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Hybrid micro/nano-topography of a TiO₂ nanotube-coated commercial zirconia femoral knee implant promotes bone cell adhesion *in vitro*

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Abstract

Various approaches have been studied to engineer the implant surface to enhance bone in-growth properties, particularly using micro- and nano- topography. In this study, the behavior of osteoblast (bone) cells was analyzed in response to a titanium oxide (TiO₂) nanotube-coated commercial zirconia femoral knee implant consisting of a combined surface structure of a micro-roughened surface with the nanotube coating. The osteoblast cells demonstrated high degrees of adhesion and integration into the surface of the nanotube-coated implant material, indicating preferential cell behavior on this surface when compared to the bare implant. The results of this brief study provide sufficient evidence to encourage future studies. The development of such hierarchical micro and nano topographical features, as demonstrated in this work, can provide for insightful designs for advanced bone-inducing material coatings on ceramic orthopedic implant surfaces.

Keywords

TiO₂ nanotube; osteoblast; orthopedic implant; zirconia; cell adhesion; osseointegration

INTRODUCTION

Zirconia (ZrO₂) was introduced as an orthopedic implant material in 1985, and is well known as one of the highest-strength ceramics suitable for medical use [1]. In particular, zirconia has been found to be a choice material for the femoral component of the total knee implant when compared to the more commonly used CoCr, due to a reduction in polyethylene wear [2]. Although zirconia orthopedic implants received a poor reputation in the late 90s due to failures found to be a result of a change in the heat treatment procedure, a much better understanding of the ceramic aging and degradation behavior and prevention techniques has been obtained [3–4], and the material is becoming popular again.

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While the current success of total knee replacement (TKR) is much improved from pioneering knee implant designs, further improvements are necessary for increased implant lifetime. Some studies have shown that the failure rate of TKR is greater in younger patients due to a higher level of activity [5]. The most common reasons for TKR revision surgery are aseptic loosening and instability [6]. The reasons for aseptic loosening are multifactorial [7]. However, in general, the source of the problem lies in osteolysis at the bone-implant interface. As such, one area for implant lifetime improvement is in strengthening the osseointegration of the implant.

Zirconia implant surfaces are chemically inert, and do not naturally form a bond with bone [8], thus cemented fixation is required. There is much controversy on the benefits of cemented versus uncemented fixation techniques of TKRs. Uncemented fixation for TKR was introduced as a method of potentially increasing implant longevity [9]. The presence of a more physiological bond between the bone and the implant is considered by some to provide a stronger device [10]. While studies in the literature often favor cemented fixation, some more recent studies suggest that there is not a significant difference between the fixation methods [10–11], and both are equally recommended in terms of implant performance. In any case, current studies investigating methods for modifying the surface properties of zirconia with the aim to achieve osseointegration naturally without the use of a bone cement. One such study utilized a CO₂ laser to introduce microstructure and higher surface energy to the surface of zirconia ceramic, which resulted in enhanced bone cell adhesion to the surface [8].

It has been well-established that the presence of nanotopography affects basic cell behavior in almost all types of mammalian cells [12]. Of particular interest are titanium oxide (TiO₂) nanotubes, since titanium is a well-known biocompatible orthopedic material. TiO₂ nanotubes have been found not only to significantly accelerate osteoblast cell growth, but also improve bone-forming functionality and direct stem cell fate. These results are especially promising for well-established titanium bone implants currently on the market, since aseptic loosening is still a relevant problem in orthopedic implants. The nanotube surface structure can essentially be added to any shape of titanium implant by a simple anodization procedure. Additionally, TiO₂ nanotubes can be grown from a thin film of titanium deposited onto another surface. This expands the possible applications to not only current titanium implants, but other types of implants as well.

In this work, a commercial zirconia ceramic femoral knee component was sputter coated with a thin film of titanium on the bone bonding (backside) surface, and further anodized to form a coating of TiO₂ nanotubes. Osteoblast viability, adhesion, and spreading was analyzed and compared on the TiO₂ nanotube coating and bare zirconia implant surface. Here we report an improvement of osteoblast cell adhesion and spreading on the TiO₂ nanotube coated implant.

EXPERIMENTAL METHODS

Apparatus and Reagents

The apparatus used for TiO₂ nanotube fabrication has been described in detail elsewhere [13]. The reagents used for each respective part of the experiment are described in the following sections.

TiO₂ Nanotube-Coated Implant Preparation

A zirconia ceramic femoral knee component was obtained from Kinamed, Inc. (GEM™ Total Knee System, Cat # 20-120-1001) and was cleaved into six pieces in a manner which maximized the sample surface area of relatively flat backside implant surface. In order to obtain regular surfaces for the experiments, the prosthesis was cut using a diamond saw into rectangular pieces measuring ~1 cm × 2 cm. The backside of the samples were then ground to create a relatively flat and even surface. Two of the pieces were set aside to be used as controls in this study, while the remaining four pieces were taken for further processing. Before film deposition, ZrO₂ implant materials were cleaned successively in acetone and isopropyl alcohol with ultrasonication. Ti thin film was vacuum-deposited using Denton Discovery 18 sputter system. Base pressure was $1\sim 10^{-6}$ torr and substrate being rotated was heated to 400 °C during sputtering. Plasma power was 200 W and Ar pressure was 3.0 mTorr. Deposition rate was 0.25 nm/s and 1 µm thick Ti film was deposited. Electrical contact for the anodization step was provided by copper tape and a lacquer protective paint. The TiO₂ nanotube surfaces were created using a two-electrode-setup anodization process. An organic-based electrochemical solution was used which was composed of 0.25 wt% NH₄F in 2 vol% deionized water in ethylene glycol. A platinum electrode (99.9% pure, Alfa-Aesar, USA) served as the cathode. The samples were anodized at 20 V for 15 min, followed by a washing step with ethanol, and an acetone soak to remove the lacquer paint. The samples were then dried at 80 °C overnight and heat-treated at 500 °C for 2 h in order to crystallize the as-fabricated amorphous structured TiO₂ nanotubes into an anatase structure. The samples used for all experiments were sterilized by autoclaving prior to use. The bare zirconia implant samples were used as a control after being chemically cleaned by acetone and isopropanol, dried and autoclaved.

Adhesion Strength Test

For adhesion strength of TiO₂ nanotube coating on the zirconia implant materials, a simple adhesion set-up was utilized. Commercial thermosetting epoxy was attached on the surface of the TiO₂ nanotubes and a hook-shaped metal wire end was tightly embedded inside the epoxy. The adhered area of epoxy was 0.2 cm². After the epoxy was fully cured, uniaxial force was applied normal to the surface. Adhesion strength was indirectly determined whether fracture occurred along the epoxy/TiO₂ nanotube interface or TiO₂ nanotube/implant interface. In result, adhesion strength was at least greater than 460 lb/in² in which epoxy was detached from TiO₂ nanotube surface with a sharp fracture interface.

Contact Angle Measurement

The measurement of contact angle for the bare implant and TiO₂ nanotube surfaces was carried out by a video contact angle measurement system (Model No. VSA 2500 XE, AST Products, Inc.).

Osteoblast Cell Culture

For this study, MC3T3-E1 mouse osteoblast (CRL-2593, subclone 4, ATCC, USA) were used. Each 1 mL of cells was mixed with 10 mL of alpha minimum essential medium (α -DMEM; Invitrogen, USA) in the presence of 10 vol% bovine serum (FBS; Invitrogen, USA) and 1 vol.% penicillin-streptomycin (PS; Invitrogen, USA). The cell suspension was plated in a cell culture dish and incubated at 37°C in a 5 vol% CO₂ environment. When the concentration of the MC3T3-E1 osteoblastic cells reached confluency, they were seeded onto the experimental substrate of interest (TiO₂ nanotubes or bare implant), which was placed on a 6-well polystyrene plate and stored in a CO₂ incubator for 24 and 48 h to observe the cell morphology and adhesion. The concentration of the cells seeded onto the substrate was 1.5×10^5 cells per well.

Cell Viability, Adhesion, and Spreading Test

Fluorescein diacetate (FDA; Sigma, USA) staining was conducted to visualize cell viability and to quantify cell spreading. At 24 h after plating, the cells on the substrates were washed with phosphate-buffered saline ($1 \times$ PBS) solution (Invitrogen, USA) and incubated for approximately 30 s with FDA stock (5 mg dissolved in 1 mL of acetone) dissolved in PBS (10 μ L/10mL), and washed once more. The samples were then inverted into new wells, visualized and photographed using a fluorescence microscope with a green filter (DM IRB, Leica Co., USA). Six fields were randomly chosen from each sample. The digital images were stored in a 672×512 pixels file and imported to a TIF format. Stored images were imported to ImageJ image processing program for digital analysis. The number of adherent cells were counted in each image. Additionally, the total cell spreading area was quantified.

SEM for Substrate and Cell Morphological Examination

After 24 h of culture, the cells on the substrates were washed with PBS and fixed with 2.5% w/v glutaraldehyde (Sigma, USA) in PBS for 1 h. After fixation, they were washed three times with PBS for 10 min each wash. The cells were then dehydrated in a graded series of ethanol (50, 75, 90, 100 vol.%) for 30 min each and left in 100% ethanol overnight. The substrates were then dried by a critical point dryer (EMS 850, Electron Microscopy Science Co., USA). Next, the dried samples were sputter-coated with palladium for examination by scanning electron microscopy (SEM). The morphology of the TiO₂ nanotubes and bare implant as well as that of the adhered cells were observed using an XL30 scanning electron microscope (FEI Co., USA).

RESULTS AND DISCUSSION

The zirconia (ZrO₂) ceramic femoral knee component obtained from Kinamed, Inc. (GEM™ Total Knee System, Cat # 20-120-1001) for modification and experimentation in this study is pictured in Fig. 1, as received. The bone-integrating (backside) surface of the femoral

knee implant is shown facing upward (the dull surface) in this photograph, while the articulating (shiny) surface is facing downward. This implant was cut into six sample pieces for use in the study herein.

Fig. 2 shows SEM micrographs of the as-received zirconia implant surface (a), and the TiO₂ nanotube coated implant surface at low and high magnification (c). Fig. 2(c) reveals the nanotopography present on the TiO₂ nanotube surface which is lacking on the bare implant surface (a). The self-assembled nanotube surface was obtained by anodizing the titanium sputter-coated zirconia femoral implant at 20 V an organic-based electrolytic solution. The nanotube pore size is ~50 nm, with a length of ~200 nm.

While the top-view of the TiO₂ nanotube surface appears slightly more porous in nature, tube morphology was verified to be present underneath a porous top layer, as seen in Fig. 2(c). This configuration of TiO₂ nanotubes with a porous surface morphology was clearly explained by Wang, et al. to be the result of an imbalance in two competitive reactions which occur during TiO₂ nanotube formation [14]. Further optimization of our anodization conditions could result in a tube-like surface morphology. However, for the purposes of this study we analyzed the porous TiO₂ nanotube surface morphology in comparison with the bare implant material.

The SEM images show that a micron-scale surface roughness is present on the as-received implant; the nanotube coated implant possesses the same microtopography, in addition to the nanotopography created by the porous nanotube surface. The surface contact angle measurements of water droplets on each surface revealed that the titanium surface coating altered the as-received implant surface to become extremely hydrophilic in nature, while the as-received implant was only slightly hydrophilic. The TiO₂ nanotube coated implant was also superhydrophilic, with a contact angle of 0° (Fig. 2(b)).

To investigate cell behavior in response to the as-received and modified implant surfaces, osteoblast cells were seeded on the comparative surfaces at a cell density of 1.5×10^5 per well. As indicated in Fig. 3, cell adhesion and spreading after 24 h of culture was found to be increased on the TiO₂ nanotube surface in comparison to the bare implant surface. The plots of the quantification of the fluorescent images (Fig. 4) clearly confirm these trends. Both the number of adhered cells (Fig. 4(a)) and cell spreading area (Fig. 4(b)) were significantly increased on the nanotube surface. These results are in agreement with our previously reported investigations of osteoblast behavior on TiO₂ nanotube surfaces when compared to flat Ti foil [15–16]. It is likely that the increase in cell adhesion and spreading is a direct result of the superhydrophilic nature of the nanotube-coated surface. Many researchers have demonstrated the positive effects of superhydrophilic biomaterials on protein and cell adhesion properties [17–18].

The higher degree of cell adhesion and spreading was further confirmed by SEM, as shown in Fig. 5. On the bare zirconia implant surface (Fig. 5(a) and (c)), the cells appear to be unable to attach to the surface. Although there are many filopodial extensions present, rarely are they in contact with the surface, as indicated by the red arrows. In contrast, the cells on the TiO₂ nanotube coated implant surface (Fig. 5(b) and (d)) are clearly integrating into the

surface, and no floating filipodia are evident. The higher magnification images of the cell edge (Fig. 5(c) and (d)) emphasize the unmistakable cell-surface interaction evident on the TiO₂ nanotube coated implant. It can be speculated that the increase in cell-surface interaction can be contributed to two key factors. Firstly, as mentioned previously, the alteration from a slightly hydrophobic surface to an extremely hydrophilic surface is likely to enhance cell adhesion and spreading capabilities. Secondly, the presence of nanotopography in addition to the bare implant microtopography may have an impact on how the cells behave. A hierarchical hybrid micro/nano-textured titanium surface has been recently considered to create an improved surface structure for osseointegration [19]. While the nanotopography can be assumed to induce an increase in bone functionality, the microtopography contributes to the mechanical interlocking ability of the surface.

The spectrum of this study was limited by the number of commercial femoral implant samples available at the present time. In order to fully evaluate cell adhesion to the surface, more extensive cell proliferation or cell survival experiments such as MTT or WST should be utilized. In addition, further studies should be performed to verify osteoblast functionality and maturation, as well as mesenchymal stem cell behavior on the surfaces in order to demonstrate the *in vitro* behavior of the two important cell types present at a bone implant interface. Furthermore, *in vivo* results should be assessed for more complete materials analysis, as well as surface roughness tests.

CONCLUSION

This study was intended to facilitate the osseointegration of zirconia femoral knee implants by providing a novel hierarchical micro- and nano-structured titanium surface coating which encourages bone cell adhesion and integration. The osteoblast cells demonstrated high degrees of adhesion and integration into the surface of the nanotube-coated implant material, indicating preferential cell behavior on this surface. The results of this brief study provide sufficient evidence to encourage future studies. The development of such hierarchical micro and nano topographical features, as demonstrated in this work, can provide for insightful designs for advanced bone-interfacing material coatings on ceramic orthopedic implant surfaces.

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Figure 1.
Photograph of the as-received commercial zirconia femoral knee implant.

Figure 2.

Physical characterization of the as-received zirconia implant and TiO₂ nanotube coated implant surfaces. (a) SEM micrographs of bare implant surface (tilted 45° angle). (b) Table with surface contact angle measurements of water droplets on the bare implant, titanium coated implant, and TiO₂ nanotube coated implant surfaces. (c) SEM micrographs of the TiO₂ nanotube coated implant surface at low magnification (left), and high magnification (right).

Figure 3.

FDA viability of osteoblast cells after 24 hours of incubation on the bare zirconia implant and TiO₂ nanotube coating on the zirconia implant. More spreading is evident on the nanotube surface which indicates greater cell adhesion. Red arrows indicate significant cell spreading.

Figure 4.

Cell number (a) and spreading area (b) after 24 hours of incubation. The bar graphs show the average $\pm$ standard error bars. The p -value after performing a t -test confirmed a statistical significance ($p < 0.005$).

Figure 5.

SEM micrographs of osteoblast cells after 24 hours of incubation on the bare zirconia implant (a, c), and TiO₂ nanotube coating on the zirconia implant (b, d). (c) and (d) are higher magnification of the cell edges on the respective surfaces. Red arrows indicate floating filopodia.