INTRODUCTION

For application to clinical implants, Ti–Zr alloys have recently been considered as available substitutes that can improve mechanical properties of Ti base implants. So far, an alloy of Ti combined with 13–17% Zr (Roxolid, Institut Straumann, Basel, Switzerland) has been developed and used in commercial products for dental applications for several years. Several studies reported that the application of Ti–1317Zr alloys under harsh conditions, for example, small-diameter implants, resulted in high osseointegration and clinical success1-4). In addition, several studies examined Ti–Zr alloys with Zr concentrations ranging from 12% to 50% and reported good biocompatibility and enhanced mechanical properties5-7). However, the reasons for choosing these compositions have rarely been reported.

One study investigated the properties of Ti–Zr alloys with various compositions, focusing on the mechanical strength of the alloys. Therein, it was revealed that Ti–Zr binary alloys with Zr concentrations from 30 to 60 mol% exhibit higher Vicker's hardness and ultimate tensile strength8). Later studies also reported higher bending strength and surface hardness for a Zr concentration of 40 wt%9,10). These studies indicated that alloying Zr with Ti could result in enhanced mechanical properties, which would be advantageous for reducing the fracture risk of implant materials.

Our previous study on Ti–Zr alloys with different compositions of Zr indicated that a Zr proportion in the range of 30–50 mol% has competitive advantages over Ti–10Zr and c.p.Ti. The aim of this study is to evaluate the biological response to Ti–Zr alloys with different compositions and their surface characteristics. Alloy surfaces are modified by sandblasting and sulfuric acid etching. As a result, similar surface structures are observed for c.p.Ti, Ti–10Zr, and Ti–30Zr, whereas Ti–50Zr does not form a micro-rough structure by the same treatment process. No significant difference is found in the viability of cells on c.p.Ti, Ti–10Zr, and Ti–30Zr, whereas lower cell attachment levels are detected on Ti–50Zr. In summary, Ti–30Zr reliably forms a micro-rough structure, which provides one evidence for its application in a new dental implant material.

Keywords: Implant, Titanium–zirconium, Alloy composition, Surface modification, Surface characteristics
The oxide layer on the surface of Zr (ZrO₂) differs from that on Ti (TiO₂). That is, the surface oxide film on Zr is stable, whereas that on Ti is relatively reactive, indicating that the oxide film on Zr is more passive and therefore more protective than that on Ti. In addition, as mentioned above, the surface hardness increases with the addition of Zr. These indicate that surface micro-roughness would be different after SLA depending on the Zr concentration. Because the results of the mechanical strength and corrosion resistance of Ti–Zr alloys are promising, an analysis of surface topography for practical applications will be the next important step (a study in this regard is being pursued by this research group). However, a comparison of the effect of SLA treatment on various compositions of Ti–Zr alloy surfaces has rarely been performed.

Accordingly, the aim of the present study is to investigate the surface characteristics, including the surface roughness of Ti–Zr alloys with a wide range of compositions (10–50 mol%) after the same treatment. In addition, we investigated biocompatibility by evaluating the level of osteoblastic-cell attachment and Ca production. Ultimately, we expect to obtain the most appropriate composition of Ti–Zr binary alloys through this series of studies.

MATERIALS AND METHODS

Specimen preparation
Ti–Zr alloys (Ti–10Zr, Ti–30Zr, and Ti–50 mol% Zr) were prepared from Ti and Zr bars. We mixed Zr with a proper mass into Ti and melted the mixture using a cold copper hearth connected to a water-cooling system in a vacuum-arc machine (Simple arc machine 76-3323, DIAVAC, Chiba, Japan) with a non-consumable tungsten electrode to form a button ingot. During melting, each ingot was re-melted 10 times to ensure complete mixing. Next, cylinders with a diameter of 8 mm were formed by casting ingots in a vacuum-casting machine (MSE-50TMD-Z, Yoshida Cast, Saitama, Japan) under the same arc-casting conditions. Subsequently, we sectioned the cylinders into disks with a diameter of 8 mm and a thickness of 2 mm using a low-speed silicon carbide abrasive cutter. Commercially pure Ti (c.p.Ti) disks were prepared with the same size as the Ti–Zr alloy samples. All specimens were mechanically ground using a #800 SiC abrasive article. We then blasted the specimens with 50 μm aluminum grit and acid-etched them in 66% H₂SO₄ at 120°C for 90 s. All specimens were ultrasonically cleaned in acetone and ethanol.

Surface characterization
The surface morphologies of the different samples were examined using a scanning electron microscope (SEM; S-3400NX, Hitachi, Tokyo, Japan). The surface roughness was examined using a color laser microscope (VK-8510, Keyence, Tokyo, Japan). We tested three points of each sample near the disk center 3,000 times with a depth resolution of 0.05 μm. Noise rejection and tilt correction were set, and the roughness was calculated using whole images of each sample. The wettability of the different samples was examined using water contact angle measurements (PG-X plus, Matsubo, Tokyo, Japan). The contact angle of water was measured on both machine-polished (before SLA treatment) and SLA-treated surface, and included five samples for each surface. The size of the pure water droplet for the contact angle measurements was 4 μL. All specimens were stored under ambient conditions for 14 and 28 days.

Osteoblast cell culture
MC3T3-E1 cells (RIKEN Bioresource Center, Ibaraki, Japan) were incubated at 37°C in a fully humidified atmosphere of 95% air and 5% CO₂ with a modified Eagle’s medium (α-MEM, Wako Pure Chemical Industries, Osaka, Japan). The medium consisted of 10% fetal bovine serum (JRH Bioscience, Shawnee Mission, KS, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA). Each substrate was cleaned with acetone for 10 min in an ultrasonic cleaning machine and air-dried on a bench (temperature, 23°C; humidity, 60%), followed by 1 h soaking in 70% alcohol for sterilization prior to placement into 24-well tissue culture plates (Iwaki Bland, AGC TECHNO GLASS, Tokyo, Japan). The cells were incubated onto each substrate placed on polystyrene dishes at a density of 5×10⁴ cells/mL.

Cell attachment
After 6 h of incubation, the medium was removed, and each plate was stained with calcine-AM (Dojindo, Tokyo, Japan). The cells were then imaged with a calcine filter using a fluorescence microscope (IX71, Olympus, Tokyo, Japan). We also counted the cells for each sample type. Three dishes were prepared, and five points on each dish were randomly selected. The number of cells was calculated from fluorescence images, which were manually analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). In total, 15 randomly selected cell fluorescence images from each sample were obtained. The cell density was calculated by dividing the average cell number by the cell area (0.09×0.07 cm²).

Ca deposition
The mineralization capability of cultured osteoblastic cells was examined by colorimetry-based quantification of Ca deposition on days 21 and 28. The cultures were washed with PBS and incubated overnight in 1 mL of 0.5 mM HCl solution with gentle shaking. The solution was mixed with o-cresolphthalein complexone in an alkaline medium (Ca binding and buffer reagent, Sigma, St. Louis, MO, USA) to produce a red Ca cresolphthalein complexone complex. Color intensity was measured using an ELISA reader at 575 nm absorbance.

Statistical analysis
All reported results were obtained from at least five independent determinations. All values are expressed as the mean±SD. One-way analysis of variance (ANOVA)
RESULTS

Surface morphology

SEM images of SLA surfaces showed similar structures on c.p.Ti, Ti–10Zr, and Ti–30Zr (Figs. 1B, D, and F). The morphologies of Ti–10Zr and Ti–30Zr presented regular microscale pits with sharp peaks, which were similar to those of c.p.Ti. However, interestingly, the morphology of Ti–50Zr (Fig. 1H) presented a completely different shape, which was a comparatively smooth surface at low magnification, and there were no sharp ridges in the high-magnification image.

Surface roughness

Figure 2 shows the roughness values of each sample modified by SLA. The value of Ra increased from c.p.Ti to Ti–30Zr, and Ti–30Zr exhibited the maximum value (Fig. 2-Ra). Meanwhile, the value of Rms showed an increasing trend between c.p.Ti and Ti–30Zr (Fig. 2-Rms). Similarly, the values of Ry and Rz for Ti–10Zr were lower than those for c.p.Ti and Ti–30Zr (Figs. 2-Ry and -Rz). In total, Ti–50Zr exhibited significant minimum values for all roughness values.

Wettability

Wettability values of c.p.Ti, Ti–10Zr, Ti–30Zr, and Ti–50Zr were measured on days 14 and 28 for machine-polished and SLA-treated surfaces. The contact angle of water on each sample clearly increased between day 14 and day 28 (Figs. 3A, B). Interestingly, the contact angle of water decreased as the Zr concentration was increased on both days for both type of surfaces. On day 14, the contact angle of water for c.p.Ti was significantly different from those for Ti–30Zr and Ti–50Zr on both type of surfaces, and the contact angle of water for Ti–10Zr was also significantly different from that for Ti–50Zr on both type of surfaces (Fig. 3A). On day 28, significant differences were detected between c.p.Ti and Ti–50Zr on both type of surfaces (Fig. 3B).

Cell attachment level

Although no significant difference was observed in the initial cell attachment level between c.p.Ti, Ti–10Zr, and Ti–30Zr, Ti–10Zr showed the highest value among all the samples (Fig. 4B). In contrast, the negative effect of a higher Zr proportion was also detected with a 25% reduction in the cell attachment level on Ti–50Zr surfaces (Fig. 4B). Fluorescence microscopy of cells stained with calcein-AM confirmed the positive and negative effects on the cells on Zr alloys depending on the Zr proportion (Fig. 4A).

Ca deposition

The total Ca deposition of Ti–10Zr was the highest, which was not significantly different from that of c.p.Ti but significantly higher than that of Ti–30Zr and Ti–50Zr on days 21 and 28 (Figs. 5A, B). The total Ca deposition of Ti–50Zr showed the lowest value compared with those of other compositions, which was 30% lower than that of Ti–10Zr on day 28 (Fig. 5B).
Fig. 3  Wettability after 14 days (A) and 28 days (B) after machine-polishing, and sandblast and acid-etch treatment. Representative images of 4 μL of water dropped onto each machine-polished surface are presented, and the differences in contact angle are evaluated. Data are expressed in mean±SD values (n=5). *p<0.05 indicates a statistically significant difference among the c.p.Ti and Ti–Zr alloy surfaces.

Fig. 4  (A) Fluorescence microscopy images of osteoblastic cells stained with calcein-AM after 6 h of culture. (B) Osteoblastic cellular attachment level evaluated by fluorescence images. Data are expressed in mean±SD values (n=5×3 disks). *p<0.05 indicates a statistically significant difference among the c.p.Ti and Ti–Zr alloy surfaces.

Fig. 5  Mineralizing capability of osteoblasts evaluated by the total Ca deposition at culture day 21 (A) and 28 (B). Data are mean±SD (n=5) for all panels. *p<0.05 indicates a statistically significant difference among the c.p.Ti and Ti–Zr alloy surfaces.
DISCUSSION

Binary Ti–Zr alloys are promising dental implant materials because they are compatible with SLA treatment, maintain the α-phase structure, and have enhanced mechanical performance (10). Figure 1 shows the surface structures of the treated c.p.Ti and Ti alloys. The samples of c.p.Ti, Ti–10Zr, and Ti–30Zr exhibited roughened surfaces with similar typical SLA-like microscale pits and sharp edges, whereas Ti–50Zr exhibited an unformed surface. This SLA-like topography produced on the surfaces of c.p.Ti, Ti–10Zr, and Ti–30Zr indicates that when the Zr atom percentage does not exceed 30%, this characteristic topography occurs regardless of the chemical composition. A similar conclusion was drawn in a previous study where Ti–15 wt% Zr was used (19). However, in this study, we reached a higher upper limit of Zr concentration for the fabrication of SLA-like surfaces. Previous XRD results show that Ti–(5–45 wt%) Zr alloys comprise a mixture of hcp α and α’ phases that occur owing to the martensitic transformation of the α phase. The lamellae and needle-like structures observed using the SEM were characteristic of a martensitic microstructure. The increase in Zr content causes an evident downshift of XRD peaks along with an increase in thinner martensitic microstructures such as lamellae and needles (17). Similarly, in another study, Ti–50 wt% Zr alloy showed a more acicular martensitic phase than Ti–16 wt% Zr (19). An additional report suggests that at higher Zr concentrations, the XRD peaks shift to lower angles for Ti–(10–70 at%) Zr alloys (11). Previously, XRD results showed that Ti–(5–45 wt%) Zr alloys were a mixture of hcp α phase and α’ phase composition, the occurrence of which could be due to the martensitic transformation from the α phase. The lamellae and needle-like structures found in their SEM results could be explained by the martensitic-phase microstructure. With increasing the Zr content, the shift of the XRD peaks to a lower angle was more obvious, and the martensitic-phase microstructures of lamellae and needles became more and thinner (17). Similarly, in another study, Ti–50 wt% Zr alloy showed more acicular martensites than Ti–16 wt% Zr (18). Our previous study also reported that the diffraction peaks of the Ti-Zr alloys matched the hcp α phase and α’ phase, with peaks shifting to the lower angle as Zr concentration (10–70 at%) increased (11). Thus, we can deduce that with increasing the Zr content, thinner martensitic microstructures (lamellae and needles) should increasingly appear on the surfaces of Ti–10Zr, Ti–30Zr, and Ti–50Zr alloys. This trend can be seen in the SEM images (Figs. 1A, C, E, and G). SLA Ti–10Zr (i.e., Ti–17.4 wt% Zr) has been successfully commercialized as Roxolid dental implant (Ti–(13–17 wt%) Zr), the surface characteristics of which have been extensively studied. Our treated Ti–10Zr reproduced a similar surface to that of Roxolid. However, Ti–50Zr presented a very dissimilar surface structure with c.p.Ti even after the same treatment, suggesting that the large change in chemistry may be responsible for the variation in the surface morphology. As mentioned above, Ti–30Zr, Ti–10Zr, and c.p.Ti exhibited similar surfaces, but Ti–50Zr had a unique appearance. We may put forward a null hypothesis that Ti–50Zr might possess a set of very different surface attributes, whereas the other three materials remain close to each other. Except for the SEM-verified surface morphologies presented herein, the remaining selected surface properties are illustrated in the following sections.

Roughness is an important surface characteristic that determines the functional activity of cells by influencing bone cell behaviors and bone apatite nucleation, and is critical for the bone–implant-interface formation (19). Ti–50Zr showed significantly lower values of Rms, Ry, and Rz and lower Ra than Ti–10Zr and Ti–30Zr, whereas there was no significant difference between c.p.Ti, Ti–10Zr, and Ti–30Zr in these surface roughness parameters. This result is consistent with the surface morphology shown in Fig. 1, where Ti–50Zr with the flattest microscale surface stands out from all the test materials. One reason for this roughness might be a difference in corrosion behavior. The immersion test in our previous study revealed a remarkable decrease in dissolved ions with addition of Zr to Ti under exposure to lactic acid (12). This result suggests that the oxide layer of the Zr differs from that of Ti, indicating that ZrO2 is more stable and resilient to dissolution. Although the acid type is different in the present study, the higher protection against the acid offered by the stable layer might have led to a lower surface roughness in Ti-50Zr alloy. Some in vitro studies indicated a positive correlation between surface roughness and cell attachment (20), which is consistent with our result of cell attachment. Namely, Ti–50Zr with the surface with the smallest roughness has fewer surface-attached MC3T3-E1 cells than c.p.Ti, Ti–10Zr, and Ti–30Zr, although the direct effect of surface roughness on regulating cell adhesion remains debatable (8).

Regarding surface wettability, our water contact angle results showed that wettability increased with an increase of Zr content regardless of the surface type (i.e., machine-polished or SLA-treated). Ti–50Zr was more hydrophilic than c.p.Ti, Ti–10Zr, and Ti–30Zr on day 14. On day 28, a significant difference remained between Ti–50Zr and c.p.Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21). As mentioned above, the property of oxide layer differs depend on the addition of Zr to Ti. The general trend observed in this study was that Ti–Zr alloys exhibited smaller water contact angles than those of c.p.Ti, consistent with a previous report (21).
study, on day 14, the SLA-treated rougher surface was more hydrophilic than the machine-polished surface for each alloy; however, this reversed on day 28. It has been reported that a Ti surface is super-hydrophilic (contact angle $<5^\circ$) immediately after acid-etch treatment; however, it gradually becomes hydrophobic due to accumulation of organic molecules over time. In the present study, hydrophilicity might have remained 14 days post SLA treatment, but wettability had decreased after 28 days compared to that of machine-polished surface, suggesting that the speed of wettability degradation might be faster on SLA-treated surface than on machine-polished surface.

Although higher hydrophilic surfaces were reported to be better for interacting with biological fluids, cells, and tissues, our results showed the hydrophilicity of the attached cell density was smaller on the surface of higher hydrophilic Ti–50Zr than on lower hydrophilic c.p.Ti, Ti–10Zr, and Ti–30Zr. It was found that higher hydrophilicity and larger roughness may attract more cell attachment. The 6 h cell density and Ca deposition on the Ti–50Zr surface were the smallest, as compared with other alloys, at both time points of days 21 and 28, illustrating its compromised cell differentiation. This result was in line with the finding that the differentiation of cells increases with the roughness, but is not consistent with the statement that greater wettability leads to better cell differentiation.

The 6 h cell density and Ca deposition on the Ti–50Zr surface were the smallest, as compared with c.p.Ti, Ti–10Zr, and Ti–30Zr. Initial cell attachment has reportedly been shown to determine the processes of cell proliferation and differentiation. The decrease in Ca deposition, which is representative of osteo-conductivity, could have resulted from a decrease in osteoblastic-cell attachment. The difference in the biological performance in our study from that in other studies might be explained by the concurrent change of multiple surface features, such as surface topography and chemical composition (except for the surface roughness and wettability). This complicates the evaluation of their effects on cell biology. However, it is noteworthy that all the examined surface characteristics showed particularity on Ti–50Zr, as compared with other materials.

We discussed the correlation between surface roughness, wettability, and cell behavior, and the influence of surface composition on cell behavior will be addressed further. The diverse post-treated surface topographies of c.p.Ti and Ti alloys are direct or indirect owing to their differences in chemical composition, which was suggested on the basis of SEM observations. This is why the impact of surface topography will not be discussed separately. It is believed that the surface composition and microstructure of the alloys play a role in the ability of the material to induce osteo-conduction, yet the surface composition alone can also influence some of the cell behaviors. Zr coating on Ti alloy enhanced the early osteogenic responses, confirming the effect of surface composition difference on biological responses. In a previous study that exclusively investigated the function of surface chemistry on initial cell attachment and early differentiation, an alloy with a composition of Ti–50Zr was found to be less efficient than other alloys and c.p.Ti. Therefore, irrespective of whether they were treated or not, in terms of basic cell behaviors, Ti–50Zr presented its inferiority compared to c.p.Ti, whereas Ti–10Zr and Ti–30Zr remained comparable to c.p.Ti. Although biological performance is regulated by multiple factors, the results of the present study consistently showed initial cell attachment level decrease with increase of Zr composition. In future, detailed biological analyses, such as proliferation and differentiation, are required with elemental analysis. In conclusion, our null hypothesis was confirmed by Ti–50Zr showing differences in surface roughness, wettability, cell attachment, and Ca deposition, whereas c.p.Ti, Ti–10Zr, and Ti–30Zr were similar.

CONCLUSIONS

Considering the comparable biocompatibility presented herein and previously found superior mechanical properties of Ti–Zr alloys to those of c.p.Ti, chemically modified (SLA) Ti–Zr alloys (Ti–10Zr and Ti–30Zr) are good alternatives to SLA c.p.Ti as dental implants. In contrast, Ti–50Zr may not be considered for clinical use. Although in vivo and clinical outcomes should be investigated further, this study provides additional evidence of the good potential of SLA-modified Ti–Zr alloys for new implant materials.

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