Comparing Biocompatibility of Titanium Orthopaedic Implants with and without an Ultra-thin Coating of TiO_2 by Ion Beam Assisted Deposition

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ABSTRACT

The evaluation of the biocompatibility of orthopaedic implants is a main factor in the validating their healing potential. Titanium (Ti) has this unique trait along with other reliable mechanical properties, which makes it a perfect choice for orthopaedic purposes. Namavar et al. employed ion beam assisted deposition (IBAD) technique to coat a thin nano-layer of titanium oxide over different surfaces in order to increase their osseointegration properties. To compare the properties of the newly engineered nano-crystalline TiO_2 surface with bulk Ti, we have studied the osteoblast functions and cellular responses on these surfaces -including adhesion, growth, and mineralization- using a human osteoblast-like cell line (SAOS-2). The correlation between surface properties and cellular responses was studied using immunofluorescence techniques and alizarin red assay. The result of this study indicated that nano-engineered TiO_2 surface is superior in supporting of cellular responses of bone-forming cells as compared to orthopaedic grade Ti.

Keywords: ion beam assisted deposition, biomaterial, nanostructure, biocompatibility, orthopaedic;

1 INTRODUCTION

Total joint replacement is the final solution for progressive bone erosion when all other treatments fail. It is a highly successful procedure that returns mobility to patients with injuries. Annually, more than one million patients in the U.S. undergo arthroplasty and the total annual Medicare expenditures for both total hip/knee arthroplasty, are around $3 billion [1]. According to Kurtz et al., the projected number of total hip and knee arthroplasties for 2030 will be over 4.4 million. Unfortunately, 10-20% of arthroplasty procedures will need revision surgery (short-term or long-term revisions) [2]. Revisions are long and complex procedures that are rarely as successful as the first operation in restoring normal function and range of motion. Thus, there is a critical need to develop better technologies for arthroplasty, in order to circumvent the need for revision surgeries. A number of different materials have been utilized in these procedures, but titanium (Ti) is considered the material of choice for orthopaedic applications because of its reasonable biocompatibility [3]. However, there is still a need to modify orthopaedic implants to improve their biocompatibility and enhance osteoblast activation to induce more/stronger bone. In recent studies, it has been shown that the morphology and structure of the surface is known to influence cellular behavior and interactions with bone [4]. Thus, if it is possible to develop a surface with these properties, then this should result in increased implant longevity. This paper addresses the ongoing research on materials for implants to evaluate adherence, survival, and growth of osteoblast-like cells on surfaces of newly engineered nano-crystal films of titanium oxide (TiO_2) compared with orthopaedic grade Ti. We have used the ion beam assisted deposition (IBAD) facilities at the Nanotechnology Laboratory at the University of Nebraska Medical Center [5] to deposit TiO_2 thin films on orthopaedic grade Ti and control surfaces such as glass and silicon.

2 MATERIAL AND METHODS

2.1 IBAD Samples

The ion beam assisted deposition (IBAD) combines an electron beam evaporation system with a simultaneous ion beam bombardment in a high vacuum environment at a base pressure of 10^{-8} Torr. IBAD processes employ energetic ions to "stitch" TiO_2 thin films with hydrophilic properties to the substrate. The ion gun supplies uniform ion beams of mixed oxygen and argon to engineer coatings with desired properties (Figure 1).

IBAD was used to produce films with 3 to 70 nm grain size. These films possess characteristics that affect the wettability and mechanical properties of the coatings [5].

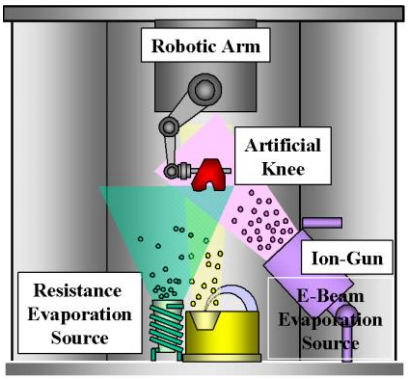

Figure 1. IBAD schematic (ref)

A typical atomic force microscopy of TiO_2 produced by the IBAD technique showed a typical roughness of 9.57 nm for a 2 micron scan size (Figure 2).

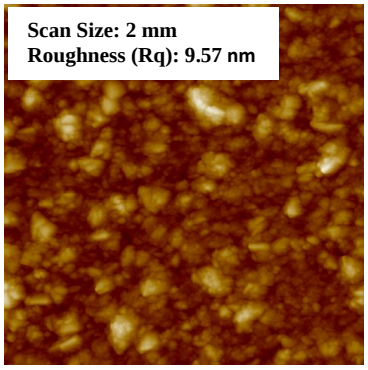

Figure 2. Atomic force microscopy (AFM) image of nano-structure TiO_2 film using IBAD

To prepare samples for experiments, they were cut into 1cm by 1cm pieces, sonicated for 2 hours in an acetone and methanol mixture (50:50), rinsed in absolute ethanol, and dried under nitrogen gas. The samples were wrapped in aluminum foil and autoclaved to ensure a sterile surface for culturing conditions.

2.2 Cell Culture

A human osteoblast-like cell line (SAOS-2) was used for this study to compare the biocompatibility of these nano-engineered surfaces with orthopaedic-grade Ti.

SAOS-2 (ATCC) cells are late mature cells and are grown in McCoy's 5A medium (ATCC) supplemented with 15% fetal bovine serum (FBS) and 1% gentamycin (Invitrogen) in a humidified 5% CO_2 atmosphere at 37°C.

Besides the worldwide availability, other advantages of using this human osteosarcoma cell line are its similarity to human osteoblast cells, high matrix mineralization capacity, and a known cytokine and growth factor profile [6]. We studied the correlation between the surface structures and the cell growth by characterizing the SAOS-2 cells with immunofluorescence and measuring the concentration of alizarin red produced over different time lines.

2.3 Immunofluorescence Techniques

To study the effect of the surface properties on cell adhesion and proliferation, immunofluorescence techniques were applied including DAPI and actin staining. DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) is a stain that emits blue fluorescent color when it binds to AT regions in DNA. Actin is a protein that forms microfilaments (the major components of the cytoskeleton), and actin staining is commonly used to determine the structure of the cytoskeleton. In this study, these stains were applied to compare short-term adhesion, long-term adhesion, and adherent cell proliferation on the different surfaces.

In the first experiment, 100,000 cells in 2ml media were seeded to each sample. Two hours after seeding, samples were washed and plated in new dishes with fresh media and incubated for 48 hours. They were fixed with 1 ml of 4% formaldehyde for 10 minutes and permeabilized with 1 ml of 0.1% Triton-X for 5 minutes at room temperature. After several washes with PBS/1%BSA buffer, the samples were stained with DAPI, fixed to slides, and coverslips with Fluormount-G were placed over the cells. The number of adherent osteoblasts was measured by counting DAPI-stained nuclei. For this purpose, DAPI-stained nuclei were scanned and counted using Metamorph software.

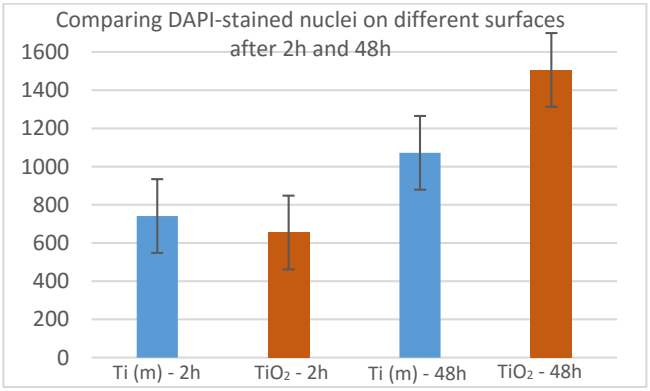

Figure 3. 100,000 cells were seeded to each sample. The number of adherent osteoblasts was measured by counting DAPI-stained nuclei after 2 hours and 48 hours which almost were the same after 2 hours but higher on nano-structure TiO_2 after 48 hours.

In the next experiment, actin stress fiber patterns were examined to compare actin remodeling at focal adhesion sites on the surfaces. In this experiment, 50,000 cells were seeded on the samples, then incubated for 48 hours. Cell morphology was visualized by microscopy after staining with actin (5:200, Invitrogen, Alexa Fluor 546 Phalloidin) and DAPI (300 nM, Invitrogen, 6-diamidino-2-phenylindole).

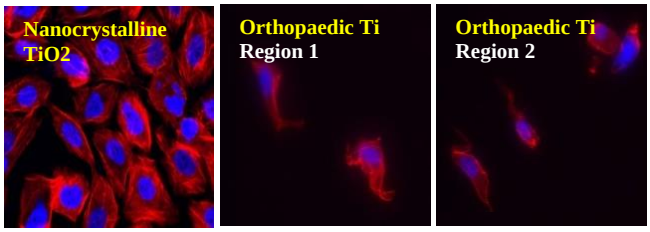

Figure 4. 50,000 cells were seeded on the samples and incubated for 48 hours. Cell morphology was visualized by microscopy after staining with actin and DAPI showing that on different surfaces there were significant differences in cell shape, and actin filament amount and orientation.

2.4 Alizarin Red Assay

Osteoblasts arise from undifferentiated precursor cells, and deposit a mineralized matrix consisting of collagen, calcium, phosphorous, and other minerals, leading to the formation of new bone. Osteogenesis can be determined by staining with alizarin red solution, which can be used to visually detect the presence of mineralization in bone tissue. Alizarin red staining is a dye that binds to calcium salts and is commonly used. In this study, alizarin red staining (Millipore Osteogenesis Assay Kit (ECM815) was used to detect calcium deposition of the SAOS-2 cells on the different surfaces (Ti and TiO_2) after 7 and 14 days. Briefly, after removing media from the samples and rinsing with PBS, samples were fixed in 10% formaldehyde and stained with alizarin red stain for 20 minutes. Later, samples were placed in 10% acetic acid for 30 minutes in order to remove the monolayer, then placed in micro centrifuge tubes, heated to 85°C for 10 minutes and centrifuged. The supernatant was neutralized with 150 µl of 10% ammonium hydroxide and the absorbance was read at 405 nm.

Day of experiment	Substrates	Alizarin red concentration average	Kruksal-Wallis p-value
Day 7	Ti (m)	4.11 ± 0.35	0.02
	TiO_2	7.98 ± 0.79	
Day 14	Ti (m)	1.91 ± 0.98	0.029
	TiO_2	8.24 ± 3.18	

Table 1. For determination of calcium deposition rates, as an indication of successful in-vitro bone formation, alizarin red quantification was applied after 7 and 14 days which showed a significant difference between nano TiO_2 and orthopaedic grade Ti.

2.5 Statistical Analysis

Data were derived from four independent experiments (n=4). Statistically significant differences between various substrates were evaluated using the non parametric kruskal wallis test (one-way ANOVA on ranks).

3 RESULTS

Counting DAPI-stained nuclei (cell numbers) showed that SAOS-2 cells were able to attach and to grow on both surfaces. The number of cells attached to Ti was slightly higher (not significant) compared to nano TiO_2. However, cells grew slowly and showed a less differentiated phenotype during growth on Ti compared to nanocrystalline TiO_2. After 48 hours, there was a greater number of cells on nanocrystalline TiO_2 compared to microcrystalline Ti. Although there was variability in different areas of biomedical grade Ti, overall, fewer cells were observed adhering to microcrystalline Ti. In addition, most of the adherent cells on the nanocrystalline TiO_2 were bigger.

Examining the actin stress fiber patterns and actin remodeling at focal adhesion sites on the surfaces showed that cells on nano TiO_2 were more spread out and the actin filaments were evident, which indicates an increase in focal adhesion patterns. Fewer cells on microcrystalline Ti were attached and those appeared smaller. Some cells had rounded up, and some had obvious nuclear fragmentation indicating apoptosis and cell death. Overall, cell survival, adhesion, and morphology showed that cells grown on the nanocrystalline TiO_2 had higher levels in both quality and quantity than those on biomedical grade Ti.

The formation of mineralization, as detected by alizarin uptake, was different on TiO_2 nano-surfaces as compared to the bulk Ti. The alizarin red concentration was significantly higher on nano samples after 7 and 14 days, which stresses a significant difference between nano TiO_2 and orthopaedic-grade Ti in mineralization and osteoconductive ability. Although all samples allowed SAOS-2 cells to attach and grow, biochemical analysis showed that SAOS-2 plated on the TiO_2 reached confluency earlier than Ti (m) and retained a differentiated state. Therefore, the TiO_2 surfaces are more likely to initiate and promote bone formation, as shown by the measurement of the mineralization process.

4 CONCLUSION

Results from immunofluorescence imaging showed that the number of the attached osteoblast-like cells and their shapes are altered on different surfaces. The actin stress fiber patterns and focal adhesion sites indicate that nano-engineered surfaces possess a more suitable substrate (roughness) for cell attachment and growth.

Calcium deposition (alizarin red) is an indicator of late differentiation and bone formation of the osteoblast-like cells, and showed a significant difference between nano and micro surfaces. Based on the results, more bone formation would be expected from cells grown on nanocrystalline TiO_2 as compared to bulk Ti.

Overall, these experiments indicated that nanocrystalline TiO_2 is superior to microcrystalline Ti in supporting

adhesion, proliferation, and differentiation of bone forming cells. It is believed that is related to the unique properties of the nano-film coatings on the surfaces. Therefore, nano-engineering the surface topology, improving the quality of surface oxide, and fabricating stoichiometric oxides may be crucial and beneficial for increasing the biocompatibility of Ti implant materials.

REFERENCES

[1] R.L. Kane, K.J. Saleh, T.J. Wilt and B. Bershadsky, "The functional outcomes of total knee arthroplasty," J.Bone Joint Surg.Am., vol. 87, pp. 1719-1724, Aug. 2005.

[2] S. Kurtz, K. Ong, E. Lau, F. Mowat and M. Halpern, "Projections of primary and revision hip and knee arthroplasty in the United States from 2005 to 2030," J.Bone Joint Surg.Am., vol. 89, pp. 780-785, Apr. 2007.

[3] F. Namavar, R. Sabirianov, D. Marton, A. Rubinstein and K. Garvin, "Biocompatibility of Titanium," in APS March Meeting Abstracts, pp. 1148, 2012.

[4] R. Sabirianov, A. Rubinstein and F. Namavar, "Enhanced initial protein adsorption on engineered nanostructured cubic zirconia," Physical Chemistry Chemical Physics, vol. 13, pp. 6597-6609, 2011.

[5] F. Namavar, C.L. Cheung, R.F. Sabirianov, W. Mei, X.C. Zeng, G. Wang, H. Haider and K.L. Garvin, "Lotus effect in engineered zirconia," Nano Letters, vol. 8, pp. 988-996, 2008.

[6] E. Czekanska, M. Stoddart, R. Richards and J. Hayes, "In search of an osteoblast cell model for in vitro research," Eur Cell Mater, vol. 24, pp. 1-17, 2012.