

Increased osteoblast cell density on nanostructured PLGA-coated nanostructured titanium for orthopedic applications

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Abstract: There are more than 30,000 orthopedic implant revision surgeries necessary each year in part due to poor implant fixation with juxtaposed bone. A further emphasis on the current problems associated with insufficient bone implant performance is the fact that many patients are receiving hip implants earlier in life, remaining active older, and that the human lifespan is continuously increasing. Collectively, it is clear that there is a strong clinical need to improve implant performance through proper, prolonged fixation. For these reasons, the objective of the present in vitro study was to improve the performance of titanium (Ti), one of the most popular orthopedic implant materials. Accordingly, the proliferative response of osteoblasts (bone-forming cells) on novel nanostructured Ti/PLGA (poly-lactic-co-glycolic acid) composites was examined. This study showed that nano-topography can be easily applied to Ti (through anodization) and porous PLGA (through NaOH chemical etching) to enhance osteoblast cell proliferation which may lead to better orthopedic implant performance. This straight forward application of nano-topography on current bone implant materials represents a new direction in the design of enhanced biomaterials for the orthopedic industry.

Keywords: osseointegration, nano-scale topography, PLGA, titanium, tissue engineering, orthopedic implants

Introduction

Each year in the United States, there are more than 200,000 total hip replacements (H-CUPnet 1999–2003) and more than 400,000 knee replacements (H-CUPnet 1999–2003). In addition, there are over 30,000 orthopedic revision surgeries in the United States required per year to simply retrieve a failed implant (H-CUPnet 1999–2003). These revisions are primarily due to infection, wear debris generated at orthopedic implant articulating surfaces, and a lack of proper implant fixation to surrounding bone. With elderly patients living actively and younger patients receiving hip implants, there is clearly a great clinical need to improve implant fixation (Bozic and Berry 2006).

To promote implant fixation, current orthopedic implants are modified with either micron-sized metal beads (Friedman et al 1996) or are altered through other techniques to establish micron-sized surface features (Jinno et al 1998; Knabe et al 2004; Takeuchi et al 2005) which serve to increase new juxtaposed bone formation. Despite these biologically-active surface treatments, these metal coated implants still have a limited ability to induce bone integration. Furthermore, the inequality between the mechanical properties of bone and the implant material permits micromotion which diminishes bone integration through shear forces at the bone-implant interface (Buser et al 1999; Webster 2001; Webster and Ejiofor 2004). These conditions necessitate

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new materials whose properties can improve implant integration with host bone to, thus, increase overall implant longevity.

One approach that may increase the lifetime of current bone implants is nanotechnology, or the use of nanostructured materials in orthopedic applications. Nanotechnology has become a prominent area of research in orthopedics since bones are naturally composed of nanomaterials. While much work has been completed on nanostructured bulk materials for orthopedics, little reports exist for nanostructured coatings on traditional bone implants. The presence of a nano-scale topography on clinically relevant materials (such as metals, ceramics, and polymers) has extensively been shown to enhance osteoblast (bone-forming cell) function (Webster et al 2000a; Webster and Ejirofor 2004).

Poly-lactic-co-glycolic-acid (PLGA) is a common resorbable coating biomaterial for orthopedic applications because it can provide a biodegradable matrix for bone cell adhesion and differentiation into calcium producing cells (Laurencin et al 1996). PLGA is FDA approved and can be manufactured as a porous material with various surface textures. A porous architecture is important for tissue engineering since many tissues, including bone, form a three dimensional structure that provides a pathway for nutrient transport and waste removal. Importantly, nanometer surface features on PLGA have been created to simulate the texture present in our own tissues (in particular bone) (Webster et al 2000a; Park et al 2005). There is a clinical need to combine these materials (namely, PLGA and Ti) to produce a biodegradable coating that promotes bone infiltration so that tissue integration occurs rapidly and robustly onto a metallic surface.

For these reasons, the objective of the present in vitro study was to create and test biologically-inspired nano-rough PLGA coatings on nanostructured Ti as an initial step in the development of a biomaterial that supports bone growth and integration. Nanorough PLGA was created through chemical etching techniques. Nanostructured Ti was created by modifying traditional anodization techniques. Anodization (or anodic oxidation) is a well-established technique that creates a biologically-inspired nanostructured roughness on common implants metals. In particular, it was hypothesized in this study that nano-scale surface features on PLGA coated on nanostructured Ti would increase osteoblast cell density compared to currently-used Ti implants which do not possess such desirable topographies.

Materials and methods

Titanium and Ti/PLGA composites

This study involved the fabrication and investigation of osteoblast cell density on three substrate types: unanodized Ti containing micron sized wells (microTi), anodized nanostructured Ti containing micron sized wells (nanoTi), and anodized nanostructured Ti containing micron sized wells coated with nanostructured PLGA created by NaOH etching (nanoTi-nanoP).

Substrate/composite preparation

Cubes of Ti (1cm × 1cm × 1cm) each with 16 micron sized wells (500 µm in diameter and 0.32 cm in depth) were fabricated by Grey Iron (Lafayette, IN, USA) using electron discharge machining (EDM). Briefly, electrical tension between an electrode and the Ti surface was used to vaporize the Ti and shape the wells on each specimen (n = 21). A set of Ti substrates was set aside without further treatment (microTi, n = 7). The remaining Ti substrates were anodized to produce nano-topography on their surfaces. Briefly, the Ti substrates were anodized in 5% hydrofluoric acid for 20 minutes with a potential difference of 10V applied across the surface that contained the micron-sized wells (Yao et al 2005). Of these 14, a set of seven were set aside without further treatment (nanoTi, n = 7).

The other seven nanoTi substrates were subjected to PLGA coatings where the PLGA was also created to have nanometer surface features through NaOH etching (nanoTi-nanoP). To produce PLGA coatings on Ti, PLGA was first prepared by dissolving a 50/50 weight percent blend of poly(lactic-co-glycolic) acid (PLGA; 12–16.5 × 10³ MW; Polysciences, Warrington, PA, USA) in chloroform at 45 °C for 20 minutes. Next, 150–250 µm diameter salt particles were added to produce a 10:90 polymer:salt ratio by weight. The salt particles were thoroughly dispersed in the polymer mixture by 20 minutes of sonication. After sonication, the mixture was poured into a glass syringe fitted with a 24 gauge needle. The needle tip was placed into each micron-sized Ti well and the PLGA injected. Once each of the wells was filled, PLGA was poured on top of the substrate to cover the remaining Ti to a depth of 2 mm. The PLGA was then cured at room temperature overnight and vacuum dried for 48 hours. To produce pores in the PLGA coating, salt was leached by soaking the composites in deionized water for 3 days, replacing the water every 3 hours. The Ti/PLGA composites were then etched in 1N NaOH for 10 minutes to produce a

nano-topography throughout the porous PLGA; the substrates were then rinsed several times to remove excess NaOH. One of each material type (microTi, nanoTi, and nanoTi-nanoP) was set aside for SEM characterization while the remaining 18 were used in cell culture experiments as described below. Unanodized Ti without wells served as controls.

Substrate/composite characterization

Scanning electron microscopy (SEM) analysis was performed on each substrate to visualize surface roughness. Each sample was gold sputter coated using a sputter coater (LADD, Williston, VT, USA) in a 100 millitorr vacuum at 5 kV and 6 mA. SEM was performed at 5 kV. Images of substrate topography were taken at 20X, 200X, 2500X, 5000X, and 10,000X so that pore geometry and surface feature size could be observed.

Cell culture and cell density assay

Previously characterized human osteoblasts (ATCC CRL 11371) with population numbers ranging from 7 to 13 were cultured in 100 mm Petri dishes (Corning; Corning, NY, USA) in Dulbecco's Modified Eagle Medium (DMEM, HyClone; Logan, UT, USA) with 10% fetal bovine serum (FBS) (HyClone; Logan, UT, USA) and 1% penicillin/streptomycin (P/S) under standard culture conditions of 37 °C in 5% CO₂ and 95% humidified air. Culturing a suitable population for these experiments was accomplished by feeding the cells every three days and splitting the populations once they achieved total confluence.

For the cell density assay, all substrates were sterilized by soaking in EtOH for 30 minutes and then exposing the materials overnight to UV light. The sterile samples were then placed in a 12 well culture dish (Corning; Corning, NY, USA). Osteoblasts were placed onto the etched glass reference, microTi, nanoTi, and nanoT-nanoP samples at a seeding density of 500,000 cells per well in DMEM supplemented with 10% FBS and 1% P/S under standard cell culture conditions. After 48 hours, unattached cells were removed by aspirating the cell culture media and rinsing three times with phosphate buffer saline (PBS). Cells were then lysed using three PBS freeze thaw cycles, centrifuged at 250g for 4 minutes, and counted spectrophotometrically (at 490 nm; Molecular Devices, Sunnyvale, CA, USA) using the CytoTox 96 assay (Promega, Madison, WI, USA). The number of cells was determined by comparing the absorbance to a

calibration curve from cell lysates taken at predetermined cell numbers. In each experiment, each group contained three samples per substrate type. Two experiments were performed (n = 6). Results on experimental samples were divided (ie, normalized) by the number of cells on the etched glass reference.

Statistical analysis

Using StatView (SAS Institute, Cary, North Carolina, USA), an Analysis of Variance (ANOVA) was performed to compare the effects of microTi, nanoTi and nanoTi-nanoP on osteoblast adhesion. A significance level of 0.05 was used for all analyses.

Results

Substrate/composite characterization

Results showed that while the micron-sized wells for microTi were placed equidistant, some of the wells were not perfectly circular (Figure 1). Ti well depths were verified by marking a needle inserted to the bottom of each well. The depth of each well was then calculated (0.32 cm) and no appreciable difference in depth was determined between samples. Results also showed from SEM images of the microTi samples an expected irregular set of micron-sized surface features with little to no nanometer surface roughness (Figures 2A and B).

In contrast, the anodized Ti substrates (nanoTi) had numerous nanoscale surface features (Figure 3). Moreover, while unetched porous PLGA samples exhibited a smooth surface at the micron and nano scale (Figures 4A and C),

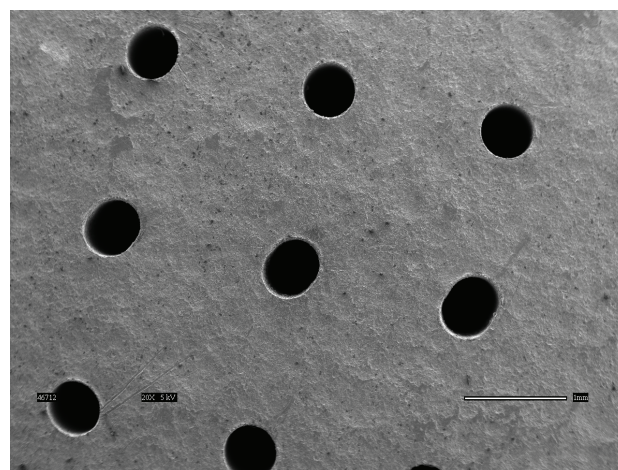
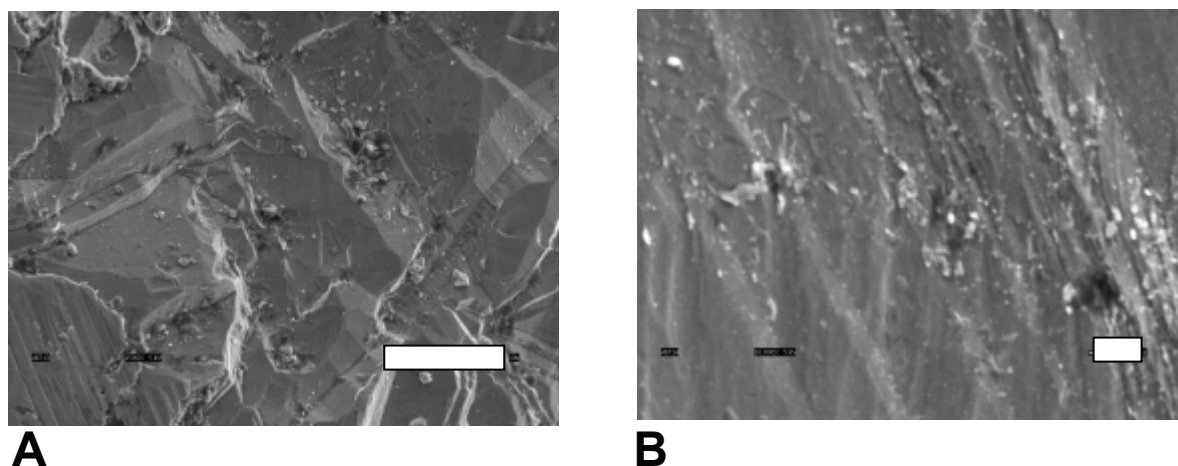


Figure 1 SEM image of micron-sized wells formed on a Ti substrate using electrical discharge machining (EDM). This is the microTi substrate. Scale bar = 1 mm.



Figures 2 (A) 2500X SEM image showing the irregular micron-sized surface features on the microTi substrate (Scale bar = 10 μm). (B) 10,000X SEM image showing the irregular micron-sized surface features on the microTi substrate (Scale bar = 1 μm).

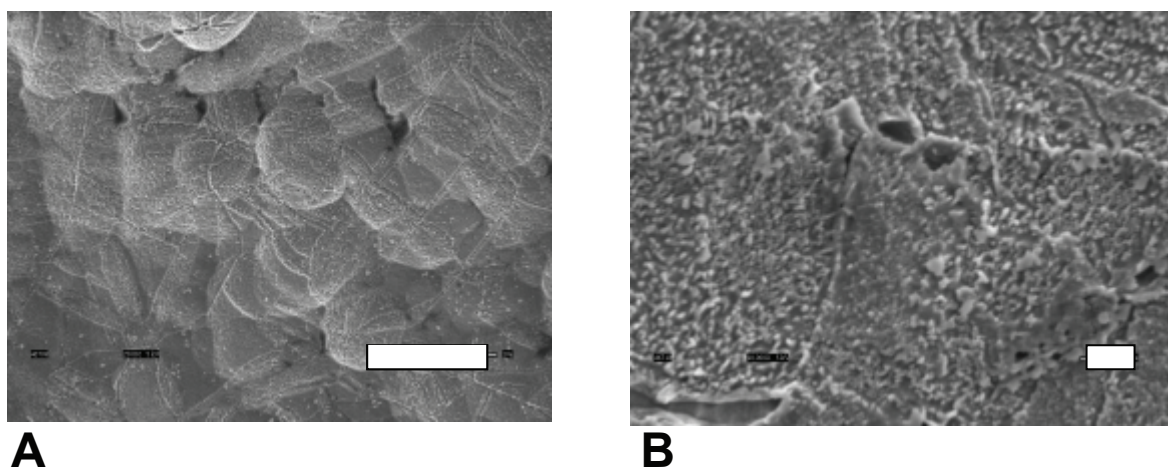
etched PLGA coated on nanoTi (nanoTi-nanoP) were full of biologically-inspired micro and nanoscale surface features (Figures 4B and D).

Increased cell density on nanoTi and nanoTi-nanoP

More importantly, the results of this in vitro study showed for the first time that when microTi was transformed into nanoTi either uncoated (nanoTi) or coated with etched nano PLGA (nanoTi-nanoP), osteoblast cell density significantly increased after 48 hours (Figure 5). Compared to each other, nanoTi and nanoTi-nanoP demonstrated no appreciable difference in cell density after 48 hours.

Discussion

The present in vitro study demonstrated that anodizing Ti and coating such Ti substrates with NaOH etched PLGA which possessed nanometer roughness promoted osteoblast cell density. Although clearly more studies are needed, these results may present promising alternative materials for orthopedic applications. Specifically, through the use of anodization, an inexpensive, easy way to modify Ti to improve its performance can be realized. Moreover, through the use of a nanostructured porous PLGA coating on Ti, enhanced bone ingrowth may occur on Ti surfaces while PLGA degrades. Since this study utilized micron-sized Ti wells filled with



Figures 3 SEM images of anodized Ti substrates. Nano-scale surface features were evident, demonstrating that anodization created a nano-topography on Ti. Ti substrates were anodized in 5% hydrofluoric acid for 20 minutes at 10V. ((A): Scale bar = 10 μm ; (B): Scale bar = 1 μm). These are the nanoTi substrates.

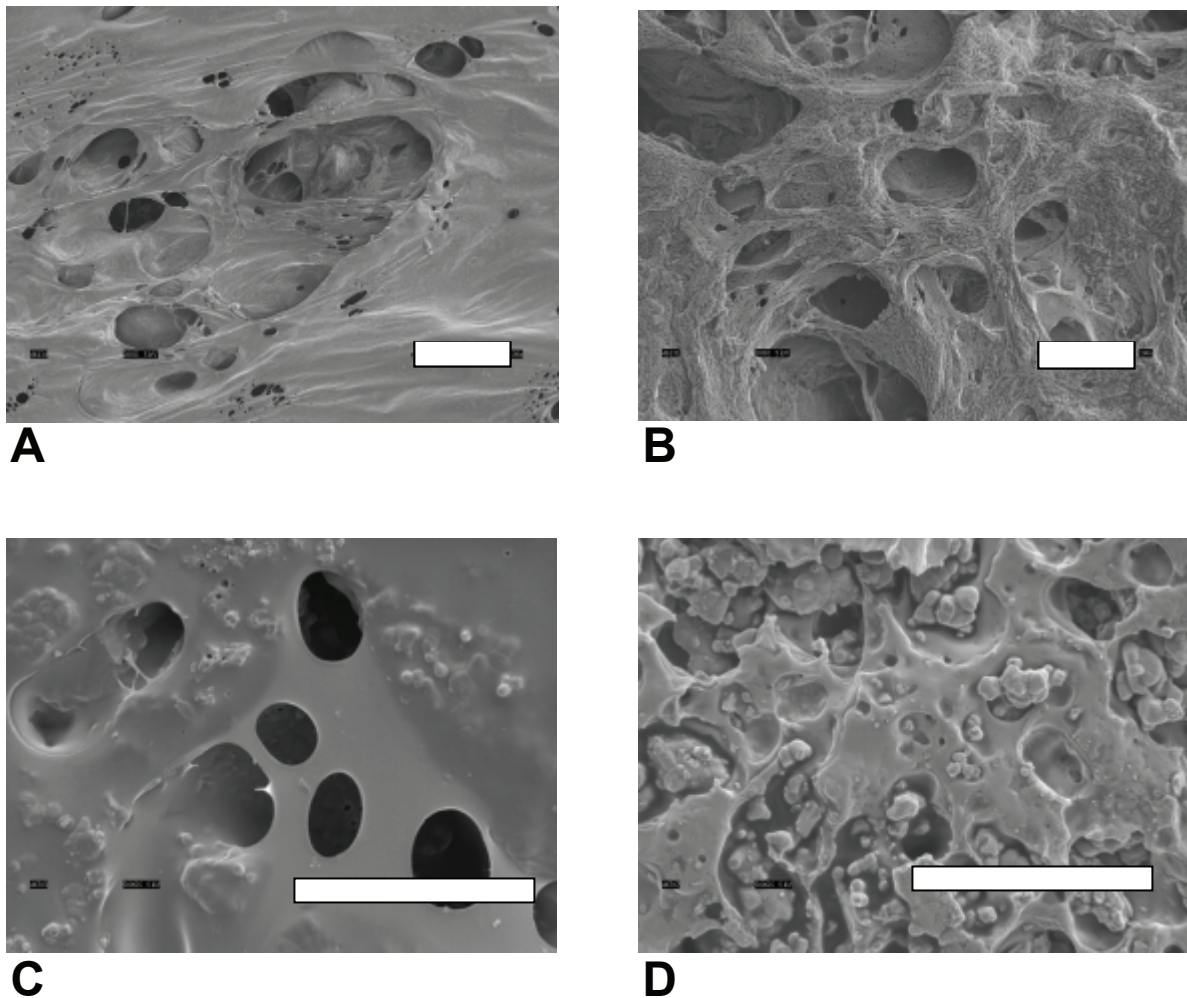


Figure 4 (A) SEM image of unetched porous PLGA coating on nanoTi (Scale bar = 100 µm). (B) SEM image of etched porous PLGA coating on nanoTi showing numerous micron-sized surface features (Scale bar = 100 µm). (C) SEM image of unetched porous PLGA coating on nanoTi (Scale bar = 1 µm). (D) SEM image of etched porous PLGA coating on nanoTi showing numerous nano-sized surface features (Scale bar = 1 µm). (B and D) are the nanoTi-nanoP substrate.

biodegradable PLGA, it is possible that even greater interlocking between newly formed bone and the implant may result. But even with this speculation, it is important to consider why osteoblast cell density was enhanced on these materials when created to have nanometer features in the present study.

Studies of cell responses on nano-surfaced metals (Webster and Ejirofor 2004), ceramics (Webster et al 2000b), and polymers (Kay et al 2002) support the concept that nano-topography increases osteoblast adhesion, proliferation, and deposition of a calcium-containing mineral. These studies suggest that nano-topographies mimic the natural bone surface better than conventional, currently-used implants. In addition, nanostructured surfaces have unique energetics to optimally interact with proteins to promote osteoblast adhesion. For example, studies have shown increased ad-

sorption of vitronectin on anodized compared to unanodized Ti which may be useful in explaining enhanced osteoblast cell densities between these two materials. In addition, altered initial interactions of proteins on etched compared to unetched PLGA have also been documented by previous studies. Specifically, greater initial adsorption of vitronectin and fibronectin on PLGA etched in a similar manner to that completed here was used to explain increased functions of chondrocytes (cartilage-synthesizing cells) on etched compared to unetched PLGA (Park et al 2005).

Although nanometer roughness may be one reason for increased osteoblast responses on nanoTi and nanoTi-nanoP compared to microTi, chemistry has also changed between the materials of interest in this study. For example, altered oxidation states of Ti may have changed osteoblast cell densities in this study. It is unclear at this time the exact

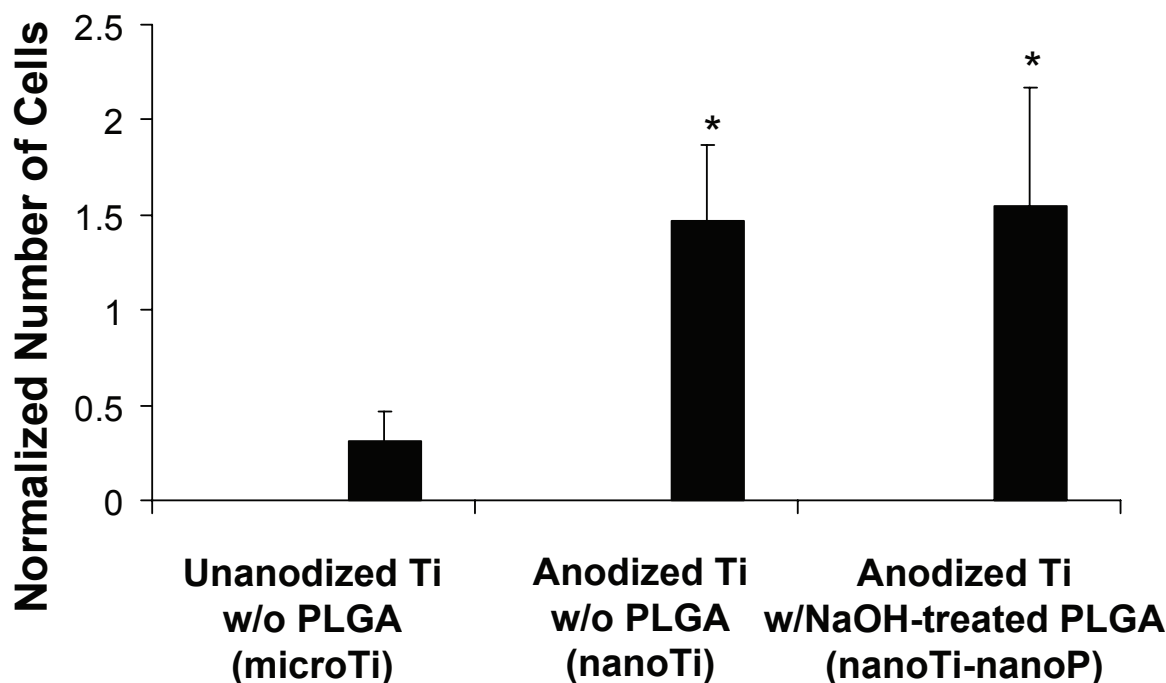


Figure 5 Increased osteoblast adhesion on nano-rough anodized Ti (nanoTi) and nano-rough etched PLGA coatings on nanoTi (nanoTi-nanoP). Data was normalized to etched glass reference. Data = mean \pm SEM; n = 6; *p < 0.05 (compared to unanodized Ti without PLGA or microTi).

reasons for greater cell density observed on nanoTi and nanoTi-nanoP.

None-the-less, to the best of our knowledge, this is the first study to look at the combined effects of anodizing Ti and etching PLGA on osteoblast cell density in a three dimensional coating structure. Applied to implants, the ability to enhance osteoblast proliferation on implant surfaces using nano-topography may increase host/implant stability and therefore decrease the chance of implant failure. Importantly, beyond etching current implant designs (like Ti and PLGA), there should be continued research in the development of a mechanically stable coating combined with optimal osteoblast responses to create better bone substitutes. A mechanically stable coating will ensure survival in a rigorous implantation process and dynamic loading conditions. Thus, coatings should be optimized through their porosity, pore size, nano-topography, and other relevant parameters to ensure that the engineered architecture is best suited to the formation of biologically and mechanically robust bone tissue at the implant interface. While this study centered on biological properties, mechanical properties can not be ignored.

Conclusions

This study showed that nano-topography can be added easily to Ti (through anodization) and porous PLGA (through

chemical etching) to enhance osteoblast cell density which may lead to enhanced orthopedic implant performance. This straight forward application of nano-topography presents a new paradigm in the design of better biomaterials for the orthopedic industry.

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