**In vitro** evaluation of H$_2$O$_2$ hydrothermal treatment of aged titanium surface to enhance biofunctional activity

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Surface modification of titanium has been extensively investigated in implant science and technology in an effort to improve its osteoconductivity. The rate of protein adsorption on titanium surfaces is known to vary depending on the chemistry, structure, morphology, and titanium-specific biological aging of the surface. It is thus desirable to modify smooth titanium surfaces of mini-implants used as orthodontic anchors immediately prior to use. In this study, we have developed a simple surface modification of titanium alloy that improves its biofunctional activity. The surface of a Ti-6Al-4V disk was modified by applying 3% H$_2$O$_2$ hydrothermal treatment using an autoclave. A nanostructured porous network TiO$_2$ was observed on the treated surface. Treated surfaces exhibited higher hydrophilicity, protein adsorption, and cell proliferation than untreated surfaces. 3% H$_2$O$_2$ hydrothermal treatment is thought to provide biofunctional activity for aged titanium surface.

**Keywords:** Titanium alloy, Surface modification, Biofunctional activity, Hydrothermal treatment, Aging

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**INTRODUCTION**

Titanium and its alloys are generally considered to be bioinert materials$^3$. Surface modification techniques such as sandblasting, acid treatment, and hydroxyapatite coating have been applied to dental implants to modify their surface chemistry, roughness, and topology. Such treatments not only increase the total surface area for osseous apposition, they also promote osteoblastic cell adhesion, spreading, proliferation, and differentiation to enhance bone formation around dental implants$^2,5$.

Recently, mini-implants have been extensively used for orthodontic anchorage because they impose minimal anatomic limitations on placement, have lower medical costs, and enable simpler placement with less traumatic surgery than dental implants$^6-8$. However, surface modifications are rarely applied to mini-implants. One reason is that mini-implants are designed to be removed without fracture at the end of their clinical application$^9$. Consequently, mini-implants are generally made of commercial titanium alloys (e.g., Ti-6Al-4V) with smooth surfaces because they have superior mechanical properties to pure titanium. However, mini-implants have lower success rates than dental implants. Thus, the application of suitable surface treatments to titanium alloy could produce bioactive surfaces that have enhanced primary stabilization.

Furthermore, the bioactivity of titanium surface decreases with time during use due to biological aging of titanium. As a result, the osteoconductivity of titanium surfaces decreases with time$^{10,11}$. Surface composition, hydrophilicity, and roughness may affect protein adsorption, osteogenic cell adhesion, and implant-tissue interaction. An in vivo study reported significant differences in the bone-to-implant contact for hydrophilic and hydrophobic titanium surfaces during the early stages (2 and 4 weeks) of bone regeneration$^{12,13}$. However, mini-implants are also sold as storable medical devices and require sterilization before implantation. It is thus desirable to modify aged surfaces immediately prior to use.

In this study, we develop a simple hydrothermal oxidation surface treatment for titanium alloys using an autoclave for sterilization in a medical clinic. The objective of this study is to characterize the morphology of the treated surface and to examine how changes in the biofunctional activity of aged titanium surface affect its in-vitro behavior.

**MATERIALS AND METHODS**

**Specimen preparation**

Ti-6Al-4V disks with diameters of 15 mm and thicknesses of 1 mm (Toho Titanium Co. Ltd., Kanagawa, Japan) were used in this study. The disks were abraded with water-cooled 800-grit SiC paper using a grinding-sliding machine (MG4000-E, Exakt Apparatebau, Norderstedt, Germany). All the disks were ultrasonically cleaned in acetone, 100% ethanol, and distilled water for 15 min. After ultrasonic cleaning, all disks were stored at room temperature in the dark place for 1 month. One month after, the half disks were randomly selected as the control group. The remaining disks were hydrothermally treated with 3% H$_2$O$_2$ solution (Yamazen Co., Osaka, Japan) and autoclaved at 121°C and 0.2 MPa for 20 min (LBS-245, Tomy Seiko Co., Ltd., Tokyo, Japan). The treated disks
were then ultrasonically cleaned in distilled water and dried in an oven at 50°C for 1 h. The treated disks were used as the test group.

**Surface analysis**

The microstructures of the outermost surfaces layer of the disks were evaluated by scanning electron microscopy (SEM; S-4000, Hitachi, Tokyo, Japan). In addition, the morphologies of the surfaces were analyzed by scanning probe microscopy (SPM; SPM-9600, Shimadzu, Kyoto, Japan). The composition of the disk surface was characterized by X-ray photoelectron spectroscopy (XPS; ESCA 5600, Ulvac Phi., Inc., Kanagawa, Japan) using surface etching with ionized argon. XPS analysis was also used to analyze the surfaces of prepared Ti sensors, where the Al Kα line (15 kV, 300 W) was used as the X-ray source. Argon ion sputtering was performed during XPS to estimate the thickness and structure of the surface layers.

**Contact angle measurements**

A contact angle (CA) meter (Kyowa CA-A, Tokyo, Japan) was used to measure the CAs of the surfaces of all samples immediately after preparation and 3, 7, and 28 days after preparation. Drops of ultrapure water were dropped onto the specimen surfaces using a syringe whose drop size can be set.

**Protein adsorption**

Bovine serum albumin, cytochrome C (Sigma-Aldrich Co., Canada), was used as a model protein. The cytochrome C concentration of the supernatant was determined by the Bradford method (Bio-Rad protein assay kit, Bio-Rad Laboratories Inc., Tokyo, Japan). For this, 500 μL of protein solution (1 mg of protein per mL of DDW) was spread over the disk. After incubating for 24 or 72 h under sterile humidified conditions at 37°C, the solution containing non-adherent proteins was removed and mixed with citric acid buffer (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 60 min at room temperature. The amount of protein removed was quantified using a microplate reader. The total amount of protein initially applied was also quantified by this method. The rate of protein adsorption was calculated as the percentage of protein adsorbed on the disk surfaces relative to the total amount of protein initially applied.

**Cell culture and growth**

MC3T3-E1 osteoblast-like cells (Riken Cell Bank, Tsukuba, Japan) were seeded on 24-well culture plates at a density of 1×10⁴ cells per well and incubated in normal culture medium α-MEM with 10% FBS and 1% penicillin-streptomycin (Wako Pure Chemical Industries Ltd.). The culture medium was changed every 4 days. The cells were incubated at 37°C in a 5% CO₂ environment.

**Cell adhesion**

After culturing for 1 or 3 h, the disks were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) at room temperature for 20 min, permeabilized in a PBS/Triton X-100 buffer (Sigma-Aldrich Ltd.) 10 mM PBS, 0.2% Triton for 30 min, and then blocked with Blocking One Histo (Nacalai Tesque Inc., Kyoto, Japan). The samples were then labeled with DAPI and rhodamine-phalloidin (Life Technologies Co., Tokyo, Japan) for 60 min. After being washed in the buffer solution, all the specimens were embedded in PBS/glycerol (Life Technologies Co.) mounting medium and examined using an Olympus U-LH100HG epifluorescence microscope (Olympus Co., Tokyo, Japan).

**Cell proliferation assay**

After cells had attached to the disks, they were cultured for 1, 4, and 7 days. The relative number of viable cells in each well at each experimental time point was determined using the cell proliferation reagent (CellTiter96®, Promega Co., Madison, WI, USA). Briefly, 100 μL of reagent was added to each well, including three wells that contained only medium for background subtraction. After incubation at 37°C for 15 min, the absorbance at 490 nm was measured using a microplate reader.

**Statistical analysis**

Data obtained by CA measurements were analyzed by the Tukey HSD test. Data on protein adsorption and cell proliferation were analyzed by the Student’s t-test. The level of statistical significance was set to p<0.01.

**RESULTS**

**Surface analysis**

Figures 1(a) and (b) respectively show SEM images of a machine-ground disk (control disk) and a 3% H₂O₂ hydrothermally treated disk (test disk). SEM observations of the surface structures on the disks revealed significant differences before and after hydrothermal treatment. The surface of the control disk exhibited a smooth line pattern, whereas the test disk exhibited a relatively irregular surface with a porous network structure. Figure 2 shows SPM images showing the nanoscale morphological changes induced on the surface by the treatment. Many irregular pits and indentations of various sizes were observed on the test surface. The surface roughnesses (Ra) of the control and test groups were measured by SPM to be 14.132 and 22.315 nm, respectively. The compositions of the surface layers of the control and test disks were characterized by XPS. A wide-scan spectrum of TiO₂ indicates the presence of Ti, O, C, and N (data not shown). The O 1s peak has two components; the peak at 531.4 eV was attributed to TiO₂, while the peak at 534.1 eV was fitted and assigned to absorbed H₂O (Fig. 3(a)). The dominant doublet component in the Ti 2p peaks of the control group corresponds to Ti 2p3/2 and Ti 2p1/2 with binding energies (BEs) of 465.8 and 460.0 eV, respectively. The
Fig. 1  SEM images of titanium alloy surfaces. (a) control and (b) test disks.

Fig. 2  SPM images of surface morphologies before and after treatment (2×2 μm). (a) control and (b) test disks.

Fig. 3  XPS spectra of surface compositions before and after treatment. (a) control disk high-resolution XPS spectra around O 1s; (b) control disk high-resolution XPS spectra around Ti 2p; (c) test disk high-resolution XPS spectra around O 1s; (d) test disk high-resolution XPS spectra around Ti 2p.
lowest energy peak was assigned to metallic Ti with an observed BE for Ti 2p3/2 of 455.2 eV (Fig. 3(b)). Compared with the test group, the O 1s peak has two components; the peak at 532.4 eV was attributed to TiOH, while the peak at 534.5 eV was fitted and assigned to absorbed H$_2$O (Fig. 3(c)). These peaks correspond to Ti 2p3/2 and Ti 2p1/2 with BEs of 460.8 and 466.6 eV, respectively (Fig. 3(d)).

Figure 4 shows XPS depth profiles of the control and test disks. In the control group, 22.6 at% oxygen was present on the surface; this is attributed to a native oxide layer on the titanium surface. The oxygen concentration decreased dramatically after the initial etching cycles. At depths greater than about 10 nm (evaporation rate: 5 nm/min), the titanium and oxygen concentrations remained almost constant at about 88 and 1 at%, respectively, until the Ti substrate interface was reached (Fig. 4(a)). In the test group, 58.6 at% oxygen was present on the surface; this was produced by H$_2$O$_2$ autoclave treatment. At a depth of about 90 nm, the concentration of titanium exceeds that of oxygen (Fig. 4(b)).

**Contact angle measurements**

The CA of H$_2$O increased with the age of titanium (Figs. 5 and 6). The CAs of both groups increased with time. The surfaces of the control group were initially hydrophilic (CA<50°), but they became hydrophobic (CA>80°) after storing for 4 weeks. On the other hand, the high hydrophilicity (CA<20°) of the fresh surfaces of the test group became hydrophobic (over 40°) after storing for 4 weeks. The test group exhibited a high hydrophilicity and it had a lower CA than the control group at each time point (n=5, p<0.01).

**Protein adsorption**

Adsorption of cytochrome C was examined under acidic conditions (pH=5.43±0.02) (Fig. 7). There were significant differences between the control and test group surfaces after 24 and 72 h of incubation (n=5, p<0.01). After 24 h of incubation, the surfaces of the test group adsorbed six times more cytochrome C than those of the control group.

**Cell adhesion**

The morphology of the adherent cells was evaluated by
Fig. 6  Age-dependent reduction of hydrophilicity and water CA on the surface before and after treatment ($n=5$).

*, $p<0.01$; significant increase between control and test group at each time point

*, $p<0.01$; significant increase between 0 and 7, 28 days in test group

*, $p<0.01$; significant increase between 0, 3 and 7, 28 days in control group

Fig. 7  Cytochrome C adsorption on titanium surface after incubation for 24 and 72 h ($n=5$).

*, $p<0.01$; significant increase between control and test group

Fig. 8  Phalloidin staining of actin cytoskeleton after MC3T3-E1 cells adhered to surface ($\times2,500$).

(a) control surface after culturing for 1 h; (b) control surface after culturing for 3 h; (c) test surface after culturing for 1 h; (d) test surface after culturing for 3 h.

Phalloidin staining to visualize the actin cytoskeleton. Test surfaces exhibited large-scale nuclear and numerical increase in cell projection compared with control surface 3 h after seeding (Figs. 8(a) and (b)).

Cell proliferation

Cell proliferation increased in a similar manner for both groups from day 1 to day 3, whereas the test group had a significantly higher cell proliferation than the control.
The test group is thought to have a 5-nm-thick oxide film. However, the bioactivity of the titanium surface (i.e., the protein adsorption capacity) decreases over time due to biological aging of titanium\(^{10,11}\).

The protein adsorption rate of titanium is correlated with the water CA, which suggests that the hydrophilicity of titanium greatly affects its protein adsorption ability\(^{12}\). In this study, the contact angle of H\(_2\)O increased with the age of titanium. The CAs of the control and test groups varied in the hydrophilic range 49–16° and the hydrophilicity of the surfaces increased after treatment in conjunction with the formation of TiO\(_2\) nanostructures. Thus, growth of the titanium oxide layer increases the surface energy, which makes the surface more hydrophilic\(^{21}\). Four-week-old titanium disks had far lower protein adsorption levels (for incubation times of 24 and 72 h) than fresh surfaces. This implies that an initial difference in the biological potential may become permanent and that it determines the subsequent biofunctional activity of titanium, which may influence osseointegration. It should be noted that the reduction in protein adsorption between the fresh and 4-week-old surfaces was greater for the test surfaces than for the control surfaces. Hori et al. have also reported the age-dependent degradation of the protein adsorption capacity of titanium\(^{11}\).

Cell adhesion is an important parameter for evaluating whether implants are suitable for medical use\(^{22}\). Thus, a stable connection between the biomaterial surface and surrounding tissue is an important prerequisite for the long-term success of implants. Consequently, strong cellular adhesion to the biomaterial surface is required. The quality of the connection between cells and the biomaterial is partially determined by the dimensions of surface topography. The cell adhesion strength mainly determines the in-vitro cyto compatibility of a surface and it may affect the in-vivo biocompatibility\(^{20}\). Cells initially adhere to implant surfaces by attaching either to a preadsorbed protein network known as the extracellular matrix (ECM) or to neighboring cells. The ECM affects cell survival, proliferation, and apoptosis by integrin-dependent activation of intracellular signaling mediators\(^{22}\).

The micro and nano scale surface topography are thought to significantly affect cell responses and activities in the establishment of osseointegration\(^{15-17}\). Furthermore, surface physicochemistry greatly affects the cell behavior on titanium surfaces\(^{15}\). The rate of protein adsorption on implant surfaces is known to differ depending on the chemistry\(^{15}\), structure\(^{19}\), and morphology\(^{20}\) of implant surfaces. Therefore, surface modification of titanium has been a primary focus of implant science and technology in an effort to improve its osteoconductivity\(^{11}\).

In this study, the surface roughnesses of the surfaces of the two groups differ significantly since the surface roughness strongly depends on the surface treatment. SEM and SPM images reveal that the control and test groups have significantly different surfaces. The control and test groups have roughnesses (Ra) of 14.132 and 22.315 nm, respectively. XPS was used to determine the chemical compositions of the sample surfaces, the oxidation state of titanium in titanium oxide layers, and the elemental distributions as functions of depth. The XPS analysis results reveal the formation of highly ordered TiO\(_2\) nanostructures on the test group, whereas the surface of control group was native TiO\(_2\). Comparison of the XPS data for the control and test groups for Ti 2p reveals that metallic titanium was detected from the XPS data for Ti 2p3/2 and Ti 2p1/2 in only the control group. The test group is thought to have a 5-nm-thick oxide film and no metallic Ti was detected in it. Comparison of the O 1s peaks reveals different TiO\(_2\) structures in the two groups: naturally generated TiO\(_2\) and TiO\(_2\) produced by H\(_2\)O\(_2\) autoclave treatment are considered to be different. In the test group, the concentration of titanium exceeds that of oxygen at a depth of about 90 nm. Thus, the H\(_2\)O\(_2\) autoclave treatment is thought to produce a thick oxide film. However, the bioactivity of the titanium surface (i.e., the protein adsorption capacity) decreases over time due to biological aging of titanium\(^{10,11}\).

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On the other hand, cell adhesion seems to be determined by wettability as well as surface topography. Surface hydrophilicity increases adsorption of ECM proteins, which results in enhanced cell responses such as osteoblastic cell attachment and differentiation relative to hydrophobic titanium surfaces\(^{23-20}\). We conjecture that MC3T3-E1 cells have greater compatibility for TiO\(_2\) nanostructures than native TiO\(_2\) surfaces due to the greater hydrophilicity (CA=16°) of TiO\(_2\) nanostructures than moderately hydrophilic (CA=49°) native TiO\(_2\) surfaces. Thus, 3% H\(_2\)O\(_2\) autoclave treatment provided a biofunctional activity surface with a moderate surface roughness in the form of a nanoporous TiO\(_2\) layer; this may contribute to the significantly enhanced hydrophilicity, protein adsorption, and proliferation of MC3T3-E1. In other words, the surfaces of treated titanium mini-implants may enhance cellular adhesion, which leads to biofunctional activity.
CONCLUSION
This present study demonstrated that surface modification of Ti-6Al-4V disks by 3% \( \text{H}_2\text{O}_2 \) hydrothermal treatment using autoclave is an easy surface modification method that forms a nanostructured TiO\(_2\) film. Moreover, this treatment reduces the degradation of the hydrophilicity and protein adsorption of titanium surfaces due to aging after surface treatment. Therefore, the 3% \( \text{H}_2\text{O}_2 \) hydrothermal treatment is thought to provide biofunctional activity to aged titanium surfaces.

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