet with a disorderly arranged biomimetic feature is suggested to be more efficient than nanotubes.

1. Introduction

Fabrication of custom-made bone substitutes has always been appealing in dentistry, orthopaedics, and joint arthroplasty clinical practices [1–5]. However, complicated and irregular anatomies make conventional manufacturing difficult because of complex cast processes and high costs of raw materials [6,7]. In addition, multiple process steps may result in imprecise products. Recently, the rapid developments in additive manufacturing (AM) have enabled the production of freeform geometries [5,8,9], allowing for custom-made and precisely controlled bone substitute architectures based on computer-aided design (CAD) data [3,10–13]. AM is more convenient in fabricating complex and custom-made structures than conventional methods based on a one-step manufacturing process [14].

Selective laser melting (SLM), one of the latest types of AM, is widely used in biomedical implants fabrication. Titanium and its alloys fabricated by SLM are expected to be promising biomaterials as bone substitutes because of their biocompatibility and biomechanical properties [15–17]. Because the bone-implant interface behaviour is important, research has focused on the biological property and surface modification of SLM titanium. Warnke et al. [4] reported that the cell proliferation on the native SLM was only 60% of that on the flat glass. However, in the research of Tsukanaka [18], no significant differences were observed between the untreated SLM titanium and flat-rolled surface, but he noted that bioactive treatment had to be applied to the SLM titanium to improve the effect on osteoblast differentiation. Pattanayak et al. [10] demonstrated that the percentage bone affinity indices of...
chemical- and heat-treated SLM titanium were significantly higher than those of untreated implants. De Wild et al. [19] compared untreated, sandblasted and sandblasted/acid-etched SLM titanium and found that the treated SLM surface provided better osteoconduction. Because the early osseointegration plays a crucial role in the long-term success of implants, it is necessary to modify and improve the surface of SLM titanium to achieve rapid and stable osseointegration in clinical settings [15,20,21].

Surface topology modifications have been widely studied in tissue engineering research; they directly interact with cells to modulate the adhesion, migration, proliferation, differentiation, and consequent bone formation [22,23]. Micro-/nano-topographies are reported to be more biomimetic and effectively increase the bioactivity of implants [24,25]. A bone tissue system consists of macrostructures including cancellous and cortical bones, microstructures including lamellae and Haversian systems, and nanostructures such as proteins, collagenous fibrils and hydroxyapatite crystals [26,27]. Compared with a simplex micro or nano-structure, a micro-/-nano-hierarchical topography better mimics such complex architectures and compositions. A surface with microscale roughness is believed to increase osteoblast differentiation and induce faster bone maturation around the implant, offering improved biomechanical interlocking stability [28]. However, it was reported to reduce cell proliferation and accordingly lead to lower bone mass [28]. With the addition of nano features, a microscale surface will be altered to a micro-/-nano-hierarchical structure, which has been demonstrated to accelerate cell functions by synergistically balancing the dilemma between cell proliferation and differentiation [27,29]. Various micro-/-nano-engineering methods were reported and utilized on the surface of titanium and its alloys to achieve better bone regeneration performance [27,30–34]. Kubo et al. [30] reported a micropit-nanodotulpe topography that performed better than micropit-only topography. Zhao and coworkers [27] developed a hybrid topography of the acid-etched microstructure with the addition of titanate nanotubes and noted a significant enhancement in multiple osteoblast behaviour types.

Based on the layer-by-layer manufacturing method, the native SLM product shows a layered and rough topography instead of a flat surface. The rough SLM surface can be used as a microscale primary substrate for the establishment of micro-/-nano-textured hierarchical topography [7]. To the best of our knowledge, there are some bioactive surface treatments applied to SLM titanium, but few studies focus on the modification of micro-/-nano-topography and its influence. In the present study, we intended to modify the surface by adding nano-porous features to the SLM microrough substrate to improve its bioactivity for better bone regeneration. At the same time, we expected to acquire some new knowledge about the difference between two nano-porous features of different arrangements.

For this purpose, we used sandblasting to modify native SLM titanium as a microrough substrate, followed by acid etching, anodization and alkali-heat treatments to create different micro or nano features, and evaluated their bone regeneration performance in vitro and in vivo. The surface treatments were chosen for the following reasons: i) they are commonly used as rapid and easily adopted methods that can be applied to irregular custom-made structures; ii) sandblasting/acid etching (SLA) is an effective modification method that has been adopted commercially [15,19]; iii) nano-porous features were reported to be dramatically improve bone performance on titanium implants, in which anodization and alkali-heat are two simple and effective methods; they can fabricate orderly and disorderly arranged nanopores, respectively [29,31,35].

2. Materials and methods

2.1. Specimen preparation

Specimens were designed by SolidWorks® 12.0 (SolidWorks Corp., Concord, MA, USA) and manufactured by an SLM machine (SLM125HL, SLM solutions GmbH, Germany) using commercial grade II pure titanium powders (average 30 μm particle size) as raw materials under 99.999 wt% argon to prevent oxidation. A fibre laser and a rectangle scanning strategy were used. The SLM processing parameters were adjusted to a laser power of 100 W, laser scanning velocity of 275 mm/s, hatch space of 130 μm, layer thickness of 30 μm, laser spot size of 83 μm and substrate heat of 140 °C. Specimens for the cell culture assay were produced as titanium discs (10 × 10 × 1 mm³) (Fig. 1.a). A titanium dome was designed for a rabbit model and proved to be simple and effective in our previous study [31]; in this study, we used a similar specimen design for the animal experiment (Fig. 1.b, c, d and e). However, some alterations were made according to this experiment. The porous scaffolds were added inside the domes. One of the reasons is that SLM is usually used for custom-made geometries, especially porous structures, and the porous scaffolds mimicked the architectures in clinical application. The other reason is that the increased superficial area of porous scaffolds make it easier to detect the BIC% difference. Because the influence of the forms of the porous structures was not the focus of this study, we used a simple cubic unit design and set the strut size to 0.2 mm and the pore size to 0.6 mm, which has been proved to be effective for bone growth [19,36–38]. After fabrication, the SLM specimens were cleaned by compressed air and ultrasonic treatment.

2.2. Surface treatment

Unetched SLM titanium discs and domes were named native-SLM and set as the control group. The other specimens were first sandblasted with 250 μm ZrO₂ particles and then immersed in an acidic mixture consisting of 40% HF and 60% HNO₃ (H₃O/HF/HNO₃: 1:4:5, v/v) for 2 min to remove the oxide layer and polish the surface of the SLM titanium. For the SIA group, the polished specimens were acid etched in a mixture of 98% H₂SO₄ and 36.5% HCl solution (H₂O:HCl/H₂SO₄: 2:4:3, v:v) at 95 °C for 15 min. Simultaneously, the polished specimens were used as the electrode anode with a titanium rod as a cathode and were anodized in a 0.5 wt% HF in distilled water solution acid electrolyte using a 20 V power supply for 45 min; this group was named the SAN group. For the SAH group, the sandblasted specimens were well stirred in 5 M NaOH solution at a temperature of 80 °C for 8 h. Afterward, all treated specimens were thoroughly cleaned ultrasonically in deionized water and autoclave sterilized before cell culture studies and animal experiments. The specimens were sealed and stored in air in dark place to avoid possible ultraviolet radiation.

2.3. Surface characterization

The surface topography and chemistry were analyzed by field-emission scanning electron microscopy (FE-SEM, Hitachi S-4800, Japan) equipped with an X-ray energy dispersive spectrometry (EDS) and an X-ray photoelectron spectroscopy (XPS, Thermo Scientific ESCALAB 250Xi, USA). Surface roughness, including the average height above the centreline (Ra) and the root mean square of Ra (Rq), were measured by a contact profilometer (SJ-210, Japan). Water contact angles were determined by the sessile-drop method on an OCA20 drop shape analysis system (DSA100, Germany).

2.4. Cell culture assay

The osteoblast-like cell line MC3T3-E1, Subclone 14, which was obtained from the Shanghai Cellular Institute of China Scientific Academy, was used in this study. MC3T3-E1 cells were cultured at 37 °C and 5% CO₂ in an α-MEM medium (Gibco, USA) containing 10% foetal bovine serum (FBS, Gibco, USA) and 1% antibiotic/antimycotic solution (Gibco, USA).
2.5. Cell viability evaluation

For the cell morphology assay, MC3T3-E1 cells were incubated with the specimen surfaces in 48-well plates at a density of $1 \times 10^4$ cells ml$^{-1}$ for 2 h and fixed in 2.5% glutaraldehyde solution for 6 h. After gradient dehydration in ethanol solutions (50%, 75%, 80%, 90% and 100%) and drying, the specimens were observed under FE-SEM.

For the cell proliferation assay, MC3T3-E1 cells were seeded onto specimens in 48-well plates at a density of $1 \times 10^4$ cells ml$^{-1}$ and cultured with an $\alpha$-MEM medium containing 10% FBS for 1, 3, 5 and 7 days. At the prescribed time points, the cells were assessed using a cell counting kit-8 assay (CCK-8, Dojindo, Japan) [31] and measured at the optical absorbance (OD) of 450 nm.

The alkaline phosphatase (ALP) activity of MC3T3-E1 cells was normalized to the total protein content. Cells were seeded onto specimens in 48-well plates at a density of $2 \times 10^4$ cells ml$^{-1}$. After 7 and 14 days of culturing in mineralized solution, 0.1% Triton X-100 were used to lyse cells at 4 °C, and the ALP activity was determined by the p-nitrophenyl phosphate (p-NPP) method using an ALP Assay Kit (Jiancheng, Nanjing, China) [31] at an absorbance of 520 nm. The total protein content was measured using a bicinchoninic acid assay kit (BCA, Beyotime, China) [31] at a wavelength of 562 nm.
2.6. Animal experiment

This study was approved by the Institution Animal Care and Use Committee (IACUC) of Sun Yat-Sen University, and the animal use protocol has been reviewed and approved by the Animal Ethical and Welfare Committee (AEWC) (Approval No. IACUC DB-16-0201). An animal model of 12 New Zealand white rabbits weighing 2.0–2.5 kg and specimens of titanium domes with porous scaffolds were used in this study. The rabbits were anesthetized with xylazine hydrochloride (0.1 mg kg\(^{-1}\); Sumianxin, Jilin, China) and pentobarbital sodium (30 mg kg\(^{-1}\); Pelltobarbitalum, Sigma, USA). As shown in Fig. 2, the surgical procedures were performed strictly under sterile conditions. After exposure of the medial calvarial bone of these rabbits, four rings were prepared in the four areas that were divided by the metopic and frontal suture, using a 6.0 mm trephine drill with sufficient saline cooling. Four titanium dome specimens with different surface treatments were implanted in the prepared ring sites at random, and then the surgical layers were stratified and sutured. All rabbits received further gentamicin injections for 4 days. The rabbits were sacrificed after 4 and 8 weeks after surgery. Specimens consisting of a titanium dome and bone tissue inside were excised and kept in 4% buffered paraformaldehyde for further evaluation.

2.7. Osteogenesis evaluation

The titanium domes with bone tissue specimens were examined by a micro-CT scanner (\(\mu\)CT50, Scanco Medical, Bassersdorf, Switzerland) under a 10 \(\mu\)m resolution protocol (voltage: 90 kV; current: 88 \(\mu\)A; 0.25 mm Cu filter; integration time: 1500 ms). Mimics® 15.0 was used to reconstruct the micro-CT images. The regenerated bone volumes were measured in terms of bone tissues inside the SLM titanium domes, and the total volume was defined as the total space inside the domes. The bone volume/total volume (BV/TV) was then calculated.

After micro-CT examination, the specimens were dehydrated in gradient ethanol and then embedded in methylmethacrylate without decalcification. After that, a 30 \(\mu\)m thickness section was sliced using a SP1600 microtome (Leica Microsystems, Wetzlar, Germany) and stained in methylene blue-acid fuchsin. A stereomicroscope (Leica M205 A, Leica Microsystems GmbH, Germany) was used to observe the overall bone formation inside the domes at 15 \(\times\) magnification, and a light microscope (AxioCam HRc, ZEISS Axio Imager Z2, Germany) was simultaneously used to observe the local bone-implant interface at 25 \(\times\) magnification. The BIC\% was calculated using the Image-Pro® Plus 6.0 image analysis system (Image-Pro Plus 6.0, Media Cybernetics, California, USA). The interest area was defined as the bone tissue inside the domes.

2.8. Biomechanical testing

To examine the bone-bonding ability of different groups, a biomechanical testing was performed using a commercial material testing system (E3000, INSTRON, Massachusetts, USA). The bottom clamping side of bone tissues and the upper side of titanium domes were severally embedded in acrylic resin (Anyang Eagle Dental Material, China) holders and firmly fixed without bending. A pull-out test was then performed at a tension speed of 0.5 mm min\(^{-1}\), and the bone-bonding ability was determined by the maximum force to failure (N).

2.9. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) and analyzed using the SPSS 13.0 software package (SPSS Inc., Chicago, USA). The level of significance was determined by a one-way ANOVA followed by a Student-Newman-Keuls (SNK) post hoc test for a multiple comparison procedure. A value of \(p < 0.05\) was considered statistically significant.

3. Results

3.1. Surface topography

The native SLM titanium substrate showed a rough waving surface consisting of printing layers, on which certain residual unmelted
titanium particles and spheres were found (Fig. 3.Aa and Ba), but no nano topographical features were observed (Fig. 3.Be). It was noted that acid led to different effects under different concentrations, treatment times and types [39]. After sandblasting, we used a mixture of 40% HF and 60% HNO₃ to polish the specimen surface for 2 min. From the SEM images, we found that the acid treatment described above did not cause severe erosion but only removed the irregularities and particle residuals; the layered surface of SLM remained (Fig. 3.Ab). Moreover, micropitted topography produced by sandblasting can be observed (Fig. 3.Ac). We did not find any concaved topography of acid etching on the surface (Fig. 3.Ad). On the microrough SLM surface, different features at the micro- or nanoscale were produced by chemical or electrochemical surface treatments. The microtopography of irregular concavities and ridges with macro and micro peaks were engraved on the SLA (Fig. 3.Bb and Bf). At high magnification, nano-porous features were formed on the SAN and SAH. The titanate nanotubes of SAN with a diameter of 50–100 nm were uniformly distributed on the microrough substrate (Fig. 3.Bc and Bg). On the surface of SAH, a three-dimensional ECM-like nanonet feature was produced (Fig. 3.Bd and Bh). Compared with the single-layered nanotubes on the SAN (Fig. 3.Ca and Cb), a hierarchical architecture of interpenetrating nanonet was readily observed on the SAH. The disorderly arranged nanonet has 50–100 nm small pores inside and 300–500 nm large pores outside (Fig. 3.Cc and Cd), more approximate to the natural ECM grid structure.

3.2. Surface characterization

To examine the basic surface characteristics, we evaluated the surface elemental composition, roughness and water contact angle of each group. The EDS results (Table 1) showed that titanium was the major element of all groups, and oxygen was found on all the surface-treated groups. In addition, 0.95% sulfur, 6.39% fluorine, 4.29% carbon and 0.49% calcium were found on the SLA, SAN and SAH, respectively. No zirconium of sandblasting residuals was found on any surface-treated groups. XPS analysis was used to determine the elemental composition of the outermost surface more accurately. Fig. 4 showed that the curves of each groups were similar. Titanium and oxygen were the main elements on the surface, which was consistent with the EDS results. The Ti2p3 peak value was approximately 458.5 eV, 459.2 eV, 458.5 eV, and 460.3 eV in native-SLM, SLA, SAN and SAH, respectively, which has been attributed to Ti⁴⁺, suggesting that the main oxide compounds of titanium was TiO₂ in the four groups. Although sulfur and calcium were tested in the EDS, they were not detected in the XPS; moreover, the quantities were quite a little, indicating that they may come from certain contaminant in air
in the process of EDS test. Fluorine may come from the acid electrolyte of HF solution, and the quantity was also relatively low.

The results of surface roughness values (Ra and Rq) are shown in Table 2. Native-SLM, with an Ra value of approximately 11.45 μm, was found to possess a rougher surface than SLA, SAN, and SAH, with Ra values of approximately 7.63 μm, 8.74 μm and 8.28 μm, respectively.

The water contact angles were measured to assess the relative wettability of each surface. Though the rough surface was reported to increase the risk of entrapment of air bubbles that may influence the water contact angle [17], however, the roughness of the three surfacetreated groups in this study were similar; we can make comparison relatively. SLA showed a hydrophobic surface with a water contact angle of approximately 131.72°, whereas SAH showed favourable hydrophilicity with a relatively lower water contact angle of approximately 31.23°.

3.3. Cell viability evaluation

We evaluated the in vitro effects on each surface by examining the adherent morphology, proliferation and differentiation activities of MC3T3-E1 cells. Cells were found to be more polygonal in native-SLM, SLA and SAN, but more elongated and unidirectional in SAH (Fig. 5a, c, e and g). The cell on the surface of native-SLM stretched slender filopodia across the titanium sphere (Fig. 5a and b). At high magnification, the pseudopods were relative single in SLA and SAN (Fig. 5d and f), whereas multiple contact points with tiny protrusions extending from the lamellipodia and wrapping around the nanonet were observed in SAH (Fig. 5h).

Fig. 6A shows the results of the cell proliferation of each group. The surface-treated groups of SLA, SAN and SAH showed significantly higher rates of cell proliferation compared with native-SLM on the 1st, 3rd, 5th and 7th days (P < 0.05). Moreover, SAN and SAH showed more proliferation than SLA as well, and SAH exhibited statistical significance at each time point (P < 0.05).

Cell differentiation was measured by the total protein contents and ALP activities after 7 and 14 days of culture, and the results are exhibited in Fig. 6B and C. In comparison, native-SLM performed poorly in terms of total protein contents and ALP activity. SAN and SAH dramatically enhanced the total protein contents compared with SLA and were statistically higher on the 14th day (P < 0.05). In the ALP measurements, SAH was more pronounced than the other three groups at an earlier stage of the 7th day (P < 0.05). SAN showed a promotion of ALP activity on the 14th day.

3.4. Animal experiments

The overall condition of the regenerated bone is illustrated by the three-dimensional reconstructions of the total bone volume in Fig. 7 and the histological images at a low magnification in Fig. 8A. In some representative images, the regenerated bone of the SLA, SAN and SAH was observed to grow into the scaffolds 4 weeks after surgery, whereas no bone was found growing into the scaffolds in the native-SLM until the 8th week. The regenerated bone volume of SAH nearly filled up the inner space of the titanium dome at 8 weeks. Fig. 9A shows the BV/TV is in the order of SAH > SLA > SAN > native-SLM (4w: native-SLM: 11.43 ± 5.03%, SAN: 11.91 ± 7.14%, SLA: 16.97 ± 4.34%, SAH: 19.60 ± 4.25%; 8w: native-SLM: 16.97 ± 2.08%, SAN: 20.63 ± 1.52%, SLA: 23.32 ± 9.10%, SAH: 24.59 ± 3.35%). SAN and SAH are statistically more significant than native-SLM at the 8th week (P < 0.05).

From the histological images (Fig. 8), we could see successively connected bone along the surface of SAN and SAH (black arrows), but the bone fragments were more scattered in native-SLM and SLA. The BIC% is in the order of SAH > SAN > SLA > native-SLM (4w: native-SLM: 6.15 ± 1.32 %, SAN: 9.18 ± 3.35 %, SLA: 9.75 ± 3.20 %, SAH: 11 ± 1.36 %; 8w: native-SLM: 14.35 ± 4.38 %, SAN: 15.13 ± 6.78 %, SLA: 18.38 ± 7.00 %, SAH: 26.5 ± 1.60 %), and SAH is significantly higher than native-SLM at both time points (P < 0.05) (Fig. 9B). The maximum pull-out force is in the order of SAH > SAN > SLA > native-SLM (4w: native-SLM: 77.19 ± 11.73 N, SAN: 81.19 ± 16.12 N, SLA: 96.21 ± 16.78 N, SAH: 103.75 ± 9.63 N; 8w: native-SLM: 101.25 ± 4.95 N, SAN: 112.04 ± 7.98 N, SLA: 113.84 ± 6.25 N, SAH: 139.33 ± 16.84 N), and SAH shows a greater significance than native-SLM and SAN at the 8th week (P < 0.05). It was noted that SAH showed a lower bone volume in the results of BV/TV compared with SLA (Fig. 8A), but it could be found higher than SLA in the results of BIC% and the pull-out force (Fig. 8B and C).

4. Discussion

There is an increasing demand for custom-made and porous architecture implants in bone substitutes applications; this has resulted in the development of the rapid prototyping SLM technique, which can conveniently fabricate materials with complex design parameters [10,40]. Titanium is one of the most commonly used materials for SLM implants. Because the bone-implant interface plays an important role in the osseointegration of materials, biofunctionalizing surface modifications are applied to modify SLM titanium to enhance its bone regeneration performance [41–44]. Recently, micro-/nano-textured hybrid topography was reported to effectively increase the bioactivity of implants [28]. In this study, we intended to improve the SLM titanium bioactivity by utilizing the microrough surface as the substrate and produce nanoporous features on it to establish an optimized micro-/nano-topography.

We produced a waving and layered surface with a roughness of 11.45 μm by the SLM technique. The special waving rough surface is different from the smooth one of conventional machined titanium. Except for the layer-by-layer SLM manufacturing process, the roughness is also

### Table 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>Titanium</th>
<th>Oxygen</th>
<th>Carbon</th>
<th>Sulfur</th>
<th>Fluorine</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>native-SLM</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SLA</td>
<td>78.77</td>
<td>20.28</td>
<td>–</td>
<td>0.95</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SAN</td>
<td>64.11</td>
<td>29.50</td>
<td>–</td>
<td>–</td>
<td>6.39</td>
<td>–</td>
</tr>
<tr>
<td>SAH</td>
<td>51.69</td>
<td>43.53</td>
<td>4.29</td>
<td>–</td>
<td>–</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Fig. 4. XPS survey spectra of the specimens: (1) native-SLM; (2) SLA; (3) SAN; (4) SAH.
influenced by the partially melted particles \cite{45,46}. As shown in the SEM images (Fig. 3.Aa), we can clearly observe the layered structure; however, we also found some unmelted titanium particles attached to it, which may produce defect to the surface \cite{10}. It was reported that the partially melted particles may come from the metallic powders that were blown into the laser melted zone by the argon gas in the build chamber, or the

<table>
<thead>
<tr>
<th>Samples</th>
<th>Ra (μm)</th>
<th>Rq (μm)</th>
<th>Contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native-SLM</td>
<td>11.45 ± 1.41</td>
<td>14.16 ± 1.66</td>
<td>86.97 ± 2.65</td>
</tr>
<tr>
<td>SLA</td>
<td>7.63 ± 1.14</td>
<td>9.42 ± 1.34</td>
<td>131.72 ± 2.07</td>
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<tr>
<td>SAN</td>
<td>8.74 ± 1.45</td>
<td>10.63 ± 1.67</td>
<td>78.73 ± 8.37</td>
</tr>
<tr>
<td>SAH</td>
<td>8.28 ± 1.33</td>
<td>10.07 ± 0.70</td>
<td>31.23 ± 3.90</td>
</tr>
</tbody>
</table>

Ra (average height above centreline); Rq (root mean square of Ra).

Fig. 5. SEM images showing cell morphologies at low magnification (a: native-SLM; c: SLA; e: SAN; g: SAH) and pseudopods at high magnification (b: native-SLM; d: SLA; f: SAN; h: SAH) after 2 h of culturing on the surface of each group.

Table 2
Roughness and water contact angle measurements of specimen surfaces (n = 6, mean ± SD).
one that flowed from the powder bed to the build because of small vibrations of the wiper [46]. To optimize the microstructure of the substrate, we used sandblasting to eliminate the particles and subsequent acid picking to polish the irregularities. After pretreatment, the residuals were eliminated, and a neat waving microrough substrate was retained. Without partially melted titanium particles, the roughness decreased to approximately 8 μm after surface treatment.

The in vitro and in vivo experiments in our study shows that SAN and SAH, which were modified with micro-/nano-textured hierarchical features, performed better in terms of cell morphology, proliferation, differentiation, bone-implant contact and bone-bonding interaction, compared with native-SLM with no additional features and SLA with a microtopography only. Microscale roughness will increase the mechanical stability of osseointegration, so the microrough surface of native SLM titanium seems to be an appropriate substrate. In our previous study, we compared a similar nanonet micro-/nano-topography on commercial pure titanium with machined titanium [31]. We found that in the animal experiment, the pull-out force of micro-/nano-topography was significantly higher than that of the flat surface in the 4th week (P < 0.05). With a similar animal model, in this study, the bone-bonding force of nanonet was not significant compared with native-SLM in the 4th week (P > 0.05). We suggest that the native rough surface of SLM titanium contributes to the mechanical interlock in the early osseointegration. However, Tsukanaka et al. [18] demonstrated that although SLM rough surface may be beneficial for early mechanical stability, the original surface was ineffective for osteoblast differentiation and had to undergo a bioactive treatment to promote their bone formation. This is consistent with our results. All surface-treated groups surpassed native-SLM, and the micro-/nano-groups are superior to the microtopography group because the nano features play an important role in modulating cell differentiation.

Owing to the better cell differentiation of micro-/nano-topography, SAN and SAH formed better osseointegration and led to higher BIC% and bone-bonding force than native-SLM and SLA. It has been reported that micro-/nano-hybrid topographies enhanced bone regeneration more effectively than either microtopography only or nanotopography only [29–31]. Micro-/nano-topography better mimics natural bone tissues that consist of a hierarchical level of compositions [22]. In addition, micro-/nano-hierarchical topographies are suggested to promote bone formation by the synergistic functions on both the micro- and nanoscale, with the microtopography influencing the initial cell adhesion and proliferation, whereas the nanotopography regulating protein conformation and signal transduction to further enhance osteogenic cell differentiation [27,47]. Thus, the micro-/nano-topography modification is appropriate for and effective in SLM titanium.

A microrough surface with different nano features can induce different cellular responses. In our study, two different nano porous features, nanotubes and nanonet, were created by anodization and alkali-heat treatment on a similar waving microrough topography, named SAN and SAH, respectively. As shown in the SEM images (Fig. 2B), nanotubes on the SAN are homogenously distributed in a single hierarchy, whereas the nanonet on the SAH shows heterogeneous textures at a multiple level with small pores inside and large pores outside. The biological evaluation results showed that SAH performed better than SAN in nearly all the tests. Dalby et al. [48] reported that a disorderly arranged nanopits feature performed better in affecting cell differentiation compared with one that is orderly arranged, suggesting the importance of biomimetic design of natural tissues. In vivo, the cells are enveloped in a nanoscaled structure called native ECM, which regulates cell-interacting features physically in the context of multiple-length scales. ECM consists of three-dimensional intricate mesh network of fibrous proteins in a relative disordered structure. From a biomimetic viewpoint, the disorderly orientated and three-dimensional nanonet of

Fig. 6. Cell viability measured based on the cell proliferation (A) after 1, 3, 5 and 7 days of culture, total protein contents (B) and ALP activity (C) after 7 and 14 days of culture. *P < 0.05 compared with native-SLM; **P < 0.05 compared with SLA; ***P < 0.05 compared with SAN.
SAH is more approximate to the natural ECM compared with orderly arranged nanotubes. Efforts were made by researchers to reproduce ECM-like nanoscale architectures, from which it was reported to increase the cell-substrate contact area by approximately 400% [49,50].

It is noticed that cells are more elongated on the SAH but polygonal on the SAN (Fig. 5). In addition, we observed that the protrusions of lamellipodia on the SAH grew into the net and wrapped around it, causing a tighter connection and larger area of contact. Surface topography directly interacts with cells, so it functions as an analogue to the ECM. When a cell interacts with ECM, it initializes integrin clustering and subsequently forms focal adhesions via a positive feedback mechanism [51]. Simultaneously, actin filaments are inclined to synthesize and move along the direction of minimum external pressure, inducing cell pseudopods stretching. The stimuli of the biophysical cues is carried to the nucleus through cytoskeleton, and gene expression is regulated accordingly [52]. Although the mechanism still remains largely unknown regarding the details (shape, height, length scale) of different topography functions on the cells, it is recently elucidated that nano features modulate the mechanotransduction cell pathway through the adjustment of focal adhesion (FA) [22]. From the SEM images, nanonet of SAH induces more contact area and tighter connection of the cells and pseudopods than SAN. We speculate that SAH topography may surpass SAN in some cell functions by adjusting the focal adhesion and cell morphology through a certain mechanotransduction. The reason for the difference in pseudopods between SAH and SAN may be attributed to the different diameters and shapes of their nanopores. The uniform nanotubes of SAN have a diameter of 50–80 nm in our study, whereas the nanonet of SAH has a diameter ranging from 50 to 500 nm; the larger the diameter of the nanonet, the better matching of the size of cell pseudopods. Moreover, the three-dimensional network presents a hierarchical architecture with the pores opening in multiple directions, from which the pseudopods are easier to stretch into. On the other hand, it is noted that the SAH exhibited higher protein contents than SAN on both the 7th and 14th days; however, SAH dramatically enhanced ALP activity on the 7th day, whereas SAN had a promotion on the 14th day. This indicates that the types of protein contents synthesized on the SAH and SAN may be different; each functions at different times in modulating the cell differentiation, and the nanonet of SAH enhances cell differentiation at an earlier stage. However, it is still unclear regarding what role the arrangement or the length scale of the nano pores plays in the regulation or how much influence each factor has in the function. Specific details must be clarified in future studies.

In the animal experiment, SAH performed the best with the highest BV/TV in the micro-CT test, the most BIC% in the histological test and the largest maximum pull-out force in the biomechanical test. The results are consistent with the cell viability evaluation. We also found that although SAN had a lower bone volume than SLA, it had a higher BIC% than SLA. BIC% reflects a direct interaction between the bone and implant, so it better represents the result of cell growth state on the

![Fig. 7. Three-dimensional reconstruction images of the regenerated bone architecture inside the titanium dome of each group (n = 3) at 4 and 8 weeks in some representative specimens.](image-url)
The BIC% of SAN is in accordance with the cell proliferation result. Moreover, the histological results showed that in SAH and SAN, regenerated bone extended successively along the titanium dome inner surface from the bottom side to the top and grew along the strut into the scaffold; this caused better connectivity of regenerated bone compared with native-SLM and SLA. Yavari et al. [53] reported a similar result in which a topography with a nanotubes feature had less regenerated bone mass than the one modified by alkali-acid-heat treatment but resulted in a more stable construct with a higher bone-bonding force, implying the importance of well-connected bone in the scaffold. These findings suggest that nanotopographies may influence the mechanotransduction and guide a sequential growth process of bone regeneration. However, Yavari [53] also demonstrated that a topography established by anodization surpassed one treated by acid-alkali in terms of bone-bonding force, which is inconsistent with our result. In our study, SAH had a higher BIC% and a higher pull-out force than SAN, which is in line with the results of our cell experiment. The reasons may be attributed to the different topographies and the different animal models in the two studies. Alkali chemical treatment forms different topographies according to the treated time and reagent concentration [31]. We observed a more interconnected nanonet in our study. The importance of interconnection of porous structures has been demonstrated in many studies at the micro or macroscale [37]; in this study, we suggest that the connectivity is also important in nano-porous features. However, the author hypothesized that the mechanical force was transmitted through the macroscale scaffolds that guided the bone regeneration. In our study, the bottom part of the titanium domes is a solid design; this may also lead to the difference.

**Fig. 8.** Histological section images of each group (n = 3) at 4 and 8 weeks in some representative specimens (methylene blue-acid fuchsin stained; red: bone): A: overall histological section images under a stereomicroscope at 15× magnification, showing total ingrowth of regenerated bone, black arrow: successively growing bone along the surface and well-connected; B: local images under a light microscope at 25× magnification, showing osseointegration at the interface of the bone-implant.

**Fig. 9.** Statistical results of the regenerated bone volume/total volume (BV/TV; A; n = 3), percentage of bone-implant contact (BIC%; B; n = 6) and the maximum force to failure of pull-out test (C; n = 3) in the animal experiment of each group. *P < 0.05 compared with native-SLM; **P < 0.05 compared with SLA.
Surface chemistry will also influence bone formation on the materials surface. The results of XPS shows that the experimental groups have similar elemental compositions in terms of TiO₂. In this study, the major difference in the surface chemistry is the wettability of each group. SAH was relatively observed to be more hydrophilic than the other groups. This can be attributed to the function way of the alkali treatment; it reacts with titanium and creates an amorphous sodium titanate layer, which releases Na⁺ ions and contributes to the formation of Ti-OH groups that promote the formation of a bone-like apatite [39]. In the animal experiment, we observed higher levels of newly bone volume in SAH. It has been elucidated that a hydrophilic surface can enhance the protein absorption and human blood coagulation and facilitate the mineralization [29,54].

5. Conclusions

The SLM titanium surface has been modified by creating optimized micro- and nano-topographies in this study. Two nanotopographies of orderly arranged nanotubes and disorderly arranged nanotetrahedrons were created by anodization and alkali-heattreatment, respectively, on a waving micro-/nano-SLM surface. These two micro- and nano-textured hierarchical topographies showed enhanced cell functions relative to micro-topography only of SLA with significantly higher proliferation on the 1st, 3rd, 5th and 7th day (P < 0.05) and higher total protein contents on the 14th day (P < 0.05). Micro- and nano-topography improved the growth of more successively regenerated bone and led to higher bone-implant contact and bone-bonding force in vivo bone formation. The BIC% of SAH was nearly double that of native-SLM and SLA. In addition, the nanotopography of SAH exhibited more osteoinduction in the cell morphology adjustment with a biomimetic design of the ECM, thus leading to more preferential cell functions and bone formation than nanotubes.

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References


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