Change in surface properties of zirconia and initial attachment of osteoblast-like cells with hydrophilic treatment

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The objectives of this study were to characterize change in surface properties of tetragonal zirconia polycrystals (TZP) after hydrophilic treatment, and to determine the effect of such changes on initial attachment of osteoblast-like cells. Roughened surfaces were produced by alumina-blasting and acid-etching. Hydrophilic treatment comprised application of immediately after blasting and acid-etching (Blast/Etch), oxygen plasma (O2-Plasma), ultraviolet light (UV). Specimens stored in air were used as a control. The water contact angle was determined and surface analysis was performed using an X-ray photoelectron spectroscopy. Blast/Etch, O2-Plasma and UV specimens showed superhydrophilicity, and these hydrophilic treatments to TZP elicited a marked decrease in carbon content and an increase in hydroxyl groups. Hydrophilic treatments enhanced initial attachment of osteoblast-like cells and a change in cell morphologies. These results indicate that Blast/Etch, O2-Plasma, or UV treatment has potential in the creation and maintenance of superhydrophilic surfaces and enhancing initial attachment of osteoblast-like cells.

Keywords: Tetragonal zirconia polycrystals (TZP), Surface wettability, Oxygen-plasma treatment, UV treatment, Initial attachment of osteoblasts

INTRODUCTION

Zirconia ceramics have drawn attention as a potential alternative to titanium (Ti), as they are not prone to graying or hypersensitivity1,3. In dentistry, tetragonal zirconia polycrystals (TZP), in particular, have shown outstanding performance in terms of mechanics, biocompatibility, and esthetics. With a flexural strength of more than 900 MPa, fracture toughness of up to 10 MPa/m0.5, and an elastic modulus of 210 GPa, they exhibit better mechanical performance, superior strength and fracture resistance than do other ceramic materials4,5. Therefore, TZP may have potential as an alternative to Ti at the implant-bone interface. However, so far, TZP have mainly been applied to the frameworks for fixed prostheses and implant abutments, and little is known about how they might be used in the implant body (fixture) itself.

Initial attachment, proliferation and differentiation of osteoblasts at the implant-bone interface play an important role in the early stages of osseointegration. In general, these cell behaviors are influenced by both the surface topography and physicochemistry of the material to which they attach6. Surface wettability is an important physicochemical property on protein adsorption and subsequent cell behavior. High surface wettability, that is high surface energy, is generally reported to promote greater cell adhesion than low surface energy7. Many in vitro studies have investigated the relationship between the hydrophilicity of a material surface and cell adhesion8-12.

Cold plasma treatment including glow discharge10,13,14 and ultraviolet light irradiation15 have been proposed as means of modifying hydrophilicity. Surface modification with cold plasma is an effective and economical way of enhancing the surface wettability due to by removal of hydrocarbon and introducing hydroxyl groups. Several studies have reported changes in surface biocompatibility in terms of cell attachment and protein adsorption with plasma treatment10,13,14,16,17. Glow discharge plasma treatment of titanium plates enhanced both hydrophilicity and initial attachment and differentiation of osteoblast-like cells16. Earlier studies confirmed that the greater the hydrophilicity of the surface, the greater the level of early-stage cell adherence, especially on superhydrophilic surfaces. Cells spread much more widely on hydrophilic surfaces than on hydrophobic surfaces10,13.

Ultraviolet light-induced superhydrophilicity of TiO2 was discovered in 199718. In this procedure, UV irradiation creates surface oxygen vacancies at bridging oxygen sites. Wetting results from alteration of surface chemistry by removal of hydrocarbon due to the photocatalytic activity of TiO219,20. Another semiconducting photocatalyst, ZrO2, also creates superhydrophilicity21,22. In addition to the surface treatments described above, chemical treatment such as acid etching also enhances the hydrophilicity of TZP surfaces by eliminating adsorbed impurities such as hydrocarbon from the atmosphere23.
Although various surface modification technologies are available for the enhancement of the hydrophilicity of Ti, such techniques are still in the developmental stage for TZP. It is believed that TZP are inherently bio-inert. Therefore, the surface physicochemical modification of TZP ceramics, especially that which would increase hydrophilicity, might enhance osteogenesis on TZP implants.

This study had two objectives: to 1) characterize change in surface properties of TZP after hydrophilic treatment; and 2) determine the effect of such changes on initial attachment of osteoblast-like cells.

MATERIALS AND METHODS
Sample preparation and surface treatment
Tetragonal zirconia polycrystals (TZP, TZ-3YB-E, Tosoh, Tokyo, Japan) were used in this study. All TZP disks (13 mm in diameter, 0.5 mm in thickness) prepared using a cutting machine were perpendicularly sand-blasted from a distance of 10 mm with 150-μm Al₂O₃ at 0.4 MPa air pressure. After sandblasting, the disks were etched with hydrofluoric acid (46%, Wako Pure Chemicals, Tokyo, Japan) for 15 min. The specimens were then cleaned ultrasonically using ethyl alcohol and distilled water, both for 15 min each (Fig.1-a). The resultant surface morphology of roughened specimens had a nano-structure created by acid-etching on an uneven surface created by large-grid blasting (Fig.1-b), and surface roughness (Ra), as measured with Handysurf E-30A (Tokyo Seimitsu, Tokyo, Japan), with a scale length of 4 mm and a cut of value of 0.8 mm, was 1.52±0.09 μm.

The roughened specimens were subjected to various types of physicochemical treatment that were not altered the surface topography, as shown in Table 1. As a control, some specimens were stored in air for 2 weeks (Air). Some specimens were stored in distilled water immediately after blasting and acid-etching for 1 day (Blast/Etch). Oxygen plasma treatment was carried out using a plasma-surface modification apparatus.
(VEP-1000, ULVAC, Kanagawa, Japan). Briefly, the specimens were introduced into the chamber of the apparatus and exposed to low-energy oxygen plasma treatment (200W, 1.5 Pa, gas flow rate 50 sccm) at room temperature for 10 min (O2-Plasma). Ultraviolet treatment was performed using a UV ozone cleaner (PC440, Bioforce Nanosciences, Sweden) for 2 h (UV). This equipment creates UV radiation with a total power of 19 mW/cm², and excitation wavelengths of 185 and 254 corresponding to ultraviolet C (UV-C), and 365 nm corresponding to ultraviolet A (UV-A).

The Air specimens were sterilized in an autoclave for 10 min at 121°C before cell culture. The Blast/Etch specimens were immersed in distilled water and sterilized in the same way as the Air specimens. Specimens prepared by plasma and UV treatment also underwent sterilization, the former by exposure to a high-vacuum atmosphere and the latter by UV sterilization.

Surface characterization
1. Surface wettability
The surface wettability of the samples was determined by contact angle measurement using double-distilled water and a contact angle meter (Phoenix α, Meiwaforsces, Tokyo, Japan). Measurements were made at 3 different locations on the sample at 3 s after application of the droplet. The distilled water contact angles at the 3 different locations on the zirconia surface were averaged. The volume of the drop was maintained at 4 μL (n=5).

2. XPS analysis
Surface analysis after physicochemical treatment was performed using an X-ray photoelectron spectroscopy (XPS; Axis-Ultra, Kratos Analytical, UK) equipped with a monochromatized AlKα X-ray source operated at 15 kV and 15 mA. Briefly, the specimen surface was irradiated with an X-ray beam, inducing ejection of electrons from the atom. The kinetic energy of the emitted photoelectrons was analyzed and their binding energy determined. Since the binding energy of electrons in the atom of origin is characteristic of the element and affected by its chemical environment, this method provides an elemental analysis and further information on functional groups. The binding energy scale for each spectrum was calibrated against the C1s peak at 285.0 eV.

Cell culture
Mouse osteoblast-like cells MC3T3-E1 (RCB1126, RIKEN Bio Resource Center, Saitama, Japan) were maintained in α-modified minimum essential medium (Invitrogen, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (PBS; Invitrogen, Gaithersburg, MD, USA). Cells were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cells were seeded into individual wells at a density of 1×10^5/cm² on TZP disks to assay initial attachment and cell morphology under each type of physicochemical modification.

Cell attachment ability
The initial attachment ability of the cells was evaluated by measuring the quantity of living cells attached to the TZP disks after 3, 6 or 12 h incubation. The supernatant of the cultured MC3T3-E1 was removed and the remaining cells washed with PBS solution. Initial attachment of cells was determined using WST-1 based colorimetry (WST-1, Roche Applied Science, Mannheim, Germany). The culture well was incubated at 37°C for 1 h with 50 mL tetrazolium salt reagent. The amount of formazan product was measured using the SpectraMax M5 (Molecular Devices, Sunnyvale, CA) at 420 nm (n=15).

Cell morphology
After 3, 6 or 12 h culture, the osteoblasts were washed twice in PBS, fixed for 30 min in 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemicals, Tokyo, Japan), and permeabilized in 1% TritonX-100 in PBS for 5 min. Cells were then incubated in PBS containing 3% BSA for 30 min to block specific epitopes. Subsequently, TZP disks were washed 3 times in PBS, after which they were dyed with DAPI (1:200, nuclei blue color, Invitrogen, Gaithersburg, MD, USA) and Phalloidin (1:100, Alexa Fluor488, actin filament green color, Invitrogen, Gaithersburg, MD, USA) for 30 min. Confocal laser scanning microscopy (LSM5DUO, Carl Zeiss, Oberkochen, Germany) was used to examine cell morphology and cytoskeletal arrangement. Areas and perimeters of actin were also quantified using an image analyzer (ImageJ, NIH, Bethesda, MD, USA) at 10 randomly selected areas per each of 5 disks.

Statistical analysis
Statistical significance of data was assessed by an analysis of variance (ANOVA), followed by Fisher’s protected least significant difference method for multiple comparisons between pairs at p=0.05.

<table>
<thead>
<tr>
<th>Code</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>Air (control)</td>
<td>Stored in air for 2 weeks (Control)</td>
</tr>
<tr>
<td>Blast/Etch</td>
<td>Blasted and acid-etched</td>
</tr>
<tr>
<td>O2-Plasma</td>
<td>Treated with oxygen-plasma (200W, 1.5Pa) for 10 min</td>
</tr>
<tr>
<td>UV</td>
<td>Treated with ultraviolet radiation (19 mW/cm², λ=185, 254, 365 nm) for 2 h</td>
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RESULTS

Surface wettability
A cross-sectional view of a water droplet on each TZP specimen after physicochemical surface treatment and contact angle are shown in Fig. 2. The Air (control) specimen had a large contact angle of $78.7 \pm 8.3^\circ$. Contact angle showed a dramatic decrease with physicochemical surface treatment, at $1.3 \pm 0.8^\circ$ on the Blast/Etch specimen and $0^\circ$ on the O2-Plasma and UV specimens. These specimens showed superhydrophilicity, in contrast to the Air specimen.

XPS analysis
Carbon content on the outermost surface of the TZP disks under XPS analysis is shown in Fig. 3. Carbon content showed a remarkable decrease in the Blast/Etch, O2-Plasma and UV specimens compared to the Air specimens. No significant differences were observed in carbon content among the Blast/Etch, O2-Plasma and UV specimens.

The O1s spectra on the outermost surface of the TZP disks with different surface treatments are given in Fig. 4-a, which shows an O1s peak at around 530.2 eV for ZrO$_2$, 531.5 eV for OH(a) and 532.5 eV for OH(b). Here, OH(a) and OH(b) indicate acidic hydroxyl and basic hydroxyl groups, respectively$^{23,24}$. Figure 4-b shows the percentage area of OH(a) and OH(b) of the typical O1s spectrum. The percentage area of both OH(a) and OH(b) increased on Blast/Etch, O2-Plasma and UV specimens compared to on Air specimens. No significant differences were observed in amount of hydroxyl groups among the Blast/Etch, O2-Plasma and UV specimens.

Cell attachment ability
Figure 5 shows cell attachment on the TZP disks after 3, 6 or 12 h incubation. Cell attachment increased at different speeds depending on the type of TZP surface. The results of the two-way ANOVA revealed significant differences with regard to both factors (surface treatment and cell culture time). Cell attachment increased with increasing cell culture time. Cell attachment in the hydrophilic surface groups (Blast/Etch, O2-plasma, UV) was greater than that in the Air group. After 3, 6 or 12 h incubation, cell attachment in the Blast/Etch specimens was greater than that in the other groups.

Cell morphology
Confocal laser scanning microscopy images of cell morphology at 12 h cultivation with dual staining with DAPI for nuclei (blue) and phalloidin for actin filaments
Actin filaments extended with time in all specimens, and cell morphology changed from round to flat by development of actin fibers. Extension of filopodia was observed on the Blast/Etch and O2-Plasma surfaces, but there were relatively fewer filopodia extending on the Air and UV surfaces.

The cytomorphometric parameters of cell area (μm²/cell) and perimeter (μm/cell) are shown in Fig. 7 and Fig. 8, respectively. On the cell area, the results of the two-way ANOVA revealed significant differences regarding both factors (surface treatment and cell culture time). Cell attachment increased with increasing cell culture time. After 3 h incubation, cell area showed no significant differences among all specimens. After 6 h incubation, cell area in the Blast/Etch specimens was significantly larger than that in the Air specimens. After 12 h incubation, cell area in the Blast/Etch, O2-Plasma and UV specimens was significantly larger than that in the Air specimens.

On the perimeter, the results of the two-way ANOVA revealed significant differences regarding both factors (surface treatment and cell culture time). The perimeter increased with increasing cell culture time. After 3 h incubation, a significant difference was observed in perimeter between the Blast/Etch and UV specimens. After 6 h incubation, no significant difference was observed in perimeter among all specimens. After 12 h incubation, the perimeter in the Blast/Etch, O2-Plasma and UV specimens was significantly larger than that in the Air specimens.

Roughened surfaces, which were blasted and acid-etched, were prepared for surface characterization and cell attachment assay. Numerous experimental reports from in vitro and in vivo studies have pointed to a more rapid bone response to roughened surfaces than to smoother polished or turned surfaces

In general, titanium constantly adsorbs organic impurities such as hydrocarbons from the atmosphere. This leads to an increase in hydrophobicity, which is referred to as an aging phenomenon. It is also reported that the atomic percentage of carbon reaches 50 at% in hydrophobic titanium surfaces stored in air atmosphere. In this study, contact angle in the Blast/Etch specimens, which were stored in distilled water immediately after blast and acid-etching, dramatically decreased to around 1°, indicating superhydrophilicity, whereas in the Air specimens, which were stored in air for 2 weeks, a large contact angle of 79° was observed. This apparent

![Fig. 4 O1s spectra on outermost surface of TZP disks.](image)
a) Typical O1s spectrum. b) Percentage areas of OH(a) and OH(b) in O1s spectra.

Identical letter shows no significant difference.

![Fig. 5 Cell attachment on TZP surface after 3, 6, or 12 h cultivation.](image)

Identical letter shows no significant difference on each culture time.
Fig. 7 Cell area ($\mu$m$^2$/cell) on TZP surface after 3, 6, or 12 h cultivation. Identical letter shows no significant difference on each culture time.

Fig. 8 Perimeter of cell ($\mu$m/cell) on TZP surface after 3, 6, or 12 h cultivation. Identical letter shows no significant difference on each culture time.
superhydrophilicity in the Blast/Etch specimens may have resulted from storage in distilled water immediately after surface preparation and a large surface area due to blast and acid-etching. First, acid-etching may clean the surface, and if immediately followed by storage in distilled water, may prevent adsorption of hydrocarbon and also enhance the formation of hydroxyl groups. This phenomenon was confirmed by XPS analyses (Figs. 3 and 4). Second, superhydrophilicity may have been enhanced by the large surface area created by roughening. This phenomenon has been described in the Wenzel model\(^{30}\), which predicts that the contact angle on a flat surface, which is \(\theta<90^\circ\), will decrease if the surface is roughened. That is, \(\cos \theta_w = r \cos \theta\) where \(\theta\) is the contact angle on a flat surface of the same nature, \(\theta_w\) is the apparent contact angle, and \(r\) is the surface roughness factor defined as the ratio of the actual wetted surface over the surface as measured on the plane of the interface (in general \(r>1\), and \(r=1\) for flat surfaces). In the present study, the Blast/Etch specimens had a nano-structure created by acid-etching on an uneven surface created by large-grid blasting (Fig. 1). The resulting increase in surface area appears to have enhanced superhydrophilicity.

The TZP surfaces treated with O2-Plasma showed superhydrophilicity. Cold plasma-surface modification with various gases generated in a high-voltage electric field at a low pressure is suitable for change in surface physicochemistry\(^{26}\). Wei et al. showed that plasma polymerization with hexamethyldisiloxane followed by oxygen plasma treatment modified surfaces\(^{13}\). The water contact angle of sample surfaces varied from 106° (hydrophobicity) to almost 0° (superhydrophilicity). Oxygen-functional groups were introduced on polymer substrates by oxygen plasma treatment. On titanium, Oxygen-functional groups were introduced on polymer substrates. Wei et al. investigated the influence of surface wettability on competitive protein adsorption and initial attachment of osteoblasts\(^{16}\). They demonstrated that initial attachment of osteoblastic cells increased with increase in surface wettability, which correlated well with Fn adsorption in the competitive mode. Thus, in a culture medium including FBS, Fn is prone to adsorb preferentially on hydrophilic surfaces, resulting in high cell attachment, whereas Alb preferentially adsorbs on hydrophobic surfaces, interfering with cell attachment. Accordingly, in the present study, Fn adsorption may have been responsible for the observed increase in cell adhesion on superhydrophilic TZP surfaces in culture media.

In addition to amount of protein adsorbed, protein conformation also affects the function of attached adhesive molecules. Adsorbed Fn shows two different conformations according to surface wettability. Antibodies tend to bind to a much greater degree to proteins with a more active conformation on a hydrophilic surface than they do to those on a hydrophobic surface. It has been suggested that an adhesive sequence such as Arg-Gly-Asp is in an active state on hydrophilic surfaces\(^{30}\). The adsorption behavior, particularly in competitive mode, of proteins on TZP surfaces with different wettability should be clarified in future study.

In the present study, the shape of the attached cells was affected by surface wettability. Cells on the hydrophilic surfaces were enlarged, with pronounced...
lamellipodia-like actin projections and a cytoskeleton within the cytoplasm; whereas the majority of cells on the Air specimens were rounded and showed no elongation of cell processes or development of cytoskeleton. When the area/perimeter ratio increased, the cell became rounder. In contrast, when the area/perimeter ratio decreased, the cell adopted a spindle-like shape. Lim et al. examined differences in cytoskeletal features of hFOB (human fetal osteoblast cell) cultured on surfaces with different surface energies by actin/integrin immunofluorescence staining. They demonstrated remarkable morphological differences between cells on hydrophilic and hydrophobic substrates at equivalent times. Cells cultured on hydrophilic surfaces treated with plasma displayed distinct, large plaques of integrins co-localized with actin stress fibers, whereas there was much less development of these adhesion structures on hydrophobic surfaces. It is clear that signaling transduction starts from integrin adhesion to the extracellular matrix, finally reaching F-actin through cytoskeletal molecules such as tensin, vinculin, talin and α-actin. The enhanced osteoblast adhesion to the hydrophilic surfaces may be associated with enhanced expression of vinculin. At 12 h incubation in this study, an interconnected morphology was observed on all surfaces, particularly those which were hydrophilic. These phenomena indicate accelerated cell proliferation on hydrophilic surfaces.

Durability of surface wettability is important for practical application in the clinic. In the present study, the surfaces that were stored in distilled water for 1 day at immediately after each type of treatment showed hydrophilicity. Thus, the surface wettability can be sustainable by controlling the immersion condition with aqueous solution. These hydrophilic surfaces may enhance initial cell attachment, indicating that this method of storage prevents the so-called aging phenomenon and ensures maintenance of high cell adhesion capability.

The findings in this study suggest that surface wettability should be taken into account in the design of new biomaterial surfaces, especially those for orthopedic TZP implants. Furthermore, the present results indicate that storage in an aqueous solution at immediately after surface modification creates and maintains superhydrophilicity and enhances initial cell attachment.

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REFERENCES


