

# Macrophage Inflammatory Response to TiO<sub>2</sub> Nanotube Surfaces

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## ABSTRACT

*It is well known that the native oxide layer on titanium (Ti) implants is responsible for its superior biocompatibility and tissue integration. Recent efforts have targeted titanium dioxide (TiO<sub>2</sub>) as a good candidate for surface modification at the nanoscale, leading to improved nanotextures for enhancing host integration properties. Here we explore the in vitro inflammatory response of macrophages to TiO<sub>2</sub> nanotube surface structures with different diameters (30, 50, 70, and 100 nm) created by a simple electrochemical anodization process. This work was designed to study the nanosize effect for controlling and optimizing inflammatory response to a Ti implant surface utilizing nanotechnology. Using intracellular staining and flow cytometry for detecting macrophage TNF cytokine expression, we have found that 70 nm diameter nanotube surfaces have the best advantage in terms of diameter size by producing the weakest inflammatory response, compared to a commercially available Ti surface without oxide modification. We also present cell-free data on free radical scavenging using the nanotube surfaces with different diameters to test the removal of nitric oxide from solution; again, our findings indicate that 70 nm titanium dioxide nanotubes exhibit optimal removal of nitric oxide from solution, making them excellent candidates for use in medical devices that would benefit from decreased inflammatory response.*

**Keywords:** Macrophage, Inflammation, Nitric Oxide, Nanotube, TiO<sub>2</sub>

## 1. Introduction

Medical devices, which are commonly used to improve the health of patients, serve to repair joints, reopen blood vessels, and trigger electrical stimuli among numerous applications. Implantation of medical devices is complicated by the creation of trauma caused by the necessary surgery which is needed for the placement of these devices [1]. The natural wound healing sequel to this surgical trauma is in turn challenged by the presence of medical devices, leading to an alternate wound healing response around the device generally termed the “foreign body response.” The foreign body response is an inflammatory response to the implanted material, and the intensity of this immediate inflammatory response to implanted devices directly impacts healing around and tissue integration of the implant, as well as down-the-road functioning of the medical device [1].

Because the body “sees” the surface of the implant, many methods to change the surface properties of biomaterials have been undertaken in hopes of 1) improving the desired native cell/tissue adhesion and overall host

integration, 2) reducing the unwanted inflammatory response of macrophages and various defense cells, and 3) eliminating the subsequent foreign body response and rejection of the medical device. One method of inquiry towards improving the integration of materials with native tissue, has been to incorporate nanotopography on the surface, which presents a surface with features that are on the same nanoscale as the *in vivo* biological materials such as biomolecules or enzymes, proteins and extracellular matrices (e.g. collagen), cell surface receptors or integrins, etc. that are nanometer in dimension. When incorporated into culture surfaces, nanotopographies can vary greatly, e.g., nanoneedles, nanorods, nanopores or nanospheres, etc.

In terms of immune cell reactions to nanotopography, previous studies on macrophages grown on zinc oxide nanorods exhibited low viability on tall, thin nanorod surfaces of approximately 50 nm diameter [2]. On the other hand, titanium dioxide surfaces with nanofeatures slightly larger in diameter (~70 nm) and shorter in height have demonstrated much better macrophage adhesion and survival, but exhibited less production of inflamma-

tory cytokines by the macrophages cultured on the nanotopographic surfaces compared to a flat surface of titanium without a nanostructure [3]. Glass nanowires have also demonstrated a trend of greater *in vitro* inflammatory response with taller than shorter nanofeatures, although the same study did not detect a significant trend for inflammatory response related to the height for polymer nanofeatures [3]. In addition, the culture of macrophages on patterned nanofeatures using several different polymers exhibited no significant differences in the release of inflammatory cytokines [4]. Overall, it appears that there is no clear trend regarding macrophage response and inflammation due to nanotopography (for review see reference [5]). Therefore further studies to elucidate the effect of nanotopography on macrophage response would be extremely valuable for understanding and controlling the inflammatory cell behavior for implantable devices.

Several labs have been investigating the use of TiO<sub>2</sub> nanotubes as a titanium surface modification to the native metal-oxide for use as an improved biomaterial surface, and many interesting cellular responses have been observed with these materials [6-10]. This type of TiO<sub>2</sub> surface nano-configuration is advantageous in regulating many positive cell and tissue responses for various applications for tissue engineering and regenerative medicine; therefore it was chosen as the primary substrate of investigation in this research on the macrophage inflammatory cell response. Previously, when comparing the effect of different diameters or inner pore sizes of TiO<sub>2</sub> nanotubes, it was found that there were distinct size regimes for controlling the behaviors of osteoblast [7], chondrocyte [11], and mesenchymal stem cells [8,10,12]. As well, a study comparing 20 nm vs. 200 nm pores of aluminum oxide revealed unique differences in the inflammatory response of macrophages [13]. Because pore size seems to play an important role in controlled cell behavior, here we investigate the potential influence of differently sized TiO<sub>2</sub> nanotubes on the *in vitro* inflammatory responses of macrophages, and we hypothesize that the geometry of the nanotube may have significant impacts on the inflammatory cell behavior.

## 2. Materials and Methods

### 2.1. Substrate Preparation

Annealed titanium foil (0.25 mm thick) was purchased from Alpha Aesar (Ward Hill, MA) and cut into 2 cm × 5 cm samples. Samples were cleaned with an ultrasonic cleaner for an hour in acetone, liberally rinsed with DI water, and dried overnight in a 60°C oven. Nanotube surfaces were prepared in a 1:7 volumetric ratio of acetic acid (99.99% purity, Sigma-Aldrich) to 0.5% w/v hydro-

fluoric acid in water (48% w/v, EM Science, USA) by anodizing 2 cm × 5 cm samples for 30 minutes at different voltages (5 V, 10 V, 15 V, and 20 V). Surfaces were then rinsed liberally with DI water for at least 30 seconds and dried in a 60°C oven overnight. Following the creation of the TiO<sub>2</sub> nanotubes, samples of these and untreated titanium were placed in a tube furnace and baked at 500°C for 2 hours to anneal the material and crystallize the fabricated amorphous TiO<sub>2</sub> to anatase phase for optimal cell culture conditions, as the effect of crystallinity has already been determined and previously reported [6, 14]. Samples were then cut into 1 cm × 1 cm pieces for cell culture, and baked again in a tube furnace at 260°C for 2 hours to ensure the degradation of any endotoxin that may have been introduced to the samples through handling. Samples were further sterilized in an autoclave prior to use in cell culture and oxygen radical elimination experiments. The presence of nanotubes was visualized by scanning electron microscopy (SEM, XL30, FEI Co., USA).

### 2.2. Primary Cell Sourcing

Pathogen-free female C57BL/6 mice, 6 to 8 weeks old, were purchased from Charles River Laboratories. Mice were maintained in the University of California San Diego animal facilities, and were given sterile water, and mouse chow for the duration of the experiments. Animal guidelines for the care and use of laboratory animals have been observed; all experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of California San Diego.

Bone marrow cells were harvested from murine tibias and femurs and differentiated into macrophage cells using previously described methods [15-17]. Bone marrow cells were flushed from long bones, and then differentiated into bone-marrow derived macrophages (BMMΦ) by incubating in complete DMEM (cDMEM: DMEM supplemented with 10% heat inactivated FBS, 10% of supernatant from L-929 fibroblast cells (ATCC, Manassas, VA), 1% penicillin-streptomycin (Gibco®, Carlsbad, CA), 0.01M HEPES buffer (Gibco®, Carlsbad, CA), 1mM sodium pyruvate (Gibco®, Carlsbad, CA), and 1% of a 100X MEM non-essential amino acids solution (Gibco®, Carlsbad, CA)). The cells were cultured for 7 days on tissue culture treated dishes, with media changes every 2 days. Adherent day 7 cultured cells were selected as mature macrophages for further studies. This protocol has been shown to produce a mature macrophage phenotype [16]. Replicates are defined as cells from different mice. A minimum of three replicates were completed for all experiments.

### 2.3. Macrophage Adhesion and Growth on Substrates

Mature macrophages were removed from culture surfaces by rinsing with non-cationic PBS (Gibco®, Carlsbad, CA), soaking for 5 minutes in non-cationic PBS, and finally scraping with a cell scraper. Cell suspensions were counted, and 500,000 cells were seeded into each well of a 24-well plate containing one 1cm X 1cm model material in each well in the complete DMEM. Cells were allowed to adhere and proliferate for 24 hours. Samples were then rinsed 3 times with PBS, and fixed by soaking in a solution of 4% paraformaldehyde in PBS for 20 minutes. Nuclei were stained using DAPI (1:1000, Chemicon) in PBS overnight and rinsed 3 times with PBS. Substrates were mounted onto glass slides using Fluoromount-G (Southern Biotech), visualized and photographed using a LEICA DM IRB microscope. Five random fields were imaged from each sample, and nuclei were counted using Image J software (NIH).

### 2.4. Scanning Electron Microscopy (SEM) for Cell Morphological Examination

After 24 hours of incubation, the cells on the substrates were washed with PBS and fixed with 2.5 w/v% glutaraldehyde (Sigma, USA) in PBS for 1 hour. After fixation, they were washed three times with PBS for 15 minutes per wash. Then the cells were dehydrated in a graded series of ethanol (50%, 70%, 90%, and 100% v/v) for 30 minutes each and left in 100% ethanol until they were dried with a critical point dryer (EMS 850, Electron Microscopy Science Co., USA). Next, the dried samples were sputter-coated with metal for SEM (scanning electron microscopy) examination. The morphology of the adhered cells were observed using SEM (XL30, FEI Co., USA).

### 2.5. Macrophage Cytokine Expression Following Exposure to Experimental Substrates

Measurement of intracellular TNF in all cell types from all substrate cultures were conducted by plating cells at sub-confluent levels in tissue culture polystyrene (TCPS) dishes (used as a control), and in 24-well plates with 1 cm × 1 cm substrates with 1 μL/mL of monensin (ebiosciences, San Diego, CA). In a separate TCPS dish, 5 μg/mL LPS (E. coli LPS, Sigma-Aldrich, St. Louis, MO) was added for a positive control. The cells were incubated for 8 hours under normal culture conditions. The treatment in this protocol stops the export of cellular products, thus allowing for the buildup of cytokines within the cell. The cells were removed from the culture surface, fixed and permeabilized in suspension using a fixation and permeabilization kit (ebiosciences, San Diego, CA) and finally stained for intracellular tumor necrosis factor,

TNF- $\alpha$  (clone MP6-XT22, rat IgG1). Antibodies were purchased from eBioscience (San Diego, CA) as direct conjugates of FITC, and PE, respectively. Data acquisition and analysis for this study were done using a FAC-Scan (BD Biosciences, Mountain View, CA), CellQuest TM software (BD Biosciences, San Jose, CA), and WinMDI software (Joseph Trotter, The Scripps Research Institute, San Diego, CA). Data presented represent at least three replicates, with each replicate of cells coming from a separate mouse.

### 2.6. Surface Interaction with Nitric Oxide

To investigate the nitric oxide quenching properties of difference surfaces, an NO donor DPTA-NO was used to make a solution of known NO concentration, and a 500 μL sample of this solution was then subjected to each of the surfaces for 15 minutes. Samples in duplicate were taken from each well and a Griess reagent was used to stabilize the NO concentration by converting it into nitrite (NO<sub>2</sub>). The measured concentration of NO<sub>2</sub> in each of the extracted samples correlates to the remaining concentration of NO after the surfaces have scavenged some of free radicals. The concentration of the total nitrate was determined from the absorbance at wavelength  $\lambda = 550$  nm by using a UV-Vis spectrophotometer (BiomateTM 3, Thermo Electron Co., USA) and calculated with the aid of a dilution standard curve.

### 2.7. Statistical Significance

All bar graphs are displayed as the mean  $\pm$  standard error. Sigma Plot software (2001) which specializes in scientific data analysis and presentation, was utilized for demonstrating statistical significance for the assays. One-way ANOVAs were performed using the pairwise multiple comparison procedure.

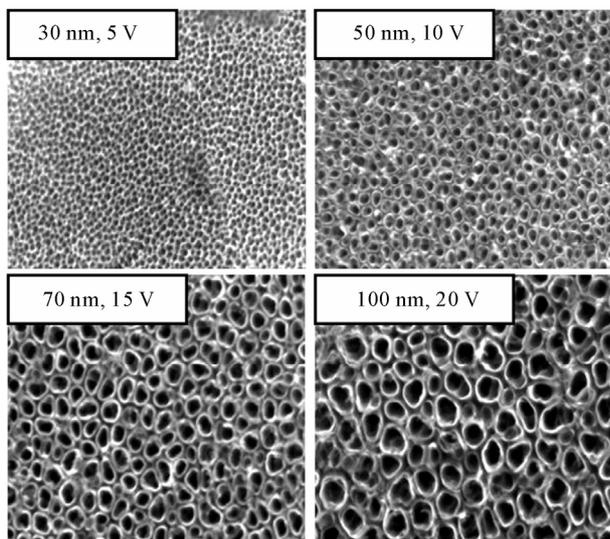
## 3. Results

### 3.1. Substrate Properties Are Consistent with Previously Published Results

The experimental TiO<sub>2</sub> nanotube surfaces appear similar to previously fabricated surfaces [6-8,11,12,18], and have similar size characteristics and discrete uniform geometries (**Figure 1**). **Figure 1** shows highly ordered, vertically aligned nanotube structures with different diameters fabricated by varying the anodization potential. Applied voltage for creating nanotube surfaces with different diameters were from the same as in previous studies: 5 V = 30 nm, 10 V = 50 nm, 15 V = 70 nm, 20 V = 100 nm.

### 3.2. Macrophage Adhesion to Substrates Following 24 Hours of Culture

As seen in **Figure 2**, the number of adhered macrophages



**Figure 1. Physical characteristics of titanium dioxide nanotube surfaces.** SEM images of nanotube surfaces and measurements of nanotube physical dimensions are reproducible and consistent with previous reports.

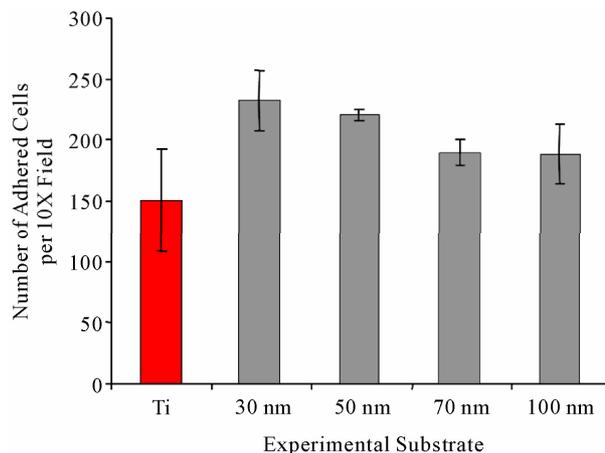
to unmodified commercial Ti (with thin native oxide layer) and anodized TiO<sub>2</sub> nanotube substrates is independent of oxide structure. The average number of cells per 10X microscopic field ranged from 150 on titanium to 230 on 30 nm TiO<sub>2</sub> nanotube surfaces, with the anodized TiO<sub>2</sub> surfaces generally supporting more cell adhesion. Although there was a general trend of a reduction in the number of adherent macrophages as the nanotube size increased, this was not statistically significant. No significant differences in cell adhesion among the substrates were observed.

### 3.3. Macrophage Morphology on the Experimental Substrates

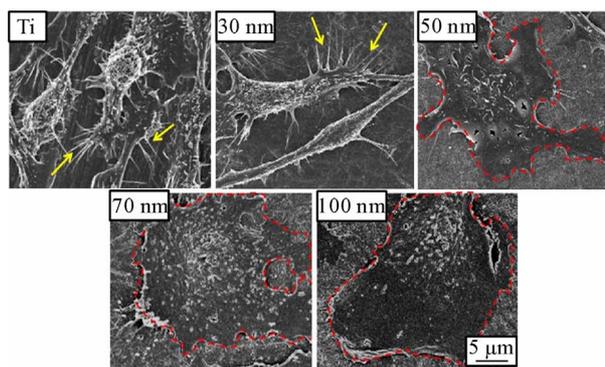
**Figure 3** illustrates SEM micrographs of macrophages on the Ti and TiO<sub>2</sub> nanotube surfaces after 24 hours of incubation, which is an adequate time to reveal the acute inflammatory response on the different surfaces. The reaction of the cells to the surfaces can be seen from cell-spreading, ruffled membranes, and extended filipodia. On the smaller 30 nm diameter and flat Ti, there seemed to be more filipodia extensions, with a spiky appearance of the cell, as shown by the arrows in **Figure 3**. Larger spreading areas with less filipodia are seen on the 50 - 100 nm TiO<sub>2</sub> surfaces. Both the cell spreading and filipodia extension are typical signs of macrophage activation.

### 3.4. Production of the Inflammatory Cytokine TNF

Tumor necrosis factor (TNF) is a cytokine involved in inflammation and is a member of a group of cytokines

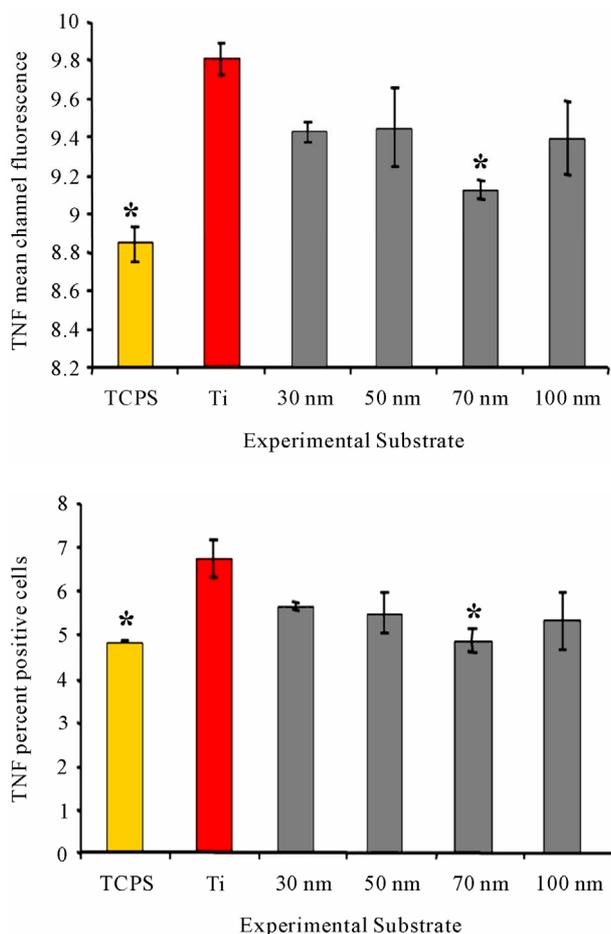


**Figure 2. Macrophage adhesion to substrates is independent of nanotexture.** No significant differences were observed in the number of adherent macrophages on substrates following 24 hours of culture.



**Figure 3. Macrophage morphology.** The SEM images show macrophages on the experimental surfaces after 24 hours of incubation. Arrow indicate abundant filipodia extensions on the Ti and 30 nm TiO<sub>2</sub> nanotubes. Dotted lines show cell outlines and large spreading area on the 50 - 100 nm TiO<sub>2</sub> nanotubes surfaces.

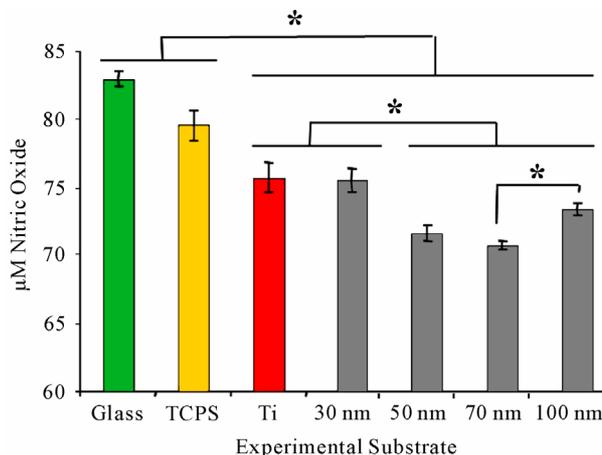
that stimulate the acute phase reaction. This cytokine was used to test the inflammatory response of the macrophages to the different surfaces. As presented in **Figure 4**, the production of TNF per cell varied on the different substrates. Mean channel fluorescence (MCF) values exhibited several significant differences for cells grown on the various substrates. MCF for cells grown on tissue culture polystyrene was 8.85, a value significantly lower than that for cells cultured on flat titanium surfaces (9.81). Cells grown on 30, 50 and 100 nm surfaces had no significant differences in MCF values compared to other surfaces (9.43, 9.45 and 9.40, respectively). However, cells cultured on 70 nm surfaces had significantly lower MCF values than flat Ti (9.13). Percent positive data show no significant differences among the culture substrates.



**Figure 4.** Inflammatory cytokine production per cell following 8 hours of culture on substrates. The production of the inflammatory cytokine TNF was dependent on the surface of the experimental substrate expressed by mean channel fluorescence and percent positive cells. \*indicates statistical significance  $p < 0.05$  compared to flat titanium surfaces.

### 3.5. Oxygen Radical Removal from Solution by Substrates

NO is generated and secreted as free radicals by macrophages as part of the natural immune response which causes a cascade of signaling for inflammation. In order to predict how the surfaces would scavenge the free radicals and lower the inflammatory response, a cell-free experiment was conducted to assess how the surfaces could clean away or remove free radicals from solution. In **Figure 5**, when compared to glass and TCPS (used as control surfaces), all substrates removed significantly more NO<sup>-</sup> from solution, with solutions exposed to glass and polystyrene having NO<sup>-</sup> concentrations of 82.9 and 79.6  $\mu$ M, respectively. Flat Ti and 30 nm nanotube surfaces had similar NO<sup>-</sup> concentrations, 75.7 and 75.5  $\mu$ M, respectively. Nanotube surfaces with diameters greater



**Figure 5.** Nitric oxide quenching by surface. The removal of nitric oxide from solutions following 20 minutes of incubation with substrates was very dependent on the surface of the experimental substrate. \*indicates statistical significance with  $p < 0.05$ .

than 30 nm performed even better, with 71.6, 70.7, and 73.4  $\mu$ M in solutions exposed to 50, 70, and 100 nm nanotube surfaces, respectively. The 70 nm surface reduced the amount of available NO<sup>-</sup> most effectively (**Figure 5**).

## 4. Discussion

We have chosen to evaluate the response of macrophages to TiO<sub>2</sub> nanotubes with different diameters, with the intention of getting a more complete understanding of the effect of nanotube size on the inflammatory system for biomedical implant purposes. Our results have shown that the nanotube size of the material studied affects the macrophage activation. The different activation states were reflected by morphological changes and secretion levels of TNF, a proinflammatory cytokine.

The results indicate that macrophage adhesion is higher on the nanostructured modified surfaces over unmodified Ti (**Figure 2**); this agrees with the general notion that nanoscale surface structuring increases cell adhesion over microstructures and commercially flat surfaces [6,7,19,20]. It could be speculated that the increased surface contact area due to the introduction of the nanostructure may be responsible for the increase in macrophage adhesion, however further research beyond the scope of this report is needed to clarify this speculation. Although there was no significant difference in adhesion relative to the size of the nanotube, it appears that the smallest diameter tends to have the greatest number of adhered cells, which is in agreement with other cell adhesion studies on the nanotube surfaces with varied diameters [8,10,12]. A recent study on macrophages cultured on nanoporous aluminum also showed an increase

in adhesion on the small 20 nm pore size in comparison to the larger 200 nm pore size [13].

Activated macrophages present a large number of morphologic, functional, and metabolic differences from normal resting cells. They are larger in size, and display pronounced ruffling of the plasma membrane, increased capacity for adherence and spreading on surfaces, increased formation of pseudopods, as well as functional differences [21]. Macrophage spreading is the preliminary step in macrophage activation and considered to be an important marker of this event [22]. The active process of spreading represents alterations including filipodia and rough plasma membranes. It appears that cells present on the experimental surfaces had activated morphologies with ruffled membranes and filipodia extensions. The Ti and 30 nm TiO<sub>2</sub> nanotubes had extensive filipodia, whereas less filipodia but greater spreading area with some membrane ruffling was observed on the 50 - 100 nm TiO<sub>2</sub> surfaces (**Figure 3**). Because cells on the Ti and 30 nm TiO<sub>2</sub> show more abundant and well-established filipodia extensions, it can be assumed that there was a higher degree of activation on these surfaces. Although less filipodia were observed on the 50 - 100 nm nanotubes, cells exhibited a larger spreading area. While the mechanism for filipodia activation (Ti and 30 nm TiO<sub>2</sub>) vs. cell spreading (50 - 100 nm TiO<sub>2</sub>) on the different nanotube diameters was not determined in the scope of this report it would be valuable in the future to shed light on this morphological phenomenon in determining the immune response.

A previous study on macrophage production of inflammatory cytokines showed higher production of many cytokines on flat Ti compared to nanostructured TiO<sub>2</sub> [3]. In the current study on TNF expression on similar nanostructured surfaces with the same chemistry but varying geometries, we observed the same general trend of lower TNF production by cells on the nanotubes, suggesting that the nanotopography itself contributes to lowering the TNF production. No statistically significant correlation was observed as a function of the diameter size, but the largest decrease in TNF values was significant for macrophages cultured on 70 nm nanotube surfaces (**Figure 4**). A possible reason for the lower inflammatory response in general of the TiO<sub>2</sub> nanotube surfaces could be due to radical quenching by the surface, a known property of TiO<sub>2</sub> [23,24], which was observed in our results with higher levels of NO<sup>-</sup> quenching on TiO<sub>2</sub> nanotube surfaces compared to the other experimental materials (**Figure 5**). Oxygen radical production is a common occurrence in inflammation [25], and the reduction of oxygen radicals at the surface of a biomaterial could reduce the local inflammatory response. Although the mechanism for oxygen radical reduction is not explained

by the extent of this report, it appears that the 70 nm diameter nanotubes are most advantageous in terms of radical reduction for decreasing the inflammatory response to the surface. Interestingly, the surfaces that had the most radical reduction or oxygen quenching, *i.e.* 50 - 100 nm TiO<sub>2</sub> nanotubes (**Figure 5**), showed the least amount of filipodia activation (**Figure 3**) and possibly a lower degree of inflammatory response of the macrophages. However, to shed further light on the complete understanding the effect of surface nanotopography on macrophage behaviors, additional studies are needed to determine the fate of the cells as well as the secretion of other growth factors.

In summary, this study demonstrated that the TiO<sub>2</sub> nanotubes with 70 nm diameter is the surface with the lowest level of macrophage morphological activation, the lowest macrophage production of inflammatory cytokines, and the greatest oxygen radical quenching capability. Hence, TiO<sub>2</sub> nanotubes in the ~70 nm diameter regime is most promising for implant surfaces for decreased inflammatory response. It should be noted that larger nanotubes in the ~70 - 100 nm range elicited a favorable response in terms of chondrocytes and osteoblast cells [7,11], mature cells also derived from mesenchymal cells in general.

While TiO<sub>2</sub> nanotubes surfaces are well known for orthopedic implant technologies, it is advantageous and beneficial to consider this type of surface nanostructure for other biomedical implant technologies including vascular stent applications. In our previous work exploring the possibility of utilizing TiO<sub>2</sub> nanotubes as a possible Ti or NiTi surface modifications in arterial stents, we have found that the TiO<sub>2</sub> nanotube surface structuring is excellent for the growth, mobility, and endothelialization of vascular luminal surface [18]. Collectively, the reduction of inflammation and the quenching of oxygen radicals shown in the present study bolster the potential use of TiO<sub>2</sub> nanotubes for stents, especially because nitric oxide synthase has been shown to co-localize with atherosclerotic plaques [26].

## 5. Conclusions

The present work shows that by changing the diameter of TiO<sub>2</sub> nanotubes, in the range of 30 - 100 nm, it is possible to modulate the macrophage and inflammatory response. In addition, these findings open the possibility of exploiting some of the beneficial material properties of TiO<sub>2</sub> as oxygen radical scavengers. In general, it was found that TiO<sub>2</sub> nanotubes surfaces had lower macrophage activation, decreased levels of TNF cytokine expression, as well as increased ability to quench free radicals, resulting in lower inflammatory effects compared to conventional Ti. The nanotube surface with ~70 nm di-

ameter has the greatest effect in reducing the inflammatory response. This study emphasizes the role of nanotopography in dictating inflammatory cell responses and demonstrates that nanotopography can be utilized to control the inflammatory likelihood of medical implants.

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