Osteogenic Potential of Additively Manufactured TiTa Alloys

Erin G. Brodie,* Kye J. Robinson, Elizabeth Sigston, Andrey Molotnikov, and Jessica E. Frith*

ABSTRACT: The only alloy currently utilized for additive manufacture of bone implants, Ti−6Al−4V, has a high elastic modulus and bioinert surface, potentially inducing stress shielding and hindering osseointegration. Low-modulus materials with bioactive surfaces could significantly reduce implant failure rates by improving the interaction between implants and the surrounding bone. In this study, laser powder bed fusion Ti25Ta and Ti65Ta alloys, highlighted previously for their low modulus, were assessed for their surface osteogenic potential, using human bone marrow mesenchymal stromal cells (hBMSCs). Polished metallic substrates were utilized to avoid the effects of surface topography on cell fate and highlight the chemical effect of the Ta content. Electron-dispersive X-ray and X-ray photoelectron spectroscopy revealed surface Ta enrichment on the polished TiTa substrates. XPS measured Ta oxide contents of 8.0 and 16.5 at. % for the Ti25Ta and Ti65Ta alloys, respectively. In vitro testing revealed increased alkaline phosphatase activity and mineralization of hBMSCs on the TiTa alloys compared to the Ti−6Al−4V control and only minor differences in biological behavior between the Ti25Ta and Ti65Ta alloys. It was concluded that the Ti25Ta composition, with a lower Ta content but equivalent biological response, was the most promising composition for additively manufactured bone implants.

KEYWORDS: tantalum, titanium, additive manufacturing, osteogenesis, biomedical

1. INTRODUCTION

Titanium alloys, specifically Ti−6Al−4V ELI, are widely used for implants because of their favorable mechanical properties (specific strength and fatigue performance), biocompatibility, and corrosion resistance. However, it is also well established that Ti−6Al−4V is not an ideal replacement for bones because of its high elastic modulus (114 GPa). The mismatch of elastic modulus between metal implants and bones results in stress shielding, causing bone resorption and implant loosening. Furthermore, it contains elemental Al and V, which are both toxic if released into the body.

New titanium alloy systems, such as TiTa, TiNb, and TiZr, avoid the use of toxic elements and possess relatively low elastic moduli of 60−80 GPa, nearly half that of Ti−6Al−4V. The Ta, Nb, and Zr additives act as β-phase stabilizers, reducing the elastic modulus, and also provide solute solution strengthening, increasing the strength of the base Ti. While other strengthening mechanisms can result in a decrease in ductility, the β-Ti alloys retain good ductility and hence fatigue life, an important mechanical requirement for cyclic loaded implants. However, implant performance can be even further improved by investigating the cellular interactions between the material surface and surrounding tissue. New bioactive materials provide enhanced functionalities such as antibacterial or improved osteogenic capabilities.

Osseointegration is key to providing bone implant stabilization. Failure to form a strong bond between the implant and the bone results in implant loosening and revision surgery. Contact osteogenesis or bone formation at the implant surface depends upon the physiochemical properties of the implant surface and can be enhanced on titanium implants through surface modifications. Methods for improving bone bonding behavior are separated into surface chemical changes, such as hydroxyapatite coatings or alkaline and acid bathing, and surface topographical changes, such as nanotube formation and microtextures.

Ta is referred to as a “bioactive” material as it has been shown to promote cell adhesion, proliferation, differentiation, and mineralization in vitro and in vivo. Ta has been used to improve implant integration as a coating for Ti implants and also in the pure form, achieving clinical success in hip, knee, spinal, and dental surgeries. While the precise cellular mechanisms of this biological advantage remain unknown, it has been hypothesized that Ta can increase osteogenesis because of enhanced surface wettability, enhanced cellular signaling pathways (such as WNT and TGF-β), and enhanced integrin binding. The difficulty in
identifying the pathway for improved osteogenic behavior of Ta surfaces is compounded by studies which compare Ti and Ta produced by different manufacturing methods, often with very different topographic features which go uncharacterized.15,16,20,26–29

The implant industry currently adopts additive manufacturing methods, such as laser and electron beam powder bed fusion, to create bespoke implant geometries based on the individual patient bone structure.30,31 3D design freedom and narrow manufacturing tolerances allow for the creation of complex and fine image features, such as lattice structures, which facilitate fluid flow, essential for osseointegration.32–34 Laser powder bed fusion (L-PBF) also facilitates the manufacture of TiTa alloys by removing the need to melt Ti and Ta simultaneously on a large scale, which requires multiple arc melting and inversion steps to achieve homogeneity.35 Instead, AM feedstocks consist of micron-sized elemental powders, which when mixed allow a wide range of compositions for investigation.36–38

Within the range of TiTa alloy compositions, the Ti–25 wt % Ta and Ti–65 wt % Ta alloys have been highlighted as the most mechanically beneficial for bone implant applications. When quenched from the melt, each alloy consists of a fully α- or β-crystal structure, respectively, both of which result in the lowest achievable elastic modulus for the TiTa alloy system of 65–70 GPa.39–45 As L-PBF processing causes similar fast cooling rates, the Ti25Ta and Ti65Ta compositions were deemed optimal for low-modulus additively manufactured bone implant materials. Each composition has been successfully L-PBF-manufactured in previous work,36–37 with the Ti65Ta alloy work being published in future. The Ti25Ta and Ti65Ta alloys retain their low elastic modulus when produced by L-PBF and show promising fatigue behavior because of their retained high ductility. A minor volume (1–2%) of unmelted Ta particles remains in the matrix of each alloy after processing, which were shown to not contribute to mechanical failure. However, the reason for utilizing TiTa alloys is not only to provide a low elastic modulus and suitable mechanical properties for stress-loaded implants but also to increase bone implant integration through the bioactivity of Ta. As the osteogenic potential of the TiTa alloy system is yet to be investigated, it is yet unknown whether the TiTa alloys provide an osteogenic potential over the commonly used Ti–6Al–4V. Furthermore, when comparing the low-modulus Ti25Ta and Ti65Ta alloys, it is yet unknown whether the higher Ta content in the Ti65Ta alloy contributes significantly to improved osteogenic behavior, enough to outweigh the drawbacks of its higher weight and cost.

In this study, Ti25Ta and Ti65Ta compositions were manufactured using L-PBF processing. Substrates for biological testing were prepared from the printed material by polishing to mirror finish, after which any remaining surface topography was characterized using surface profilometry. The surface and oxide layer chemical compositions were characterized using electron-dispersive X-ray (EDX) and X-ray photoelectron spectroscopy (XPS) before the osteogenic potential of the alloys was investigated in vitro through cell culture of human bone marrow mesenchymal stromal cells (hBMSCs). hBMSCs were chosen for this study because of their differentiation capacity and ability to produce quantifiable products [alkaline phosphatase (ALP) activity and mineral], used to directly compare the osteogenic potential of the different substrates. Measurements of cell attachment, proliferation, morphology, ALP activity, and mineralization were analyzed and compared to a Ti–6Al–4V control to determine the suitability of the L-PBF TiTa alloys for bone implant applications.

2. MATERIALS AND METHODS

2.1. Sample Fabrication. Ti25Ta and Ti65Ta alloys were prepared from mixed commercially pure titanium (TLS Technik, Germany) and tantalum (TEKNA, Canada) powders to the appropriate weight percent compositions. Mixed powders were used in this study as prealloyed TiTa powders are not yet commercially available. Mixed powders are used extensively to explore new alloy systems for additive manufacturing39–44 however, they can result in reduced homogeneity compared with their prealloyed powder counterparts. By optimizing the processing parameters in previous works,45 it was demonstrated that matrix homogeneity could be achieved, with minimal remaining Ta particles. The Ti–6Al–4V ELI powder was supplied by Falcon Tech Co., Ltd. (Wuxi, China). L-PBF was conducted on a Concept Laser Mlab cusing machine (Concept Laser GmbH, Germany) under an argon atmosphere. The optimal printing parameters for Ti–6Al–4V were provided by Concept Laser.

Rectangular prisms of 5 mm (x) × 5 mm (y) × 15 mm (z) were printed and subsequently cut into 3 mm (z) thick substrates. The upper 25 mm2 plate surface was mechanically ground and polished for each substrate using 0.04 μm OPS colloidal silica solution to remove any surface topography and ultrasonically cleaned in ethanol. The samples were then left to oxidize at room temperature for greater than 24 h before surface analysis. Prior to biological experiments, the metallic substrates were sterilized by 30 min of soaking in 80% ethanol within a biosafety cabinet.

2.2. Surface Profilometry and Imaging. The surface roughness of the metallic substrates was analyzed after polishing using a Wyko NT1100 optical profilometer (Veeco) in the vertical scanning interferometry mode by scanning an area of 0.93 mm × 1.2 mm. Five areas were scanned on each surface, and the average surface roughness (Rz) and average peak-to-trough height (Rq) values were recorded. The 1–2 μm deep surface chemical composition of each substrate was measured by elemental mapping over an area of approximately 1 mm². A JOEL 7100F FEGSEM was used, equipped with an EDX detector.

2.3. XPS Analysis. XPS measurements were performed on a Thermo Scientific Nexsa X-ray Photoelectron Spectrometer using a monochromated Al Kα source (1486.68 eV, 6 mA, 12 kV). Instrument calibration was checked by performing high-resolution scans (50 eV pass energy) on a clean Ag standard (Prescan: Ag 3d5/2 368.36 eV, 0.863 full width at half-maximum (fwhm); postscan: 368.44 eV, 0.863 fwhm). All scans were performed using an analysis area of 400 × 400 μm. Surveys scans (0–1350 eV) were obtained using a pass energy of 150 eV, and high-resolution scans with window ranges of 18–27 eV were measured with using a pass energy of 50 eV. Depth profiling was carried out using Ar+ etching (500 eV) to further investigate the oxide layer and the underlying alloy surface. Etching was carried out in 10 s intervals between which spectra were recorded for a total of etch time of 320 s. The internal flood gun was used in the “charge compensation mode”; however, because of the conductive nature of the sample, no charging was observed. All data obtained were analyzed using CasaXPS version 2.3.18. For further fitting parameters and methods, see XPS Supporting Information S1–S3.

2.4. Mesenchymal Stromal Cell Culture. hBMSCs (Lonza) were cultured in basal media (D-MEM-low glucose, Gibco), supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin antibiotic, in a humidified incubator at 37 °C and 5% CO2. In this study, to account for the heterogeneity in behavior of hBMSCs, passage six cells were used from five independent donors. This study utilized hBMSCs as they are commonly used to model osteogenic response for new implant
materials and tissue scaffolds.16,44−48 MSCs are critical for implant success in the body as they are largely responsible for bone repair and remodeling as well as enhancing generalized wound healing. hBMSCs are also primary human cells, which are more predicative of an in vivo or clinical response than nonhuman or immortalized cell lines, such as L929 and SaOS2s.

2.5. In Vitro Adhesion, Proliferation, and Morphology. The metallic substrates were placed in a 48-multiwell plate and seeded at a density of $1 \times 10^4$ cells per cm$^2$ in 300 μL of media. Triplicates were used for each alloy. A schematic of the testing procedure is shown in Figure 1. The metallic substrates supporting hBMSC cultures were moved into a clean well plate after 4 h. Cell adhesion to and proliferation on the metallic substrates were assessed at 4 h, 3 day, and 7 day time points, using an MTS assay (Cell Titre 96 Aqueous One Solution Cell Proliferation Assay, Promega) and fluorescence imaging. At each time point, the metallic substrates supporting hBMSC cultures were moved from culture conditions to a clean well plate and were incubated in an MTS working solution (1:5 dilution of the MTS reagent in culture media) for 3 h at 37 °C. 100 μL of this solution was then transferred to a 96-multwell plate, and the optical absorbance was measured at 490 nm (Thermo Scientific Multiskan Spectrophotometer). The samples were fixed for morphological analysis by incubating in 4% paraformaldehyde (15 min, room temperature), permeabilized using 0.1%-Triton-X-100 (10 min, room temperature), and stained with Actin Red 555 ReadyProbes Reagent (Molecular Probes) and Hoechst 33342 (Thermo Scientific) (1 h, room temperature). An inverted fluorescence microscope (Nikon Eclipse Ti) was used for imaging, and the cell morphology was assessed using ImageJ software. Cell circularity was calculated by ImageJ software using the Analyze Particles tool, which utilizes the cell area and perimeter to produce a circularity index of 0−1 (0 indicating a straight line and 1 indicating a perfect circle). The cell morphology was analyzed only at the 4 h and 3 day time points as by the 7 day time point, cells had become confluent and hence could no longer spread fully.

Figure 1. Experimental schematic showing L-PBF of the TiTa alloys, followed by substrate preparation and material and biological characterization.

Figure 2. (a) Surface roughness values ($R_a$) and (b) average peak-to-trough height ($R_z$). Graphs show individual measurements from different regions of each substrate ($n = 5$), with bars representing the mean ± standard deviation (SD). (c) Surface profilometry maps and (d) backscattered images of Ti−6Al−4V, Ti25Ta, and Ti65Ta substrates.
2.6. In Vitro Osteogenesis Assays. Separate 48-multiwell plates were used for the p-nitrophenol (pNP) assay and the mineralization study. Each study had both a basal media and osteogenic media condition, and triplicates were used for each alloy. The osteogenic media was composed of basal media supplemented with 1% β-glycerophosphate, 0.1% ascorbic acid 2-phosphate and 0.01% dexamethasone. For the osteogenic condition, cells were seeded in basal media which was replaced with osteogenic media after 24 h.

ALP activity was measured after 1 week of culture using a colorimetric pNP assay (Sigma-Aldrich) and normalized to the same-well DNA content measured through a PicoGreen dsDNA assay (Invitrogen). Samples were washed with PBS, lysed using 0.1% Triton-X-100, and mechanically scraped from the polished substrate surfaces. Lysates then underwent three freeze−thaw cycles, and half the lysate from each well was used for each assay. ALP activity was measured by spectrophotometry at 410 nm and was calculated from a standard curve. The results are displayed as pNP (nmol), normalized by incubation time (min) and DNA (μg).

Mineralization was measured after 4 weeks of culture using OsteoImage (Lonza) stain. Five fluorescence images were taken per sample using an inverted fluorescence microscope (Nikon Eclipse Ti) with consistent exposure. ImageJ software was used to quantify the fluorescence intensity of the OsteoImage dye.

2.7. Statistical Analysis. Quantitative data were analyzed for normality using the Brown−Forsythe test, with p > 0.05 indicating that the data set standard deviations were not significantly different. Following approved normalization, a one-way ANOVA test was conducted, followed by Tukey−Kramer multiple comparisons. The analysis was performed using GraphPad Prism software, and p < 0.05 was considered significant. * symbols are used to represent statistical significance between alloys, whereas # symbols are used to represent statistical significance between time points.

3. RESULTS

3.1. Surface Topographical Characterization. Surface topography is known to be a significant driver of cell morphology and fate. In order to elucidate the effect of alloy chemistry on cell behavior, the substrate surfaces were polished using 0.04 μm silica. It was hypothesized that the obtained surface topography would have a negligible effect on cell behavior. This assumption was tested by measuring and comparing the surface roughness of each substrate.

Surface profilometry measurements showed a surface roughness (R₆) of approximately 110 ± 37 nm on all samples, with no significant difference between alloys, (Figure 2a). However, a significantly higher mean peak-to-trough height (R₉) was noted on the TiTa compositions compared with the Ti−6Al−4V control (Figure 2b). Both, 1 μm protrusions and 1 μm pores were noted on the TiTa substrates (Figure 2c), and the protrusions were confirmed by BSI and EDX analysis to correspond to partially melted Ta particles (Figure 2d). Both the porosity and partially melted Ta particles can be attributed to the L-PBF processing. Minimizing these features using L-PBF parameter optimization has been explored in previous work. However, both features cover only minor areas of the substrate. The porosity was found to be approximately 0.1% for the TiTa alloys and 0.04% for the Ti−6Al−4V alloy, calculated using image thresholding of the backscattered electron images (Figure 2d). This agrees well with previous work investigating L-PBF processing of the Ti25Ta alloy, and the porosity densities calculated for all samples are still within the range expected for well-processed L-PBF Ti alloys. The Ta protrusions were found to cover approximately 1% of the substrate surface on each TiTa alloy, also agreeing with the Ta particle distribution noted in previous studies. The higher peak-to-trough height in the Ti25Ta sample when compared with the Ti65Ta sample is likely due to a slightly higher pore density and size. The Ta particles appear to be approximately 1 μm in height on both TiTa surfaces.

EDX analysis was also conducted on the metallic substrates to confirm the surface composition of each alloy, Table 1. EDX has a depth of analysis of approximately 1−2 μm, which is much deeper than the expected depth of the surface oxide layer, which is on the nanometer scale, and was analyzed by XPS. The EDX results showed that the Ti25Ta alloy surface contained 35 ± 0.2 wt % Ta and the Ti65Ta alloy surface contained 68 ± 0.2 wt % Ta. The surface of both alloys showed a higher Ta concentration than the intended nominal composition. The powder mixing error was ruled out as bulk parts printed from the same powder batch, when tested by spectroscopic chemical analysis, showed Ta contents within 1% of the nominal composition. Hence, the surface Ta enrichment was likely caused by lattice strain and preferential polishing of Ti. Surface enrichment in polished TiTa samples was also observed by Mendis et al.

3.2. Surface Chemical Characterization. Surface oxide compositions are critical in metallic implant materials as the oxide layer is in direct contact with the biological environment and hence dictates tissue compatibility. The oxide formed on the substrate surface was analyzed using XPS analysis. Survey scans (Figure 3a) indicated adventitious carbon and oxide on the surface of the alloys, as expected for a polished sample exposed to atmospheric conditions. Silicon was also observed in the survey in the form of silica based on the indicative shift of the silicon peak to a higher binding energy (103.8 eV), likely from the polishing suspension. The depth profiles (Figure 3b) showed removal of C contamination and then a slow etch through the oxide for each sample until the oxygen content plateaus. The interpolated etch time to remove half of the oxygen between the starting and final steady states decreased from 133 s for Ti−6Al−4V to 125 and 107 s for Ti25Ta and Ti65Ta, respectively. This suggests that the thickness of the oxide layer decreased with increasing amounts of Ta, agreeing with TEM observations by Mendis et al.

The depth profiles shown in weight percent excluding the nonmetallic elements (Figure 3c) showed the concentration of Ta in the oxide to be higher than the nominal (dashed line) and surface EDX compositions. An increase in Ta concentration with etch time was also observed, which is likely a sign of preferential etching of Ti.

In addition, a larger number (n = 5) of single-spot analyses were taken from over the entire surface of the metallic substrates to confirm the oxide composition. The values displayed in Table 2 showed a similar oxide oxygen content for each alloy (average = 56 at. % following corrections for impurities) and a 2.1-fold increase in the oxide Ta content between the Ti25Ta and Ti65Ta compositions, 8.0 ± 1.2 and 16.5 ± 2.4 at. %, respectively. This increase is less than what is
to be expected from the EDX analysis, where a 3-fold increase in Ta content was observed at the substrate surface of the Ti65Ta substrate when compared with the Ti25Ta substrate, 12 to 36 at. %.

Figure 3. (a) Representative surveys for the surface of each investigated alloy (Ti−6Al−4V, Ti25Ta, and Ti65Ta), showing major metallic element peaks, the oxygen peak, and surface contaminant peaks (e.g., Si and C). (b) XPS depth profiles showing the atomic percent of the major elements for each alloy \((n = 2)\), displaying removal of the C contaminant and oxide etching. (c) Corresponding weight percent depth profiles of only the metallic elements for the alloys, highlighting the surface Ta enrichment in the TiTa alloys. The dashed lines represent the nominal composition, while the dotted lines represent the EDX-measured surface composition.
3.3. hBMSC Adhesion and Proliferation. Successful implant integration depends on the ability of cells to colonize the implant surface through adherence and proliferation. To determine whether there were any differences in cell adhesion in response to the TiTa alloys, the number of attached hBMSCs after 4 h of culture was assessed via an MTS assay (Figure 4a) and counting of imaged nuclei (Supporting Information S4). hBMSCs attached equally well to each alloy, with no significant difference observed between the number of attached cells measured by the MTS measurements or nuclei counts. Cell proliferation was assessed by normalizing the MTS absorbance readings by the initial 4 h absorbance to calculate a fold increase. On average, across all donors, there was a 2-fold increase at 3 days and a 6-fold increase at 7 days (Supporting Information S5). Improved early onset proliferation, important for subsequent osseointegration, was noted on the Ti65Ta alloy at 3 days (Figure 4b); however, this trend was only observed for one of the five hBMSC donors.

Table 2. Relative Atomic Concentration of Elements in the Oxide Layer, Determined by Spot Analyses (n = 5)

<table>
<thead>
<tr>
<th></th>
<th>Al 2p (at. %)</th>
<th>O 1s (at. %)</th>
<th>Ta 4d (at. %)</th>
<th>Ti 2p (at. %)</th>
<th>V 2p (at. %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti—6Al—4V</td>
<td>10 ± 2.4</td>
<td>56.4 ± 2.6</td>
<td>8.0 ± 1.2</td>
<td>32.8 ± 2.8</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>Ti25Ta</td>
<td>57.0 ± 4.2</td>
<td>16.5 ± 2.4</td>
<td>16.5 ± 2.4</td>
<td>28.7 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Ti65Ta</td>
<td>54.7 ± 3.3</td>
<td>16.5 ± 2.4</td>
<td>16.5 ± 2.4</td>
<td>28.7 ± 3.0</td>
<td></td>
</tr>
</tbody>
</table>

No significant difference in proliferation rate was observed for any alloy at 3 days and 7 days.

Equal cell attachment and proliferation were further confirmed by imaging of cell nuclei (Figure 4c). As the new TiTa alloys showed equal attachment and proliferation characteristics to Ti—6Al4V, the TiTa alloys are likely as capable as the Ti—6Al—4V standard material to support implant surface cell colonization.

3.4. hBMSC Morphology. Cell spreading and morphology are important indicators of cell fate. Analysis of spreading provides information on the ability of a surface to support cell adhesion and spreading, while shape characteristics can provide indications of improved osteogenic differentiation capacity.56 To assess the geometry of the hBMSCs cultured on the different alloys, the nucleus and actin cytoskeleton of the hBMSCs were stained and imaged at 4 h and 3 days. The 4 h time point was chosen to capture the cell morphology at a very early stage of attachment, while the 3 day time point was used to assess the morphology once the cells had become established on the substrate surface.

At 4 h, the cells had begun to spread on all alloy compositions and were still trapezoidal, rather than spindle-shaped, and had actin filaments concentrated on the cell periphery (Figure 5a). Lamellipodia were observed, indicating that the cells were still actively spreading or moving. The spread area of the cells was equivalent on all the surfaces (Figure 5b), but the circularity index was significantly higher for hBMSCs on TiTa surfaces compared to Ti—6Al—4V, indicating that these cells were more rounded (Figure 5d and Supporting Information S6). This may suggest that the cells on the TiTa surfaces were slower to spread than those on Ti—6Al—4V.

After 3 days, hBMSCs on all surfaces were fully spread and there were no longer any significant differences in circularity between alloys, (Figure 5e). The average circularity index at 3 days (0.19) was lower than that at 4 h (0.23), reflecting the decrease in regularity of the cell shape as the cells reached an elongated steady-state conformation. Established actin fibers could be seen bridging the entire length of the cell, indicating a state of tension. Analysis of the cell spread area at 3 days showed that cells on the TiTa alloys had a significantly larger cell area than those on Ti—6Al—4V (Figure 5c and Supporting Information S7). Generally, cells that spread over a larger area and have a less regular morphology are more likely to undergo osteogenesis.56—58 A higher cell area may also be indicative of a higher number of integrin binding sites, given the larger surface—substrate interaction area. Such an increase in integrin binding sites and the subsequent focal adhesion formation has been correlated with improved osteogenic differentiation.54,57,59

3.5. Osteogenesis. Given the differences in cell spreading and shape, osteogenic potential was investigated to determine whether these differences in cell morphology would translate into an osteogenic outcome. Osteogenic differentiation of hBMSCs cultured on the different alloy substrates was assessed by measuring ALP activity and mineralization. ALP activity
plays an important role in bone formation and was assessed at 7 days as an early marker for osteogenesis. Mineral content was assessed at 4 weeks as a mature marker.

ALP activity and mineralization were both significantly increased on the TiTa alloys compared with the Ti−6Al−4V control for all donors which displayed an osteogenic response (Figure 6a,b). There was some variation in efficiency between hBMSC donors, highlighting the importance of conducting osteogenesis studies over several different hBMSC lines. Similar trends in ALP activity and mineralization were observed for three donors as the magnitude of differences was greater for donors with a stronger osteogenic response (Supporting Information S8). High ALP activity is an early indication of commitment to osteogenic differentiation and has

Figure 5. (a) Fluorescence images of the cell morphology, showing the actin cytoskeleton (red) and nuclei (blue). (b,d) Cell area and (c,e) circularity measured at 4 h and 3 days for Ti−6Al−4V, Ti25Ta, and Ti65Ta alloys. Each data point represents one cell (n > 100).

Figure 6. a) ALP activity and (b) mineralization measured by the pNP assay and OsteoImage assay, respectively. (c) Fluorescence images showing mineral formation. In (a), each data point represents the ALP activity for each individual sample within the triplicate. In (b), each data point represents the mineral intensity from multiple readings (n > 5) from all viable substrates.
been consistently observed on Ta surfaces\textsuperscript{13−16,18,29,44} but never on TiTa alloys.

Mineral formation was also analyzed via fluorescence staining of hydroxyapatite with OsteoImage (Figure 6c) and quantified by image intensity analysis (Figure 6b). All alloys displayed the ability to support mineral formation; however, the stained mineral intensity on the TiTa compositions was significantly higher than that on the Ti−6Al−4V samples. This correlates with the ALP activity measurements and suggests that increased osteogenic commitment observed on the TiTa surfaces at day 7 leads to increased osteogenesis overall and enhanced mineralization at 4 weeks.

Remarkably, there was no statistical difference in ALP activity or stained mineral intensity between the Ti25Ta and Ti65Ta compositions, and the difference in osteogenic potential between the TiTa and Ti−6Al−4V alloys was much greater than that between the Ti25Ta and Ti65Ta compositions. The similar osteogenic potential of Ti25Ta and Ti65Ta suggests that despite the weight content of Ta in the Ti65Ta alloy being almost 3 times greater than that in the Ti25Ta alloy, there is unlikely to be any significant improvement in osteogenic response and hence implant stability and functions.

4. DISCUSSION

New alloys are required for stress-loaded bone implants in order to reduce implant failure. Implant loosening can be reduced by developing alloys with a similar elastic modulus to bones, reducing stress shielding, and by creating bioactive implant surfaces, improving implant-bone integration. Here, we explored new low-modulus additively manufactured TiTa alloys to assess whether the bioactive Ta component could increase osteogenesis of hBMSCs compared with the current gold-standard implant material, Ti−6Al−4V. It is well known that surface characteristics, including both topography and chemistry, affect cell attachment and differentiation.\textsuperscript{49,61} The purpose of our study was to understand the effect of the alloy composition on hBMSC fate separately from the many topographic techniques than can be used to enhance osteogenesis. Hence, to avoid any effects of topography on cell behavior, each sample was ground and polished to a mirror finish using 0.04 μm OPS colloidal silica solution. Although polished samples are not directly representative of implant surfaces, removal of topography was required to separate any osteogenic behavior caused by the alloy composition from that caused by the surface topography.

Polishing of the TiTa alloys resulted in the excavation of partially melted Ta particles, which covered approximately 1% of the substrate surface area (Figure 2). Furthermore, pores were found to cover approximately 0.1 and 0.04% of the surface of the TiTa and Ti−6Al−4V substrates, respectively. The particles and pores were randomly dispersed, 1−3 μm in height/depth, and did not affect the overall surface roughness (R\textsubscript{s}). Overall, these pores and protrusions did not have a statistically significant effect on the overall roughness; however, cells are able to detect features on the micron scale.\textsuperscript{57,65} For example, pores of 5−10 μm diameters, similar in size to the pores observed here, in polystyrene interconnected porous scaffolds were shown to increase osteogenic behavior when compared with a smooth surface.\textsuperscript{66} Furthermore, multiple studies have shown osteogenic markers of ALP, osteocalcin, and osteopontin to be enhanced by 1−3 μm surface protrusions, while smoother surfaces enhanced the cell number and proliferation.\textsuperscript{63−68} However, in all these studies, the topography was in the form of an entire surface texture, which often contributed to a significant surface roughness reading.\textsuperscript{63−66} In this study, statistically, only 1 in 40 hBMSCs, with an average spread area of 2,500 μm\textsuperscript{2}, will encounter a single topographical feature observed on the TiTa surfaces. In addition, as there was no increase in cell attachment or proliferation on the smoother Ti−6Al−4V sample, the effect of these protrusions on cell behavior is likely negligible. Hence, it can be concluded that the pores and protrusions observed on the TiTa substrates are unlikely altering cell fate.

Chemical analysis of the substrate surface using EDX showed an enrichment of Ta at the surface compared with the nominal compositions (Ti25Ta = 35 wt % Ta and Ti65Ta = 68 wt % Ta). The Ta enrichment was not caused by a powder mixing error as bulk parts printed from the same powder batch were tested by spectroscopic chemical analysis and showed Ta contents within 1% of the nominal composition. As EDX is a surface analysis technique, it is more likely that surface Ta enrichment occurs. As the samples in this study were mechanically polished, it is possible that the Ti matrix was preferentially polished away because of its lower hardness. This is supported by the raised remaining Ta particles seen on the TiTa substrate surfaces (Figure 2c). Despite not observing the remaining Ta particles, Mendis et al.\textsuperscript{54} also observed Ta enrichment on the surface of mechanically polished conventionally manufactured TiTa samples. Mendis et al.\textsuperscript{54} suggested that because of the differences in enthalpy of oxidation of Ti and Ta, Ta undergoes migration to the surface to form a stable oxide layer. Surface enrichment is also thought to occur because of the lattice strain theory, where substitutional atoms with largely different atomic radii, such as Ta and Ti, diffuse to release lattice strain.\textsuperscript{55}

The difference in Ta content measured by XPS for Ti25Ta (8.0 at %) and Ti65Ta (16.5 at %) did not reflect the same 4-fold atomic increase in Ta content intended through design of the nominal compositions (Table 3). This indicates that the nominal Ta content and oxide Ta content have a nonlinear correlation. Hence, as the potential osteogenic advantage of Ta can only be obtained by the interaction of cells with Ta in the oxide, Ti65Ta does not show as great an improvement to osteogenic potential as anticipated. The quantitative results of the cell morphology and osteogenesis showed no significant difference between the Ti25Ta and Ti65Ta cell responses, supporting this hypothesis. As both the Ti25Ta and Ti65Ta alloys can obtain a low elastic modulus value of approximately 65 GPa\textsuperscript{35,37} and show a similar biological response \textit{in vitro}, the

<table>
<thead>
<tr>
<th>composition</th>
<th>Ti25Ta (Ta at.%)</th>
<th>Ti65Ta (Ta at.%)</th>
<th>fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>nominal (bulk)</td>
<td>8.1</td>
<td>33.0</td>
<td>4.1</td>
</tr>
<tr>
<td>EDX (&lt;2 μm of the surface)</td>
<td>12.0</td>
<td>36.0</td>
<td>3</td>
</tr>
<tr>
<td>XPS (&lt;10 nm of the surface)</td>
<td>18.6 (8.0\textsuperscript{a})</td>
<td>36.5 (16.5\textsuperscript{a})</td>
<td>2 (2.1\textsuperscript{a})</td>
</tr>
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</table>

\textsuperscript{a} at. % including oxygen content.
TiTa alloys did not significantly increase the number of attached cells or enhance proliferation, when compared to Ti–6Al–4V. However, the majority of these studies investigated the behavior of osteoblast or preosteoblast-type cells, rather than MSCs. It is possible that there are cell-type-specific variations in material response on Ta surfaces. As successful integration of an implant is dependent on cell migration and colonization of the implant surface, the TiTa alloys will likely perform equally well as Ti–6Al–4V in this regard. The lack of significant variation in cell proliferation and attachment indicates that these factors are unlikely to drive increased osteogenic behavior in this study.

The cell morphology analysis suggested that the hBMSCs had a stronger initial interaction with the TiTa alloys than the Ti–6Al–4V standard material because of the larger observed spread area. Stronger initial cellular-material interactions with Ta may have caused an increase in integrin production, which allowed more adhesion to the cell and enhanced spreading on the TiTa compositions. The role of increased integrin signaling on MSC osteoblastic differentiation is well documented. The formation of α5β1 integrins and their localization to focal adhesion sites are integral to the initiation of osteogenic signaling. In support of this hypothesis, Lu et al. investigated equally polished Ti and Ta surfaces and found that cells cultured on Ta surfaces had higher α5β1 integrin expression. They hypothesized that as the electronic band gap of Ta oxide is larger than that of Ti oxide, electrons are less likely to pass from a Ta-rich oxide to α5β1 integrins, which can also act as semiconductors in the wet state. Hence, the integrin function is less likely to be disrupted on Ta oxide surfaces. Alternatively, the electronic configuration of the metal oxides may also affect protein absorption to the metal surface, which in turn alters the integrin binding functionality.

However, surface wettability also contributes to the cell spread area. Lu et al. observed no difference in wettability between the Ti and Ta substrates, while a trend of increasing wettability was observed with increasing Ta content in this study, with contact angle measurements (Supporting Information S9). The increased wettability in this study may have contributed to the higher cell area and in turn enhanced osteogenesis. Higher cell tension, noted in established actin filaments of cells with enhanced spreading, also enhances osteogenesis. To pinpoint the cause behind the increased osteogenic potential of Ta and TiTa alloys, the attachment mechanism could be further elucidated through blocking of integrin production after the cell has initially attached or through cellular force imaging, such as that developed by Brockman et al. Future work characterizing the absorbed protein layer on the TiTa alloy surfaces could help to elucidate the mechanism of improved osteogenesis.

5. CONCLUSIONS

In this study, new low-modulus alloys Ti25Ta and Ti65Ta were manufactured using L-PBF. In vitro testing using hBMSCs was performed to determine whether the TiTa alloys displayed an improved osteogenic response compared with the industry standard material, Ti–6Al–4V. All metallic substrates were polished to remove effects of topology on cell fate. The TiTa alloys displayed enhanced ALP activity and mineralization, when compared with Ti–6Al–4V, indicating that the improved osteogenic capacity of Ta was retained in the L-PBF TiTa alloys. There was no significant difference in biological behavior noted between Ti25Ta and Ti65Ta alloys, attributed to a nonlinear representation of Ta in the surface oxide when compared with the nominal composition. Both the L-PBF Ti25Ta and Ti65Ta alloys are suitable low-modulus alternatives to Ti–6Al–4V to enhance bone-implant interactions for stress-loaded bone implants; however, the lighter and cheaper Ti25Ta alloy is the most favorable.
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REFERENCES

