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Physicochemical and biological evaluation of SLM-manufactured Ti-10Ta-2Nb-2Zr alloy for biomedical implant applications

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Abstract

Titanium alloys, such as Ti-10Ta-2Nb-2Zr (TTNZ), are promising biomaterials due to their excellent biocompatibility and low Young’s modulus. The TTNZ samples herein were manufactured by selective laser melting and the novel material was evaluated as a dental implant in vitro and in vivo. The microstructure, mechanical properties, electrochemical behaviour, cytotoxicity, haemocompatibility and osteogenic differentiation were systematically investigated. Based on the tensile test results, the as-printed TTNZ samples had an elongation of 20.23% ± 1.95%, an ultimate tensile strength of 646.61 ± 24.96 MPa and a Young’s modulus of 23.72 ± 1.18 GPa. According to the biocompatible value, the as-printed TTNZ sample exhibited no cell cytotoxicity and it showed even better cell adhesion ability than that of the as-printed Ti-6Al-4 V and wrought Ti-6Al-4 V samples. The haemolysis percentage of the as-printed TTNZ sample was 0.629% ± 0.363%. Moreover, the as-printed TTNZ sample facilitated protein adsorption and osteogenic differentiation of human osteoblast-like (MG-63) cells in vitro. The in vivo data also demonstrated the histocompatibility of the as-printed TTNZ. In summary, the as-printed TTNZ developed in this study demonstrated good biocompatibility, low stress shielding, excellent ductility and great osteogenic differentiation. These results indicated that as-printed TTNZ alloys can be promising for end-use human biomedical applications.

1. Introduction

Implantable devices used in dentistry need to possess suitable mechanical and physicochemical properties as well as excellent biological properties. Titanium (Ti) and its alloys are considered the gold standard for biomaterials and are widely used for dental implants and orthopaedics due to their appropriate characteristics [1].

Although dental implants made of commercially pure Ti (CP-Ti) or Ti-6Al-4 V (Ti64) have excellent mechanical properties, corrosion resistance and biocompatibility, the stiffness is compatible with that of bone, which may cause stress shielding effects [2], and lead to detrimental resorptive bone remodelling and the premature failure of the implant [3]. The Young’s modulus of commercial dental implants ranges from 112–115 GPa, while that of cortical bone ranges from 10–26 GPa [4]. However, the porous lattice structure of Ti64 could effectively reduce the Young’s modulus [5, 6]. Ti64 releases vanadium (V) and aluminium (Al) ions to the surrounding biological environment, which results in adverse effects [7–9]. Therefore, it is desirable to develop alloys with a decreased Young’s modulus and improved biocompatibility for biomedical applications.

It is well accepted that the Ti-Ta-Nb-Zr alloy system is one of the promising choices for next-generation orthopaedic implants. First, this type of alloy is composed of non-toxic elements, such as tantalum (Ta), niobium (Nb) and zirconium (Zr), and their Young’s moduli have a closer match to human bone than CP-Ti or Ti64 [10, 11]. Eisenbarth et al evaluating the toxicity of Ti, Ta, Nb, Al...
and molybdenum (Mo), suggested that pure materials of Ta, Nb and Zr were selected to comply with the demand for greater biocompatibility and low toxicity [12]. In addition, Ta and Nb had good osteoconductivity in vivo [13]. Zr is expected to be an effective element for solution strengthening [14]. Moreover, a new Ti-Ta-Nb-Zr alloy has shown excellent cytocompatibility, compatible physicochemical properties and positive bioactivity, demonstrating that it is a promising choice for implant use [15, 16].

Additive manufacturing (AM), so-called 3D printing, directly enables the fabrication of a complex physical 3D hierarchical architecture from models designed with computer-aided design (CAD) [5], including direct metal laser sintering [17, 18] and selective laser melting (SLM) [6, 19]. SLM is one of the most recent AM techniques. Moreover, prior studies have emphasised fabricating biomedical components and scaffolds of CP-Ti or Ti64 [20–22]. During recent decades, the evolution of the SLM technique has substantially broadened the field of applications for Ti alloys and enabled implants to be manufactured more efficiently. Traini et al first introduced SLM into implant dentistry with a detailed illustration that showed a Young’s modulus that was lower than that for materials produced by conventional manufacturing means [18].

The SLM dental implants used in previous studies were made of Ti64 powder. As mentioned above, there are some adverse effects from Ti64 powder. Recent research in biomedical Ti, such as Ti-30Nb-5Ta-3Zr and Ti-35Zr-28Nb, have resulted in the implants being successfully manufactured by SLM, which proved that they possessed low modulus, good biocompatibility and osteogenic differentiation [23, 24]. But their high content of rare metal elements leads to them being expensive. The first aim of the present study was to extend the AM of dental implants and assess the physicochemical properties of selective laser melted specimens. The biological performance of the assemblies was systematically investigated with respect to surface hydrophilicity, protein adsorption and osteogenic behaviour. The second aim was to evaluate the bioactivity and biocompatibility of the selective laser melted specimens in vitro or in vivo. Overall, this study offers an alternative and promising functional selective laser melted Ti alloy for applications involving dental implants.

2. Materials and methods

2.1. Sample preparation and characterisation

An as-cast Ti-10Ta-2Nb-2Zr (TTNZ) alloy ingot was prepared using the electrode induction-melting gas atomisation method. Figure 1 shows the size and morphology of the TTNZ alloy powder. The chemical composition of the powder is shown in table S1 (stacks.iop.org/BMM/15/045017/mmedia). The samples were designed by SolidWorks® 12.0 (SolidWorks Corp, Concord, MA, USA) and manufactured by an SLM machine (SLM Solution, SLM 280, Germany). Both selective laser melted TTNZ (as-TTNZ) and Ti64 samples were fabricated under high-purity air conditions with environmental oxygen concentrations of no more than 80 ppm. The laser scanning speed was 700 mm s⁻¹. The laser power was set at 170 watts and the spot size was 100 µm. The chamber constraints were 280 × 280 × 365 mm. All samples were ultrasonically cleaned in ethyl alcohol for 15 min. The samples were analysed by x-ray diffraction (XRD).

The as-TTNZ microstructure was observed by a field-emission scanning electron microscope (FESEM) (FEI QUANTA 200, FEI, Netherlands). The sample was polished with SiC abrasive papers (up to
5000 grit) and etched by Kroll’s reagent to show the microstructure.

2.2. Mechanical properties
The hardness was determined by using Vickers hardness number. The indenter (Micro Hardness Tester, Chuming Optical Instrument Corporation, Shanghai, China) was controlled by the instrument and imposed 0.25 kgf for 15 s. The test was performed in a randomly selected sample at ten randomly distributed points, and the average was taken as the hardness value.

The tensile tests and the elastic modulus were evaluated by a Sansi universal materials testing machine (Sansi, Shenzhen, China) at room temperature. The length and size of the samples were designed based on ISO 6892-1-2009 (the samples were cylinder-shaped with a size of 20 × Φ3 mm).

The rate of load application for the tensile test was 2 mm min⁻¹. Three specimens were tensile tested.

2.3. Surface characterisation
The chemical composition was determined by x-ray fluorescence. Surface wettability plays a vital role in protein adsorption, adhesion and the proliferation of cells [25], and the wettability was investigated by the contact angle (CA) of 2 µl ultrapure water. The measurements were evaluated immediately after applying the water droplet (SinDin Precise Scientific Instruments Co. Ltd, Dongguan, China).

The surface roughness parameters (average roughness and Ra) of the specimens were evaluated by a profilometer (SJ-210, Mitutoyo, Japan).

2.4. Corrosion characteristics
The corrosion characteristics of the as-TTNZ were measured by polarisation curves and electrochemical impedance spectroscopy (EIS), which were measured in artificial saliva (NaCl 0.4 g l⁻¹, KCl 0.4 g l⁻¹, CaCl₂ 0.6004 g l⁻¹, NaH₂PO₄ 0.2H₂O g l⁻¹, KSCN 0.300 g l⁻¹, Na₂ S 0.9H₂O 0.005 g l⁻¹ and urea 1.000 g l⁻¹) at 37 °C ± 0.5 °C. The pH of the test solution was adjusted to 7.0 by adding sodium hydroxide [26]. The specimens were polished with 1000-grit SiC paper and embedded in paraffin. The specimens were immersed in the artificial saliva at 37 °C ± 0.5 °C for 0.5 h after being stabilised for 24 h. A saturated calomel electrode was used as the reference electrode, and a 1.5 × 1.5 cm platinum electrode was used as the counter electrode. The polarization was calculated for 2000 s, while the EIS was calculated for 900 s at a scan rate of 1 mV s⁻¹. The specific EIS parameters were evaluated with ZSimpWin 3.0 software.

2.5. In vitro cell cytotoxicity and differentiation
2.5.1. Cell viability
The samples were fabricated into 10 × 10 × 2 mm pieces. The cell proliferation and differentiation of the as-TTNZ and Ti64 (as-Ti64) and the wrought Ti64 (WTi64) were evaluated by using human osteoblast-like cells (MG-63), which can be regarded as a model for studying biocompatibility and biological activity. The MG-63 cells were cultured at an initial density of 4 × 10⁴ cells per 100 µl to measure the proliferation. Cells were seeded on the sample surfaces (n = 3) in 24-well plates with a droplet size of 50 µl. The MG-63 cells were cultured in an alpha modified Dulbecco’s eagle’s medium and supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin, all of which were from Gibco, at 37 °C in an atmosphere of 5% CO₂ and 95% air, as previously mentioned.

For cell proliferation, the number of viable cells was evaluated by Cell Counting Kit-8 (CCK-8, Biosharp, China). The sterilised samples were cultured in 24-well culture plates. The cell viability was evaluated with the CCK-8 after 5 d of culturing.

2.5.2. ALP activity
The MG-63 cells were seeded on the sample surfaces (n = 3) in 24-well plates at a density of 2 × 10⁴ cells ml⁻¹ and cultured at 37 °C in an atmosphere of 5% CO₂ and 95% air, and they were measured 7 d after being incubated in an osteogenic differentiation medium. The culture was fixed with 4% paraformaldehyde and stained with an alkaline phosphatase (ALP) kit. The optical density (OD) absorbance values at 405 nm were obtained to determine the ALP activity.

2.5.3. Cell morphology
The MG-63 cells were cultured on the WTi64, as-Ti64 and as-TTNZ samples at a density of 2 × 10⁴ cells cm⁻². After being cultured for 2 h, the cells were fixed with 4% paraformaldehyde and washed with PBS three times and then they were dehydrated in a gradient ethanol/distilled water mixture (50%, 60%, 70%, 80%, 90% and 100%) for 15 min [27]. The samples were observed by SEM after being dried and sputtered with Au.

2.6. Protein adsorption assay
After ultrasonic cleaning in 75% ethyl alcohol for 15 min, the protein adsorption onto the samples was evaluated by a BCA Protein Assay Kit (Biosharp, China) and conducted according to the directions provided by the manufacturer. The total protein concentration was indicated by the OD at 562 nm, which was calculated by using a PerkinElmer Enspire 2300 device. Each test was performed in triplicate.

2.7. Haemolysis rate
Peripheral blood was obtained from healthy volunteers after consent and ethical allowance forms were signed. The procedure was performed as previously described [27]. The OD was evaluated at 545 nm, and
the haemolysis was measured according to the following equation:

\[
\text{Haemolysis (\%)} = \frac{OD(\text{test}) - OD(\text{negative})}{OD(\text{positive}) - OD(\text{negative})} \cdot 2.8.
\]

2.8. In vivo biocompatibility

2.8.1. Acute systemic toxicity and blood analysis

Ten mature male Kunming mice with a body weight of 17–23 g (SPF grade) were obtained from the Experimental Animal Center of Central South University. After being held in the laboratory for one week, the mice were randomly divided into two groups. The mice were injected via the caudal vein, with five mice in the extract (as-TTNZ) group. The material extracts were prepared at a ratio of 1.25 cm\(^2\) of sample material per 1 ml 0.9% sodium chloride solution of extract vehicle and were soaked for 72 h at 37 °C. The control group was injected with 0.9% sodium chloride solution (NS). The injection standard was 50 ml kg\(^{-1}\).

All tested animals were observed at 24, 48 and 72 h after injection, and symptoms of slight, moderate or marked toxicity and death were recorded. After 72 h of observation, each mouse was exsanguinated via eyeball extraction, and blood was collected for haematology (BC-2800vet, Mindray, Shenzhen, China) and biochemistry assessments (Chemray 240, Rayto, Shenzhen, China).

2.8.2. Oral mucosa irritation test

Five adult male golden hamsters with a body weight of 111–155 g (SPF grade) were obtained from the Experimental Animal Center of Central South University. After being anaesthetised by intraperitoneal injection of 2% pentobarbital, the as-TTNZ discs were sewn into their right cheek pouches, while the CP-Ti discs were sewn into their left cheek pouches as a positive control. The discs were sewn tightly without oppression. After 14 d, the hamsters were euthanised, and the mucosal tissue was cut, fixed in 4% formaldehyde, and stained with haematoxylin and eosin (H&E).

2.9. Statistics analysis

SPSS version 22 (IBM, Chicago, IL, USA) was used for the statistical analysis. All quantitative results are expressed as the mean ± standard deviation (SD). The analysis of variance was used to compare group differences, and a \(p\)-value < 0.05 was considered statistically significant for all tests.

3. Results

3.1. Powder properties

An SEM image of the TTNZ powder and particle size distribution (supplementary material) are shown in figure 1(a). The medium diameter of the TTNZ powder was 43.5 \(\mu m\) and its shape was nearly spherical. The concentration of the TTNZ powder is shown in the supplementary material.

3.2. Surface and microstructure characterisations

The resulting microstructure of the as-TTNZ sample is shown in figure 1(a). The selective laser melted samples consisted of an as-TTNZ solid solution matrix with unmelted TTNZ particles. Figure 1(b) shows that the XRD pattern of the as-TTNZ indicates that it comprised the hexagonal \(\alpha\) phase. The addition of Ta, Nb and Zr did not change the microstructure of the Ti, which remained as a hexagonal \(\alpha\) phase. Both the microstructure of the as-TTNZ and XRD pattern indicated a hexagonal \(\alpha\) phase.

The surface roughness was represented by the Ra values, which are summarised in figure 2(a). Among the three samples, the highest Ra was obtained for the as-Ti64 sample. The measured Ra values
for the WTi64, as-Ti64 and as-TTNZ samples were 0.111 ± 0.015, 0.126 ± 0.019 and 0.200 ± 0.031, respectively.

The CA was evaluated by the sessile drop method, as shown in figure 2(b), to measure the surface wetting ability of the as-TTNZ surface. The WT64 surface was found to be slightly hydrophilic, with a CA of 79.376° ± 9.602°, while with CA values of 62.628° ± 1.553° and 44.317° ± 2.424° that were obtained for the as-Ti64 and as-TTNZ surfaces, respectively.

### Table 1. Comparison of the mechanical properties of the as-TTNZ with three other Ti alloys in [28, 29].

| Sample  | UTS (MPa) | YS (MPa) | Elongation (%) | Young’s Modulus (GPa) | Microhardness (HV) | Reference |
|---------|-----------|----------|----------------|-----------------------|--------------------|-----------|
| CP-Ti   | 550       | 483      | 15             | 102                   | —                  | [29]      |
| WTi64   | 930       | 860      | 10             | 110                   | 410.56 ± 17.62     | [28]      |
| as-Ti64 | 1165.69 ± 107.25 | 1055.59 ± 63.63 | 6.10 ± 2.57 | 131.51 ± 16.40 | 421.89 ± 9.96 | [28, 30] |
| as-TTNZ | 646.61 ± 24.96 | 638.60 ± 28.61 | 20.23 ± 1.95 | 23.72 ± 1.18 | 320.60 ± 27.82 | This work |

Figure 3. Fracture morphologies after tensile testing for the (a) as-Ti64 and (b) as-TTNZ samples and the (c) stress–strain curve for the as-TTNZ sample.

3.3. Mechanical properties
The tensile properties of the CP-Ti, WTi64, as-Ti64 and as-TTNZ samples are shown in table 1. The as-TTNZ sample exhibited a yield strength (YS) of 638.60 ± 28.61 MPa. The CP-Ti, WTi64 and as-Ti64 samples exhibited YS values of 483, 860 and 1055.59 ± 63.63 MPa, respectively; also, their ultimate tensile strength (UTS) values were 550, 930 and 1165.69 ± 107.25 MPa, respectively. The as-TTNZ sample showed a Young’s modulus of 23.72 ± 1.18 GPa, while the Young’s modulus values for the CP-Ti, WTi64 and as-Ti64 samples were 102, 110 and 131.51 ± 16.40 GPa, respectively. The as-TTNZ sample exhibited an elongation of 20.23% ± 1.95%, while the elongations for the CP-Ti, WTi64 and as-Ti64 samples were 15%, 10% and 6.10% ± 2.57%, respectively. In this study, the necking phenomenon could be easily seen in the as-TTNZ sample.

The fracture morphology after tensile testing was observed by FESEM, as shown in figure 3. The presence of fine dimples and voids indicated the ductility of the as-TTNZ.
Table 2. Polarisation curve and EIS fitting results for the bulk samples immersed in artificial saliva at 37 °C ± 0.5 °C.

| Samples   | OCP (mV) | Ecorr (mV) | Icorr (Icorr/Acm⁻²) | L     | R1 (Ω cm²) | Q     | N     | R2 (Ω cm²) |
|-----------|----------|------------|---------------------|-------|------------|-------|-------|------------|
| WTi64     | −0.51    | −0.46      | 1.044 × 10⁻⁷        | 1.00  | 1×10⁻²²    | 87.2  | 2.86×10⁻⁵| 0.90×10⁵   |
| as-Ti64   | −0.46    | −0.46      | 9.636 × 10⁻⁸        | 2.283 | 1×10⁻²⁶    | 105.1 | 2.45×10⁻⁵| 0.82×10⁵   |
| as-TTNZ   | −0.34    | −0.46      | 9.591 × 10⁻⁸        | 2.11  | 1×10⁻¹⁶    | 106.7 | 3.36×10⁻⁵| 0.92×10⁵   |

Figure 4. (a) Nyquist curves, (b) potentiodynamic polarisation curves, (c) Bode plots and (d) OCP curves of the WTi64, as-Ti64 and as-TTNZ samples.

Figure 5. (a) In vitro biocompatibility of the samples: i. SEM micrographs illustrating the MG-63 cell morphology cultured on the WTi64, as-Ti64 and as-TTNZ sample surfaces; and ii. CCK-8 assay of the MG-63 cells on the WTi64, as-Ti64 and as-TTNZ sample surfaces after 5 d of incubation. There was no significant difference among them. (b) Haemolysis percentage of the WTi64, as-Ti64 and as-TTNZ samples. There was no significant difference among them.

3.4. Corrosion property
The corrosion properties of the as-TTNZ samples in artificial saliva were evaluated with polarisation curves and EIS, while the WTi64 sample was regarded as a reference. The value of the corrosion parameters and fitting result for the EIS data are shown in table 2. The open circuit potential (OCP) reflects the thermodynamic equilibrium at the interface of the metal and solution as a function of time. As can be seen in figure 4, after 2.4 × 10³ s immersion, the OCP of as-TTNZ sample was higher than that of the others. The corrosion potentials (Ecorr) for the WTi64, as-Ti64 and as-TTNZ samples were the same, while their exchange current densities (Icorr) were 1.044 × 10⁻⁷ Acm⁻², 9.636 × 10⁻⁸ Acm⁻² and 9.591 × 10⁻⁸ Acm⁻², respectively. According to an equivalent circuit fitted EIS plot (table 2), the as-TTNZ sample showed an elevated resistance for both the R1 and R2 parameters.

3.5. In vitro biocompatibility
The cell viability and adhesion of the MG-63 cells are shown in figure 5. The cell viability of the WTi64, as-Ti64 and as-TTNZ samples was not significantly different from that of the control group when cultured for 5 d, indicating that the as-TTNZ sample exhibited excellent biocompatibility. The morphology of the MG-63 cell adhesion on the surface of the samples is shown in figure 5(a). The MG-63 cells adhered to the surface of the as-TTNZ sample and exhibited full spreading with the shape of polygonal, pseudopodium and peripheral ruffles, consistent with the cell viability result. This indicates excellent biocompatibility with the surface of the
as-TTNZ sample compared with the morphology of the WTi64 and as-Ti64 samples.

3.6. Histocompatibility
All the samples had values less than the haemolytic level of 5%, demonstrating non-haemolytic properties according to ISO 10993-4. The haemolysis rate of the as-TTNZ alloy was the lowest herein (figure 5(b)), indicating better haemolysis performance than that of the other samples.

3.7. Protein adsorption
The protein adsorption capability of the WTi64, as-Ti64 and as-TTNZ sample surfaces was observed by using model protein BSA. According to the data summary shown in figure 6(a), the protein adsorption level of the as-Ti64 surface was significantly higher than that of the WTi64 surface among all of the observed surfaces. Moreover, compared with that for the as-Ti64 and WTi64 surfaces, the as-TTNZ surface had a significantly increased level of protein adsorption.

3.8. ALP activity
The ALP activity was taken as an osteogenic differentiation marker at an early stage and a phenotypic marker of osteoblasts; the ALD activity was measured after 7 d of incubation. After 7 d of incubation, MG-63 cells incubated with the as-TTNZ sample showed significantly higher ALP activity than those incubated with the as-Ti64 and WTi64 samples (figure 6(b)). The findings indicated that the as-TTNZ sample showed superior osteogenic differentiation.

3.9. Acute systemic toxicity test and blood analysis
Within the observation times, there was no statistically significant difference in the weight change over 72 consecutive h among each group (figure 7). The activity, diet and excretion of the mice were normal.

There were no statistically significant differences in the haematologic and biochemical assessments (tables 3 and 4).

3.10. Histological assessment
After being sewn into the cheek pouches of golden hamsters and left for 14 d, the two different materials showed the same phenomenon: no dysplasia, they were neatly arranged with clear structures, and there was a slight inflammatory cell infiltration (figure 8).

4. Discussion
The CA of the as-TTNZ sample was 44.317° ± 2.424°. Lim et al [31] showed a positive relationship between the surface roughness and CA. Previous studies have shown that the wettability and surface roughness emerged as key factors for cell adhesion and protein adsorption [25, 32].

The tensile properties are shown in table 1. Although the YS and UTS of the as-TTNZ sample were lower than those for the WTi64 and as-Ti64 samples, the values are still higher than those for the CP-Ti Grade 4 [29]. Notably, the elongation of the as-TTNZ sample was 20.23% ± 1.95%. The presence of fine dimples and voids in the tensile test fracture morphology (figure 3) indicated the ductility of the as-TTNZ sample. Compared with that of the as-Ti64 sample (a), the fracture morphology presented flat planes with atomic steps, indicating brittleness of the as-Ti64 sample. The as-TTNZ showed similar ductile behaviour [23]. The excellent elongation was considered to be linked to the presence of Ta [33]. Moreover, the increased elongation was a trade-off for the decreased YS. Herein, the Young’s modulus plays a pivotal role in biomedical implant materials by inhibiting bone atrophy and accelerating bone remodelling [14, 34]. The as-TTNZ sample exhibited the lowest Young’s modulus among the
samples herein, and this is preferred for reducing the stress shielding. The Young’s modulus of the as-TTNZ sample was 23.72 ± 1.18 GPa, which matches that of the cortical bone (15–30 GPa), whereas that of the others was above 100 GPa [28]. It is well known that the mechanical characteristics of alloys are due to the microstructure, which depends on the kind and number of alloying elements. Generally, β Ti alloys exhibit a low Young’s modulus. However, Hao et al reported that the α′′ and β phases have nearly the same elastic modulus [35]. The α′′ phase should have existed in the as-TTNZ sample, and that may be lead to low elastic modulus of as-TTNZ. The decreased Young’s modulus of the α-phase as-TTNZ might be due to the Zr and Ta reducing the Young’s modulus when they are alloyed with Ti, as indicated in previous studies [36, 37]. In addition, the microstructure and fracture surface morphology exhibited non-melted particles, ductile dimples and undesired porosity, as shown in figures 1 and 3; the non-melted particles

Table 3. Haematology values of the male Kunming mice (n = 5).

| Group   | WBC (×10^9 l^-1) | RBC (×10^12 l^-1) | HGB (g l^-1) | PLT (×10^9 l^-1) | LY (%) | MO (%) | NEUT (%) |
|---------|------------------|-------------------|--------------|-----------------|--------|--------|---------|
| NS      | 3.88 ± 1.38      | 6.33 ± 1.8        | 127 ± 15.73  | 660.40 ± 235.29 | 84.66  | 12.72  | 18.68   |
| as-TTNZ | 4.00 ± 0.19      | 6.92 ± 2.14       | 135.60 ± 7.50| 966.20 ± 348.73 | 79.88  | 3.66   | 12.72   |

Note: Values expressed as the mean ± SD.

Abbreviations: WBC, white blood cell count; RBC, red blood cell count; HGB, haemoglobin concentration; PLT, platelet count; LY, percent of lymphocyte; MO, percent of monocyte; and NEUT, percent of neutrophilic granulocyte.

Table 4. Clinical biochemistry values of the male Kunming mice (n = 5).

| Parameters          | NS               | as-TTNZ          |
|---------------------|------------------|------------------|
| ALB (g l^-1)        | 17.79 ± 0.49     | 16.93 ± 0.63     |
| ALP (U l^-1)        | 102.01 ± 7.48    | 107.96 ± 28.22   |
| ALT (U l^-1)        | 25.89 ± 8.30     | 17.24 ± 1.39     |
| AST (U l^-1)        | 37.26 ± 6.51     | 40.88 ± 8.58     |
| Cr (µmol l^-1)      | 92.27 ± 62.64    | 40.12 ± 14.78    |
| DBIL (µmol l^-1)    | 25.57 ± 15.03    | 13.92 ± 3.58     |
| TBA (µmol l^-1)     | 5.38 ± 1.14      | 4.29 ± 0.10      |
| TBIL (µmol l^-1)    | 31.46 ± 17.21    | 18.59 ± 4.33     |
| TP (g l^-1)         | 31.50 ± 3.20     | 28.69 ± 0.84     |
| UA (µmol l^-1)      | 147.56 ± 59.29   | 115.16 ± 10.20   |
| γ-GT (U l^-1)       | 2.50 ± 0.59      | 2.20 ± 0.26      |

Note: Values expressed as the mean ± SD.

Abbreviations: ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cr, creatinine; DBIL, direct bilirubin; TBA, total bile acid; TBIL, total bilirubin; TP, total protein; UA, urea; and γ-GT, γ-glutamyl transpeptidase.

Figure 7. Body weight changes of male Kunming mice in the acute systemic toxicity study.
and undesired porosity may trigger the decreasing of Young’s modulus [38].

Consequently, this new as-TTNZ alloy, which demonstrated a low Young’s modulus, good ductility and neutral strength, is a very promising choice for biomedical implants.

According to the equivalent circuit fitted EIS plot (table 2), the as-TTNZ sample showed an elevated resistance in both layers (R1 and R2), which indicated the high stability of a passive layer on the sample placed in artificial salivary conditions. Both the polarisation and EIS data were consistent with the result that the as-TTNZ sample displayed passive behaviour and good electrochemical corrosion properties. As observed in the literature, the corrosion properties were linked with the formation of a protective oxide layer, such as Nb2O5 [24], ZrO2 [24] and Ta2O5 [23]. The semiconducting behaviour of the oxide layer on Ti alloys may contribute to the enhancement of the histocompatibility, which prevented fibrinogen from being transferred into the material [39], as evidenced by the histocompatibility results.

The histocompatibility results also correlated with the capacity of protein adsorption on the biomaterial surface, which contributed to cellular membrane extension [40]. For albumin adsorption, physicochemical characteristics of the sample surface, such as the chemical elements, topography and wettability, play a major role in cell attachment and spreading [41]. As mentioned above, the as-TTNZ sample possessed improved hydrophilicity and exhibited strong cell attachment. In addition, it is well documented that Ta and Zr have positive effects on osteoblast-like cell proliferation and attachment [42, 43]. The surface presence of Ta and Zr may not have contributed to cytotoxicity. Furthermore, it was reported that surface roughness is beneficial for osteoblast proliferation and adhesion [44]. The increased cell adhesion ability of human osteoblast-like MG-63 cells that resulted from increased roughness values indicated that the increase in the Ra value might be attributed to the cell adhesion ratio.

In general, the high absorbed mass values of albumin promote cell attachment [45]. The MG-63 cells on the surface of the as-TTNZ sample exhibited an improved extension of the pseudopodia, which was correlated with the adsorption of the protein. Thus, the conclusion can be drawn that the as-TTNZ alloy is a suitable substrate for cell attachment. Furthermore, the albumin surface coverage on the hydrophobic surface was much smaller than that on the hydrophilic surface. Thus, the adsorption and interaction of the albumin molecules were much stronger on the hydrophilic surface than on the hydrophobic surface [46].

Cellular responses, such as cell adhesion, proliferation and differentiation, are prominently correlated with surface properties (e.g. the surface morphology, roughness, hydrophilicity and surface energy) [47]. To evaluate the proliferative activity of pre-osteoblast, the cell growth was measured by CCK-8 assay. The number of metabolically active cells at the studied time point was not significantly different between the analysed samples and the others. This result is consistent with the research by Raluc on Ti-Nb-Ta-Zr alloys [48]. Most studies have focused on the bioactivity of metal biomedical implants, while fewer have focused on the biocompatibility. The change in the manufacturing process may cause different properties.
and impact the biocompatibility. Thus, we tested the biocompatibility of an as-TTNZ alloy both in vitro and in vivo. The cytotoxicity of the as-TTNZ sample using osteoblasts (MG-63 cells) was similar to that of the WTi64 and as-Ti64 samples. Furthermore, host-response tests, such as acute systemic toxicity and oral mucosa irritation tests, were performed. According to the guidelines for systematic toxicity, the as-TTNZ material showed no mortality or any systemic toxicity evidence. Moreover, the results of the serum chemistry and haematology parameters showed no significant differences. No significant changes in the immune cells were observed. These results indicated that the as-TTNZ sample did not induce systemic toxicity or inflammatory reactions in animals. With regard to foreign matter, this work first studied the oral mucosa irritation test of the as-TTNZ to determine whether it would induce inflammatory and allergic reactions of the buccal mucosa. Generally speaking, pro-inflammation was associated with both delayed bone healing and bone loss [49]. Previous studies have revealed the gene and protein of the pro-inflammatory cytokines and chemokines on Ti-Ta-Nb-Zr alloy in vitro, and the results suggest that the Ti-Ta-Nb-Zr alloy induced a wound healing response, rather than profound inflammation. In the present work, haematology assessment of the inflammatory cells was evaluated by blood analyses. The results indicated that as-TTNZ did not induce systemic toxicity and inflammatory reactions in animals. Besides, neither CP-Ti nor as-TTNZ caused irritation of the buccal mucosa. It was demonstrated that the biocompatibility of the as-TTNZ was as good as that of CP-Ti.

It is essential for a candidate biomedical material to significantly promote bone matrix maturation and mineralisation. In this work, quantitative measurements were used to estimate the in vitro osteogenic differentiation of cultured MG-63 cells on WTi64, as-Ti64 and as-TTNZ substrates. According to the ALP activity results, a possible explanation for this observation is that the surface roughness might impact the osteogenic differentiation of human osteoblast-like MG-63 cells [50]. In addition, the Nb ions released from the as-TTNZ alloy displayed a higher ALP activity than that of the other samples herein [51]. Moreover, the oxide layer (such as TiO₂, ZrO₂ and Ta₂O₅) that was exposed on the Ti alloy surface might enhance osteointegration via osteogenic differentiation [40, 52].

5. Conclusion

The present research demonstrated the promise of a novel TTNZ alloy manufactured by SLM for future use in dental implants. The Young’s modulus and elongation of the selective laser melted samples were 23.72 ± 1.18 GPa and 20.23% ± 1.95%, respectively. In addition, the cell viability of the WTi64, as-Ti64 and as-TTNZ samples was not significantly different to that of the control group, while the cells fully spread on the surface of as-TTNZ. In vivo, the absence of acute systemic toxicity, inflammatory response and allergic reaction under the test conditions indicated that the as-TTNZ would be safe and feasible for biomedical end-use human applications. Furthermore, the haemolysis rate of the as-TTNZ sample was 0.629% ± 0.363%, which demonstrated its potential use in dental implantology. The findings of the substantial increase in protein adsorption and osteogenic differentiation suggested that SLM-manufactured Ti-10Ta-2Nb-2Zr, taken together in further long-term in vivo studies, will hold promise to serve as a new orthopaedic application.

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