Effect of O2-Plasma Treatment on Surface Characteristics and Osteoblast-Like MG-63 Cells Response of Ti-30Nb-1Fe-1Hf Alloy

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In this study, the effect of O₂-plasma treatment with various powers on the surface characteristics and also the cell response of the Ti-30Nb-1Fe-1Hf alloy were investigated. Surface characteristics of Ti-30Nb-1Fe-1Hf alloy were evaluated by X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray powder diffractometer (XRPD) and wettability test. The results show that the surface roughness of the alloy decreases after O₂-plasma treatment, and also with increased plasma power. By XPS analyses, both Ti valence states of Ti⁴⁺ and Ti⁵⁺ as well as Nb valence states of Nb⁵⁺, Nb⁶⁺ and Nb⁷⁺ can be detected in the oxide films. Also, the concentrations of TiO₂ and Nb₂O₅ increase with increasing O₂-plasma power. The results also show that the contact angle decreases as the alloy is modified by O₂-plasma treatment, and therefore, the treated alloys can be expected to be more hydrophilic. On the other hand, for the evaluation of cell response, cell (MG-63) culture was performed. The results show that the oxidation effect on the alloy surface brought about by O₂-plasma treatment enhances the spreading of cells. In addition, in vitro tests suggest that cell spreading on Ti-30Nb-1Fe-1Hf alloy is similar to that on Ti-6Al-4V alloy, whereas, cell adhesion on Ti-30Nb-1Fe-1Hf alloy is better than that on Ti-6Al-4V alloy.

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1. Introduction

Titanium and its alloys have been widely used in medical fields, such as for dental and orthopedic implant material applications, due to their excellent mechanical properties, corrosion resistance and biocompatibility.¹,² Ti-6Al-4V alloy, the most commonly used titanium alloy, is a suitable biomaterial for surgically implanted applications. However, certain metallic ions, such as aluminum (Al) and vanadium (V), may dissolve from the implanted Ti-6Al-4V alloy and diffuse into the human body, resulting in harmfulness of both soft and hard tissues.³ Some studies reported that Al ions may accumulate in the human brain and cause Alzheimer’s disease,⁴,⁵ while V ions may cause severe cytotoxicity.⁵ Furthermore, because of a much higher elastic modulus of the Ti-6Al-4V alloy (around 110 GPa) than that of the human bone (around 10–30 GPa), the stress shielding effect will occur as Ti-6Al-4V alloy is implanted into the skeleton of the human body.⁷ For the afore-mentioned reasons, new types of Ti alloys need to be developed which not only should exclude the bio-toxic elements, such as Al and V, but also should possess high strength and low elastic module. It had been reported that niobium (Nb) and hafnium (Hf) exhibit good biocompatibility and osteoconductivity.⁸–¹⁰ In addition, Hf has been found to possess the advantage of enhancing strength, while at the same time reducing the elastic modulus of the Ti alloys.¹¹,¹² In an authors’ previous study,¹³ a specific Ti-alloy, i.e., Ti-30Nb-1Fe-1Hf, had been developed, which exhibits relatively high strength and low modulus (UTS: 914 MPa, 0.2% Proof stress: 862 MPa, E: 62 GPa). In this study, Ti-30Nb-1Fe-1Hf alloy was used as the substrate material for evaluating the effect of surface treatment or modification on the surface characteristics and biocompatibility of this specific alloy.

Good biocompatibility of titanium and its alloys can be attributed to the naturally formed oxide film on their surfaces.¹⁴ However, the oxide film formed is rather thin and may contain numerous defects.¹⁵ In addition, the surface characteristics of the implant materials will influence the nature of interactions between implants and tissues.¹⁶,¹⁷ Therefore, improved osseointegration through appropriate surface treatment or modification of the implants is a vital aspect concerning the development of biocompatible implant alloys.

Surface treatments, e.g., thermal oxidation¹⁸–²⁰ and anodization,²¹ had been performed with results showing improvement of characteristics of oxide layer. Unfortunately, some drawbacks are with these surface treatment methods. For instance, for thermal oxidation process, relatively high treatment temperature of some 600 °C are required, and also the possible occurrence of impurities contamination in oxide films. For anodization process, the oxide films may be less pure due to the electrolyte residence. Due to the fact that the purity of the oxide layer is an important factor in attaining better biocompatibility, the glow discharge plasma treatment method (Ar- and O₂-plasma) was employed in this study, which is a common technique for cleansing and modifying the implant surfaces.²²,²³ and also offers a dry, clean, one-step and in situ procedure. Furthermore, the potential of glow discharge plasma treatment for sterilizing implants had also been mentioned.²⁷ It has been well recognized that the effect of plasma treatment on any given alloy depends on plasma power, gas composition and treatment time.²²,²³,²⁵,²⁶,²⁸ In this study, O₂-plasma treatment was utilized to modify the alloy surfaces, and the effect of plasma power (at a fixed treatment time of 20 minutes) on the surface characteristics of Ti-30Nb-1Fe-1Hf alloy will be investigated. In addition, the cell response of the treated alloys will be evaluated by culturing the specimens with MG-63 cells. The aims of this work are to realize how the O₂-plasma treatment can alter the surface...
characteristics of Ti-30-1Fe-1Hf alloy, and to study the effect of surface modification on the cell response.

2. Materials and Methods

2.1 Materials

In this study, the substrate material used for investigation is Ti-30Nb-1Fe-1Hf alloy. Ti-30Nb-1Fe-1Hf ingots were prepared using pure titanium (99.7 mass% in purity), pure niobium (99.8 mass% in purity), pure iron (99.98 mass% in purity) and pure hafnium (99.98 mass% in purity). The weighed charge materials were placed in a water-cooled copper crucible and then melted using a non-consumable tungsten electrode arc in a vacuum chamber. The melting chamber was first evacuated and then purged with argon. An argon pressure of 0.1 MPa was maintained throughout the melting process. The ingots were re-melted four more times to achieve chemical homogeneity. The solidified Ti-30Nb-1Fe-1Hf ingots were homogenized at 1000°C for 6 h in a vacuum of higher than 0.27 Pa, and then hot-rolled at 750°C into plates with a thickness of some 2 mm. The final rolled plates were then annealed at 700°C for 1 h, and then furnace cooled to room temperature.

2.2 Specimen preparation

1 × 1 cm² flat specimens were cut from the rolled and annealed plates and then were ground with silicon carbide papers up to 2500 grit. The ground specimens were then processed by Ar- and O₂-plasma treatments (Plasma reactor, AST, Boston, MA, USA); in which specimens were first cleansed by Ar-plasma for 10 min in a plasma reactor, and then oxidized by O₂-plasma for 20 min. Two different O₂-plasma powers were employed (80 W and 280 W) to study the effect of plasma power on the surface characteristics of the alloy. After Ar-O₂ plasma treatments, specimens were annealed for 10 min in a plasma reactor. For the purpose of discussion, the specimens were designated as TN, TN-80 and TN-280 for Ti-30Nb-1Fe-1Hf alloy without plasma treatment, with Ar- and O₂ plasma treatments with power of 80 W, and 280 W, respectively.

2.3 Surface analyses

The surface morphology and roughness of both non-treated and O₂-plasma treated specimens were analyzed by scanning electron microscopy (SEM, Philips, XL-30) and atomic force microscopy (AFM, Digital Instruments, Dimension-3100 Multimode). The AFM probe scanned over an area of 5 × 5 μm², with 512 scans performed at a scanning rate of 1 Hz in the tapping mode. The phases formed on the surface were analyzed by X-ray powder diffractometer (XRPD, MAC Sience, MXP18, JAPAN) at 40 kV, 150 mA, fixed incident angles of 0.5°, scanning rate of 2 deg/min and in the scanning range of 30°-75°, and also by X-ray crystallography using a Ni-filtered Cu Kα radiation source. Phases were identified by matching their characteristic peaks with those in the files of the Joint Committee on Powder Diffraction Standards (JCPDS). The compositions of the surface layer of the specimens without and with O₂-plasma treatment were analyzed by X-ray photoelectron spectroscopy (XPS, England, Thermo VG-Scientific, Sigma Probe) with a monochromatic Al Kα (1486.6 eV) source operating at 108 W (15 kV), which could examine a surface area of 400 μm in diameter. In all cases, the surface contaminations were cleansed by sputtering with an Ar⁺ ion gun (3 keV, 1 μA) at an angle of 45° for 1 min. In addition, the morphologies of oxide layer for various treated alloys were observed by field emission gun-SEM (FEG-SEM, LEO1530, England), and the thicknesses of the oxide layers were measured by secondary ion mass spectrometer (SIMS, France Cameca, IMS-4F). For identifying the aqueous wettability of the treated alloys, the contact angles, determined by using drops of ultrapure distilled water, were measured using a FTA-32 video contact angle system (First Ten Angstrom Inc., USA) at room temperature.

2.4 Cell culture

In order to evaluate the cell response of the Ti-30Nb-1Fe-1Hf alloy without and with O₂-plasma treatment, specimens TN, TN-80 and TN-280 were cell (MG-63) cultured, and then the surface morphology and the adhesion of the cells were analyzed by SEM. For comparison purpose, cell culture was also performed on untreated Ti-6Al-4V alloy. The MG-63 cell suspension with a 2 × 10⁴/cm² density was employed. The culture medium consists of DMEM containing 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin, and 100 units/ml of penicillin. The procedures for cell culture are as follows: Firstly, the specimens were placed at the bottom of a 24-well plastic plate, and were exposed to the ultraviolet (UV) ray for 24 h. Subsequently, the specimens were washed several times using Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL) and phosphate-buffered saline (PBS, pH 7.4, Gibco). Finally, the specimens were cell cultured for 4 h, 8 h, 24 h, and 48 h, respectively. The plate was placed inside a culture chamber at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After culturing, the specimens were gently washed with PBS three times, and then fixed with two different solutions. The specimens were first fixed with 2% paraformaldehyde and 2.5% glutaradehyde (GA) in 0.2 M cacodelate for 30 min at 4°C, and then washed three times with 0.2 M cacodelate at 4°C every five minutes. The specimens were then fixed with 1% OsO₄ in 0.1 M cacodelate for 60 min at room temperature, and then again washed three times with 0.2 M cacodelate at 4°C every five minutes. After the second fixation, the specimens were dehydrated sequentially in solutions with 70%, 80%, 90%, 95% and 100% alcohol. After dehydration, HMDS (1,1,1,3,3,3-hexamethyl-disilazane) was added to each well and then dried. Finally, a thin gold layer was coated on the surfaces.

3. Results and Discussions

Figure 1 shows the SEM images of the surfaces of specimens TN, TN-80 and TN-280. The surface morphology of TN exhibits an irregular shape with numerous protuberances, as shown in Fig. 1(a). On the other hand, little protuberances, except for numerous pits and/or cavities, can be detected in TN-280 (Fig. 1(c)). Figure 2 shows the surface roughness of specimens as a function of plasma treatment power by means of AFM measurement. It is clear that the surface roughness decreases as the plasma power increases.
The surface roughness values of TN, TN-80 and TN-280 are 82.8 nm, 65.3 nm and 47.1 nm, respectively. A relatively rough surface found in TN is due to the original polishing effect. The surface roughness is improved after plasma treatment and this is because, during O$_2$-plasma treatment, electrical gas discharge takes place which generates energetic ions, facilitating physical bombardment on the alloy surface. Plasma treatment reduces the surface roughness is in agreement with that reported in several studies. 

Figure 3 shows the cross-sectional SEM images of Ti-30Nb-1Fe-1Hf alloy both without and with plasma oxidation. It appears that TN-280 has a thicker oxide layer than TN. In addition, the thicknesses of oxide layers formed by plasma oxidation treatment with various plasma powers were measured by SIMS, with the results presented in Fig. 4. The thickness of oxide layer of TN-280 (~340 nm) is thicker than that of TN-80 (~240 nm), which in turn is thicker than that of TN (~170 nm). The results clearly indicate that the thickness of oxide layer increases with increasing plasma power. During O$_2$-plasma treatment, oxygen diffusion and subsequent formation of oxides account for the increase in thickness of the oxide layer. Similar results have also been reported by Aronsson et al. on pure titanium.
The measurement results regarding the aqueous wettability test in terms of contact angle are presented in Table 1 and are also shown in Fig. 5. As indicated in Fig. 5, the contact angle is substantially reduced as the specimens were treated with O\textsubscript{2}-plasma, implying that the wettability of the alloy increases with O\textsubscript{2}-plasma treatment, and hence, better hydrophilic can be expected. Similar results had also been reported in some works\textsuperscript{23,29,30). However, little difference in contact angle was obtained by varying O\textsubscript{2}-plasma power.

The XRPD spectra of TN, TN-80 and TN-280 are shown in Fig. 6. The results indicate that the phase present is entirely of \textit{β}-Ti in TN and TN-80, while TiO\textsubscript{2}, Ti\textsubscript{3}O\textsubscript{5} and Fe\textsubscript{2}O\textsubscript{3} peaks can be detected in TN-280. In addition, XPS analyses were conducted to detect the presence of iron oxide. Figure 7 shows the pattern of Fe 2p in XPS spectra for different plasma treatment conditions. No peak of iron oxide was detected in specimen TN. After plasma oxidation treatment, the presence of Fe\textsubscript{2}O\textsubscript{3} was confirmed according to the Handbook of X-ray Photoelectron Spectroscopy.\textsuperscript{31) Moreover, the inflective intensities of titanium oxide peaks of TN-280 are higher than those of TN, indicating that plasma oxidation induces the formation of titanium oxides and iron oxides.

On the other hand, chemical binding states of the surface oxide layers of the Ti-30Nb-1Fe-1Hf alloy without and with O\textsubscript{2}-plasma treatments were analyzed by XPS. Figure 8(a) shows the Ti2p spectra for different plasma treatment parameters. The Ti2p peak is a doublet peak consisting of the Ti2p\textsubscript{3/2} and Ti2p\textsubscript{1/2} peaks. For specimen TN, the main binding energy for Ti2p\textsubscript{3/2} is at 456 eV, which is assigned to TiO. The other binding energy for Ti2p\textsubscript{1/2} is located at 462 eV, which corresponds to TiO\textsubscript{2}. After plasma oxidation treatment, for specimens TN-80 and TN-280, the binding energies for Ti2p\textsubscript{3/2} and Ti2p\textsubscript{1/2} are at 459 eV and 465 eV, respectively, both energies correspond to TiO\textsubscript{2}. It then can be concluded that when the alloy was treated with O\textsubscript{2}-plasma, the Ti2P\textsubscript{3/2} and Ti2P\textsubscript{1/2} peaks changed from TiO to TiO\textsubscript{2} and Ti\textsubscript{3}O\textsubscript{5} to TiO\textsubscript{2}, respectively. By using the computer assisted Gaussian-Lorentzian peak model to curve fit the element spectra, Fig. 8(b) can be derived from Fig. 8(a). Figure 8(b) shows the results of curve fitting for the Ti2p spectra for three different plasma treatment conditions. In Fig. 8(b), the binding energies at 455.9 eV, 456.8 eV and 458.6 eV correspond to Ti\textsuperscript{2+}, Ti\textsuperscript{3+} and Ti\textsuperscript{4+}.

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Table 1 Results of contact angle measurements for TN, TN-80 and TN-280.

<table>
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Fig. 4 The thickness of oxide layer of TN, TN-80 and TN-280.

Fig. 5 Contact angle analyses for TN, TN-80, and TN-280.

Fig. 6 XRPD spectra for TN, TN-80 and TN-280.

Fig. 7 Fe 2p XPS spectra for TN, TN-80 and TN-280.
2p_{3/2} emissions, respectively. And, binding energies at 460.2 eV, 462 eV and 464.3 eV also correspond to Ti^{2+}, Ti^{3+} and Ti^{4+} 2p_{3/2} emissions, respectively. Accordingly, for the surface oxide layers of the non-treated specimens, Ti^{2+}, Ti^{3+} and Ti^{4+} chemical states are present in Ti2p spectra, while Ti^{2+} and Ti^{3+} chemical states in Ti2p_{1/2} spectra. After O_{2}-plasma treatment, the valence state of Ti^{4+} increases, while Ti^{2+} and Ti^{3+} decrease, indicating that the proportion of TiO_{2} increases while TiO and Ti_{2}O_{3} decrease with increasing O_{2}-plasma power in both the Ti2p_{3/2} and Ti2p_{1/2} spectra. The results demonstrate that the oxygen atoms tend to form TiO_{2} as the plasma treatment energy is increased.

Regarding the Nb, as shown in Fig. 9, different valence states of Nb^{2+} (203.7 eV), Nb^{4+} (205.2 eV) and Nb^{5+} (207 eV) are evident in specimen TN. After plasma treatment (both TN-80 and TN-280), the surface valence state of Nb^{2+} decreases, Nb^{5+} (208.2 eV and 210.2 eV) increases, and Nb^{4+} remains more or less unchanged, implying that the proportion of Nb_{2}O_{5} increases, while NbO decreases, by O_{2}-plasma treatment. The present results clearly indicate that plasma oxidation tends to promote the formations of TiO_{2} and Nb_{2}O_{5}.

The chemical state of O spectra and the corresponding O1s spectra are shown in Figs. 10(a) and 10(b), respectively. As shown in Fig. 10(a), the binding energy of O1s spectra of specimen TN is at ~532 eV, that corresponds to O_{2}. The binding energies of specimens TN-80 and TN-280 are quite similar, but shift slightly to a lower energy as compared to that of specimen TN. In addition, the intensity of O_{2} chemical state increases not only by O_{2}-plasma treatment, but also with increasing O_{2}-plasma power. By using the computer assisted Gaussian-Lorentzian peak model to curve fit the element spectra, Fig. 10(b) can be derived from Fig. 10(a).
Figure 10(b) shows two peaks (531 eV and 532 eV) for the O1s spectra for different plasma treatment conditions. According to the Handbook of X-ray Photoelectron Spectroscopy, the binding energies of 531 eV and 532 eV can be assigned to metal oxide and hydroxide, respectively. Furthermore, the peak at 531 eV can be further assigned to titanium oxide according to the published data. However, the possibility of the existence of niobium oxide could be considered. On the other hand, the appearance of the peak at 532 eV may indicate that some OH groups are likely to be bonded to the surface oxide. Based upon the above discussion, O2-plasma treatment leads to the formation of titanium oxides and possibly niobium oxides that contain some OH groups on the alloy surface.

In order to study the effect of plasma oxidation on cell response of Ti-30Nb-1Fe-1Hf alloy, in vitro tests were conducted by culturing the specimens with MG-63 cells in short-term. For comparison purpose, untreated Ti-6Al-4V alloy was also tested. Figure 11 shows the SEM images of the cells that had been cultured for 24 h. As can be observed in Figs. 11(a) and 11(b), cells in circular shape are present in both TN and TN-80, but the extension of filopodia and the formation of membrane are more apparent in TN-80 than in TN. On the other hand, the cells on TN-280 (Fig. 11(c)) exhibit more spread and contain more extended filopodia when compares with those in TN-80. The SEM images of the cells after being cultured for 48 h are shown in Fig. 12. In all three specimens, the cells exhibit significant flattening and spreading. However, in both specimens of TN-80 and TN-280, in addition to flattening and spreading of the cells, numerous filopodia along the cell boundaries formed, with a higher degree with a higher plasma power. Furthermore, dorsal ruffles on the surface of cells of TN-80 and TN-280 can also be observed, but not in TN. Based upon the above observations, we conclude that alloys treated with a higher plasma power can attain better spreading of MG-63 cells on the alloy surface. Figure 13 compares the cell formation on the untreated Ti-6Al-4V alloy and TN-280 after 48 h cell culture. Figure 13(a) shows an enlarged cell on Ti-6Al-4V alloy, in which an extended and flattened cell with numerous filopodia along the cell boundaries is evident, that is similar to the cell morphology of TN-280 (Fig. 12(c)). The SEM images of cell morphology of Ti-6Al-4V and TN-280 after 48 h cell culture are shown in Figs. 13(b) and 13(c), respectively. The cells exhibit better adhesion on TN-280 than on Ti-6Al-4V, indicating that for Ti-30Nb-1Fe-1Hf alloy, when treated with proper plasma oxidation, can obtain better cell adhesion than Ti-6Al-4V alloy.

By XRD and XPS analyses of the O2-plasma treated alloys, the authors have demonstrated that O2-plasma treatment can induce the formation of TiO2 and Nb2O5 on the alloy surfaces. The formations of TiO2 and Nb2O5, together with the presence of pits and/or cavities in a relatively smooth surface of the O2-plasma treated alloys, provide favorable environment for cells spreading and adhesion. Kasemo reported that unsaturated chemical bonds almost always exist on perfectly cleansed titanium surfaces. However, those unsaturated chemical bonds become saturated through reacting with molecules (such as contaminants, dust particles and debris) present in the surrounding environment to which the implant is exposed. Therefore, the perfectly clean surfaces with unsaturated chemical bonds on them represent a higher surface energy state than the contaminated ones. In addition, the surface energy state is related to a certain degree with the surface wettability, that is, the wettability increases with increasing surface energy. In this study, during the O2-plasma treatment, the surface contaminants were believed to be removed and a ultra-clean surface was achieved, accompanying with the formation of unsaturated chemical bonds on the treated surface. This
results in increased surface energy and therefore wettability. Due to the fact that the wettability increases with \( \text{O}_2 \)-plasma treatment, we can postulate that more unsaturated chemical bonds would be generated by \( \text{O}_2 \)-plasma treatment. In the subsequent cell culture process, the nutrient molecules of the culture medium would bond to those unsaturated chemical bonds on the specimen surface, facilitating cell spreading and adhesion. Consequently, the specimens treated with \( \text{O}_2 \)-plasma exhibit better cell spreading than the untreated specimens, and also, the \( \text{O}_2 \)-plasma treated specimens with higher plasma power exhibit better cell spreading than the specimens with lower plasma power.

4. Conclusions

In this study, the effect of \( \text{O}_2 \)-plasma treatment with various powers on the surface characteristics and also the cell response of the Ti-30Nb-1Fe-1Hf alloy were investigated. Based upon the results obtained herein, the following conclusions can be drawn:
Plasma oxidation induces the formation of titanium oxides ($\text{TiO}_2$) and iron oxide ($\text{Fe}_2\text{O}_3$). In addition, $\text{O}_2$-plasma treatment tends to transform the originally present $\text{TiO}$ and $\text{Ti}_2\text{O}_3$ oxides to $\text{TiO}_2$, and NbO to $\text{Nb}_2\text{O}_5$. Furthermore, the concentrations of both $\text{TiO}_2$ and $\text{Nb}_2\text{O}_5$ increase with increasing $\text{O}_2$-plasma power.

The results of short-term (48 h) in vitro tests indicate that the incorporation of oxygen into the surface oxide layer not only promotes smoothness of the surface, but also achieves increased wettability, and hence favors cell spreading of the $\text{O}_2$-plasma treated Ti-30Nb-1Fe-1Hf alloy. In addition, the extent of cell spreading in Ti-30Nb-1Fe-1Hf alloy is similar to that of Ti-6Al-4V, whereas the cell adhesion on Ti-30Nb-1Fe-1Hf alloy is better than that of Ti-6Al-4V alloy.

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