INTRODUCTION

Zirconia has recently become more widely used in dental materials, and is indispensable for ceramic restorations because of its good chemical and dimensional stability, mechanical strength, and toughness. Implant fixtures are among the many applications of zirconia\(^1\)\(^2\). Although titanium is the most popular material for dental implants and its use has been well documented over the long term\(^3\)\(^-\)\(^7\), its applications in this regard are hindered by reports of titanium allergies and its unnatural greyish colour. Several studies have found that titanium can induce an allergic reaction and cellular sensitization\(^8\)\(^-\)\(^11\). Moreover, thin peri-implant soft tissues can take on the greyish colour of the titanium implant\(^12\). Zirconia implants have therefore been developed as an alternative.

Calcium is known to favour osteoblast proliferation\(^13\) and improve the osseointegration of titanium implants effectively\(^14\)\(^-\)\(^19\). Calcium ions are typically incorporated into titanium by soaking the metal in a calcium solution\(^20\)\(^-\)\(^22\). This is because it is difficult to apply heat treatment to the titanium surface without oxidizing it and thus decreasing its mechanical strength. The calcium ion is also known to stabilize zirconia\(^23\), and can be incorporated into zirconia via heat treatment. Excess calcium, however, may result in the formation of cubic zirconia that displays lower mechanical strength\(^24\). Therefore, the amount of incorporated calcium is quite important to obtaining an optimally bioactive zirconia surface. In the present study, we developed a novel method of incorporating calcium ions into zirconia to improve its biocompatibility. We characterized the surface properties of the modified zirconia, and performed cell proliferation tests to evaluate the effect of modification on the surface biocompatibility.

MATERIALS AND METHODS

Materials

Two commercially available types of zirconia, yttria-tetragonal zirconia polycrystal (Cercon, DeguDent, Hanau, Germany) and ceria-stabilized zirconia/alumina nanocomposite (P-NANOZR, Panasonic Healthcare, Ehime, Japan), were used in this study (Table 1). Presintered blocks were cut into the appropriate sizes for each experiment, and sintered according to the manufacturers’ recommendations.

Characterization of modified surface

1. Sample preparation

After sintering, the zirconia surface was sandblasted with 70 µm alumina powder at 0.4 MPa, with the sand spray directed against the surface from a distance of 10 mm. These specimens were then ultrasonically cleaned for 10 min in acetone, ethyl alcohol, and distilled water, and dried in air. After the ultrasonic cleaning and drying, calcium acetate solution (0.5 mol/L) was painted onto the surface. The specimens were then fired at 1,000, 1,100, 1,200, and 1,350°C for 30 min (hereinafter abbreviated as Ca-incorporation) (Fig. 1).

2. X-ray diffractometry

The prepared samples were characterized using X-ray diffractometry (XRD; Ultima IV, Rigaku, Japan). The diffractometer was operated at a tube voltage of 40 kV and tube current of 30 mA. The data were recorded over the 2θ range 20° to 80° at a rate of 2°/min.

3. Mechanical properties

The three-point flexural strength test and Vickers hardness test were conducted to investigate the effect of calcium ion incorporation on the mechanical properties of zirconia. The specimens (20 mm long, 4 mm wide, 0.8 mm thickness) were subjected to the three-point strength test using a mechanical testing machine (Model 4481,
Table 1  Materials used in this study

| Product name (Manufacturer) | Composition | Final sintering |
|-----------------------------|-------------|-----------------|
| Cercon (DeguDent)           | 3 mol Y₂O₃-ZrO₂ | 1,350°C, 2 h     |
| P-NANOZR (Panasonic Healthcare) | 10 mol CeO₂-ZrO₂ | 1,450°C, 2 h     |

Instron Corporation, Canton, MA, USA) (n=8). The crosshead speed was 0.5 mm/min and the span length was 10 mm. The flexural strength (Fs) was calculated using the formula:

\[ Fs = \frac{3Pm}{2bh^2} \]

where \( Pm \) (kgf) is the maximum load, \( l \) (mm) is the span length, \( b \) (mm) is the width of the test specimen, and \( h \) (mm) is the thickness of the test specimen.

The Vickers hardness (HV) was measured with a micro-hardness tester (HM-101, Akashi Corp., Kanagawa, Japan). A load of 1 kg was applied for 15 s, and both indentation diagonals (mm) were measured (n=8). The Vickers hardness number was determined using the equation:

\[ HV = 0.1891 \frac{F}{d^2} \]

where \( F \) (N) is the indentation load and \( d \) (mm) is the arithmetic mean of two diagonals.

4. Field emission electron probe microanalysis

Concentration profiles were measured perpendicular to the interface using a field emission electron probe microanalyser (FE-EPMA; JXA-8530F, JEOL Ltd., Tokyo, Japan). Cross-sections perpendicular to the interface were taken and polished. To decrease the possibility of the formation of artifacts by the preparation process the specimens were polished using Ar-ion etching at an accelerating voltage of 5 kV (Cross Section Polisher SM-09010, JEOL Ltd., Tokyo, Japan). Subsequently, a thin layer of carbon was vaporized on to surface to make it conductive. The probe was operated at an accelerating voltage of 15 kV and a probe current of 50 nA, and was focused to produce an electron beam with a diameter of 0.1 µm on the specimen surface. \( \theta/2 \) Scans were performed every 10 ms, with a step size of 0.1 µm.

Evaluation of biocompatibility

1. Sample preparation

Specimens (15 mm in diameter, 1.3 mm thickness) were prepared and subjected to one of the following treatments: polished (hereinafter abbreviated as Pol), sandblasted (hereinafter abbreviated as Sa), subjected to Ca-incorporation at 1,100°C (hereinafter abbreviated as Ca-inc), and soaking in a simulated body fluid (SBF) at 37°C for 1 week after being modified by Ca-incorporation at 1,100°C (hereinafter abbreviated as Ca-incSBF). The Pol were first ground on both sides with diamond disks (35 µm) using a polishing machine (Ecomet3, Buehler, USA). Next, they were finished with a polishing cloth, using 9 µm and 3 µm diamond particles and 0.6 µm colloidal silica as abrasives. The Sa were sandblasted with 70 µm alumina powder at 0.4 MPa. The SBF was prepared using reagent-grade chemicals to a final composition of 137.8 mmol/L of NaCl, 4.2 mmol/L of NaHCO₃, 3.0 mmol/L of KCl, 1.0 mmol/L of K₂HPO₄, 1.5 mmol/L of MgCl₂·6H₂O, and 2.5 mmol/L of CaCl₂·2H₂O in ultrapure water and buffered at pH 7.2 with tris(hydroxymethyl)-aminomethane [(CH₂OH)₃CNH₂] and 1.0 mol/L HCl.

2. SEM observation

The surfaces of Pol, Sa, Ca-inc, and Ca-incSBF were examined via FE-EPMA with an accelerating voltage of 15 kV after Pt coating.

3. Mechanical properties of Ca-incSBF

The three-point flexural strength test and Vickers hardness test were conducted for Ca-incSBF (n=8).

4. Surface wettability

The wettability of each surface was investigated by measuring the contact angle of distilled water and calculated with \( \theta/2 \) methods. The surfaces tested were...
Pol, Sa, Ca-inc, and Ca-incSBF, in addition to Pol after soaking in SBF (Pol-SBF) and Sa after soaking in SBF (Sa-SBF). The contact angle was measured at three different locations on each specimen; the average of these three values was reported as the contact angle of the specimen. The volume of the drop was 5 µL and measurements were made at 15 s after injection of the drop onto the specimen (n=5).

5. Cell proliferation test
Cell proliferation was evaluated on the surface of Pol, Sa, Ca-inc, and Ca-incSBF (n=4). Mouse osteoblast-like cells (MC3T3-E1, Calvaria, mouse; DS Pharma Biomedical Co., Osaka, Japan) were prepared and passage-cultured. MC3T3-E1 cells were cultured in Alpha-Eagle's Minimal Essential Medium (Invitrogen Japan KK, Tokyo, Japan) containing 10% foetal bovine serum and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cells were suspended at 1×10⁵ cells/mL in each medium and seeded at 1,000 µL/well. The cell proliferation was measured by the WST assay using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan; OD at 450 nm) at 1 day and 9 days after incubation. The specimens were sterilized in ethylene oxide gas before cell culture.

Statistical analysis
All data were statistically analyzed by using Tukey’s method. Probability values for the significance of differences between values were calculated at a significance level of 5%.

RESULTS

X-ray diffractometry
Figure 2 shows XRD patterns of the two zirconia samples (Cercon and P-NANOZR) after painting with calcium acetate and firing at 1,000, 1,100, 1,200, and 1,350°C for 30 min. In the case of Cercon, firing at 1,000°C formed CaO, firing above 1,100°C produced CaZrO₃, and upon firing at 1,350°C, CaZrO₃ disappeared and cubic zirconia was formed. P-NANOZR showed nearly the same behaviour as Cercon, although smaller amounts of CaO, CaZrO₃, and cubic zirconia were formed. Unlike Cercon, P-NANOZR contains alumina, which did not react with calcium ions and was still present in the specimens after the final firing at 1,350°C.

Mechanical properties
Figure 3 shows the three-point flexural strength of the specimens after Ca-incorporation firing at 1,000, 1,100, 1,200, and 1,350°C for 30 min. No significant differences in strength were observed for either Cercon or P-NANOZR after firing at different temperatures.

Figure 4 shows the results of Vickers hardness testing. Cercon that was fired at 1,000°C had a slightly lower Vickers hardness number compared with that of the other specimens. In P-NANOZR, no significant differences were observed among the specimens fired at different temperatures.

Field emission type electron probe microanalysis
Figure 5 shows the results of FE-EPMA. In Cercon, calcium was detected within the zirconia surface at each temperature. Domains of 1 µm or less, corresponding to high concentrations of calcium, increased with the firing temperature and calcium was incorporated more deeply. At firing temperatures of 1,350°C, localized domains featuring a high concentration of calcium were
observed. P-NANOZR showed behaviour similar to that of Cercon.

SEM observation
FE-SEM micrographs of the zirconia surface are shown in Fig. 6. In Cercon, CaO or alternatively CaZrO\(_3\) appeared as the black regions in the Ca-inc image. Ca-incSBF was clearly different from Ca-inc, lacking the granular structures on the zirconia surface. Similarly, the FE-SEM image of the Ca-inc P-NANOZR exhibited black regions larger than typical for alumina domains. These regions appeared to correspond to CaO or CaZrO\(_3\). Soaking in SBF greatly changed the surface of Ca-incSBF P-NANOZR; after soaking, the granular structures were not observed on the zirconia surface.

Mechanical properties of Ca-incSBF
Figure 7 shows the three-point flexural strength of Ca-incSBF. No significant differences in strength were observed for either Cercon or P-NANOZR after soaking in SBF.

Figure 8 shows the results of Vickers hardness testing. No significant differences were observed for either Cercon or P-NANOZR after soaking in SBF.

Surface wettability
Contact angles are shown in Figs. 9 and 10. In both Cercon and P-NANOZR, there were no significant differences among the specimens that had not been soaked in SBF. Moreover, soaking in SBF did not produce significant changes in the Pol and Sa samples. The contact angle of the Ca-inc specimens was 64.0±7.2° on the Cercon and 46.0±6.5° on the P-NANOZR. The wettability of the Ca-incSBF was superior to that of the other specimens, at 7.5±1.5° for the Cercon Ca-incSBF and 4.2±0.6° for the P-NANOZR Ca-incSBF.
Cell proliferation tests
Figure 11 shows the results of cell proliferation tests. After 1 day, the proliferation rate on the Ca-incSBF Cercon was higher than that on the other specimens, and the proliferation rate on the Ca-incSBF P-NANOZR was higher than on Pol and Sa P-NANOZR. After 9 days incubation, no significant differences were observed among specimens of either Cercon or P-NANOZR.
Fig. 6  FE-SEM micrographs showing the surface of Pol, Sa, Ca-inc, and Ca-incSBF samples. (a) Cercon and (b) P-NANOZR.

Fig. 7  Three-point flexural strength of zirconia with surface modification after soaking in SBF. (a) Cercon and (b) P-NANOZR. Items marked with identical letters exhibited no significant difference ($p > 0.05$).

Fig. 8  Vickers hardness number of zirconia with surface modification after soaking in SBF. (a) Cercon and (b) P-NANOZR. Items marked with identical letters exhibited no significant difference ($p > 0.05$).
Fig. 9  Contact angle of distilled water on zirconia with three surface modifications, with and without SBF soaking. (a) Cercon and (b) P-NANOZR. Items marked with identical letters exhibited no significant difference ($p>0.05$).

Fig. 10 Horizontal view of water droplet on specimens with different surface modifications. (a) Cercon and (b) P-NANOZR.

Fig. 11 Proliferation of MC3T3-E1 cells on the zirconia surfaces. (a) Cercon and (b) P-NANOZR. Values marked with the same letter are not significantly different at each incubation time ($p>0.05$).

DISCUSSION

Apatite coating and calcium ion incorporation are known to be surface treatments that improve the biocompatibility of titanium; applying these treatments to zirconia requires different methods. For example, coating a zirconia surface with apatite requires the glass coating technique$^{20}$, and incorporating calcium ions into zirconia can be accomplished by heat treatment. Zirconia is more amenable than titanium to calcium ion incorporation. In the present study, calcium acetate was used as the source of Ca$^{2+}$, because of its high solubility (Table 2) and considered of wettability, which permits homogeneous and easy coating using conventional
methods. Other calcium compounds with lower solubility, such as calcium carbonate, calcium sulfate and calcium hydroxide, are more difficult to apply using conventional methods. The reason for calcium acetate was used in 0.5 mol/L, when the concentration was higher than 0.5 mol/L, the excess amount of CaO was produced and the property of zirconia surface probably became alkaline overly. It is assumed to be disadvantageous for cell proliferation. On the other hand, when the concentration was lower than 0.5 mol/L, calcium ions were little incorporated into zirconia. The firing temperature is also important, because higher temperatures may produce more CaZrO₃, lowering the mechanical strength. The reactions undergone at each temperature are assumed to be as follows.

At 150–160°C, calcium acetate decomposes to carbonate and acetone.
\[
\text{Ca(CH}_3\text{COO)}_2 \rightarrow \text{CaCO}_3 + \text{CH}_3\text{COCH}_3
\]

At 850°C, calcium carbonate decomposes to oxide and gas.
\[
\text{CaCO}_3 \rightarrow \text{CaO} + \text{CO}_2
\]

At 1,100°C, calcium oxide reacts with zirconia.
\[
\text{CaO} + \text{ZrO}_2 \rightarrow \text{CaO-ZrO}_2 \text{ solid solution} + \text{CaZrO}_3 (+\text{CaO})
\]
The XRD results shown in Fig. 2 suggest that CaZrO₃ likely decomposes above 1,350°C to produce Ca-stabilized cubic zirconia. FE-EPMA revealed local areas of high calcium concentration in zirconia at 1,350°C; these areas were assumed to be CaZrO₃ (Fig. 5). Their presence indicates a decrease in mechanical strength, corresponding to formation of cubic zirconia. To avoid forming cubic zirconia, we fired the specimens at 1,100°C after coating them with calcium acetate. Although the specimens fired at 1,100°C showed the formation of CaZrO₃, and the presence of calcium in their surface layer was confirmed using elemental analysis, their flexural strength did not decrease.

The wettability of an implant surface plays an important role in the initial responses of cells and cell kinetics. In this study, water contact angle measurements showed remarkable wettability, superior to that of other specimens, of Ca-inc surface-modified Cercon and P-NANOZR after soaking in SBF. The decrease in contact angle was consistent with the production of calcium hydroxide on the surface. The FTIR spectrum of Ca-incSBF (not shown here) exhibits OH⁻ absorption bands around 1,700 cm⁻¹. This suggests that the zirconia surface becomes modified with hydroxyl groups, which may decrease the contact angle of water with the surface. Furthermore, specimens after soaking in SBF have left open the possibility that the surface roughness of them were changed according to SEM observation (Fig. 6), it may be also related to the decrease in contact angle.

High surface wettability may also promote cell proliferation. In the present study, it is thought that Ca-ion surface modification favours the early stage of cell proliferation. The initial attachment of osteoblastic cells increases as surface wettability increases, correlating well with fibronectin adsorption in the competitive mode. Protein adsorption on modified surfaces may also be relevant to cell proliferation. Initial attachment and proliferation of osteoblasts at the implant surface play an important role in the early stages of osseointegration.

CONCLUSION

Calcium ions became incorporated into two types of zirconia surface after firing with calcium acetate. Modifying the surface of zirconia by incorporating Ca ions at the appropriate firing temperature improves the biocompatibility of the zirconia without decreasing its mechanical strength.

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