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Paper

IN VITRO RESPONSE OF OSTEOBLASTIC AND FIBROBLASTIC CELLS TO WATER-JET TREATED TITANIUM SURFACES.

A.J. Steeves, and F. Variola University of Ottawa, Ottawa, Ontario, Canada

M. Vijay, A. Tieu, W. Yan, M. Xu and B. Daniels VLN Advanced Technologies Inc., Ottawa, Ontario, Canada

ABSTRACT

Small-scale interactions between orthopaedic implants and biological systems impact how well devices will integrate in the human body. The response to these implant interfaces is fundamentally affected by the nature of the biomaterial that it is composed of, with key characteristics including surface chemistry and topography. It is generally accepted that rough implant surfaces have a more pronounced and beneficial influence on cellular activity than smooth ones. For this reason, methods to roughen metallic surfaces, such as sandblasting and acid etching, are currently used in the biomedical sector. In collaboration with VLN Advanced Technologies Inc, we propose to demonstrate the potential of Pulsed Water Jet (PWJ) as an effective method to modify the surface of titanium, the gold standard for biomedical implants, with a technique which only uses water. Inspired by previous collaborative work, we assessed the efficacy of PWJ modified titanium on the bioactivity of a fibroblastic cells (NIH-3T3), reflective of possible types encountered in current and future therapeutic implant implementations. Preliminary results have shown that PWJ modification of titanium is effective in the generation of surfaces with distinct nano- and microscale structural attributes which follow a gradient correlated with variable nozzle speeds. Preliminary results indicate cell-specific differential effects of these surfaces with larger fibroblastic NIH3T3 cells preferring balanced surfaces generated from a nozzle speed of 800 mm/s. Ultimately, the application of this environmentally-friendly and contaminant-free technique to industrial processes will permit to obtain implants that integrates in bone more rapidly while decreasing the manufacturing costs.

> KEYWORDS Titanium; Water jet; Surface characterization; Fibroblast; Osteoblast

1. INTRODUCTION

Whether due to age, lifestyle or by an accident, the replacement of teeth with dental implants has become a reliable and standard procedure with the number of prostheses designs extending beyond 1300 different implant systems.(Smeets et al.) Standard periodontal implants are composed of three main components: crown, abutment and implant (Figure 1A,B). Of critical importance is the implant ("screw") portion of the prostheses as it is the portion in direct contact with the jaw bone. The interaction between this screw and the bone ("osteo-") is the major determinant of the of implant success as this will influence how the implant will integrate/fuse with the patient tissue ("osseointegration").(von Wilmowsky et al.) In particular, numerous studies have demonstrated that the strength, and hence the performance, of osseointegrated implants is not only a function of the local bone quality and quantity (primary stability), but it also depends on whether fibrous encapsulation occurs (secondary stability).(Albrektsson et al.) Such phenomenon is produced when an interposing layer of connective tissue (e.g., collagen or fibroblastic matrix) forms between the surface of an implanted device and the surrounding bone, as a result of the wound healing process initiated by the introduction of a synthetic biomaterial. Such fibrous capsule is generally caused by micro-movements of primary unstable implants during early phases of healing, and is particularly detrimental for load-bearing devices such as the orthopedic (e.g. femoral stem) and dental (e.g. screw) devices, ultimately resulting in a compromised osseointegration.(Isaacson and Jeyapalina) In order to improve osseointegration of orthopedic and dental implants, significant efforts by the scientific community have focused on surface modification of implantable materials, in particular titanium, the gold standard in implantology.(Variola, Brunski, et al.) The goal is creating a surface capable of promoting the activity of bone cells while hampering that of fibroblasts (which represent the cells in oral gums), in order to secure strong secondary stability by eliciting bone formation and discouraging fibrosis encapsulation.(Variola, Yi, et al.) However, for dental screws, while the ability of limiting the formation of a layer of connective tissue is advantageous for the implant's osseointegration, it may also prevent, in turn, the creation of an effective biological seal at the implant-soft tissue interface. Such biological seal is considered a dominant factor from the clinical point of view to ensure the long success of peri-implant health.(Chai et al.) Peri-implantisis, a site-specific infectious disease, can in fact cause bone loss around an osseointegrated dental implant, thereby compromising its functions performance.(Prathapachandran and Suresh) For this reason, achieving the capacity of modulating the response of fibroblasts by varying the surface properties of a titanium implant promises to unlock the key to engender dental implants that simultaneously discourage fibrous encapsulation but favour the creation of an effective interface with the gum. Previous work demonstrated that water jet treated surfaces have the ability to favour the proliferation of osteoblastic cells.(Steeves et al.) However, no studies to date have addressed the potential of water jet treated surfaces to modulate the response of fibroblasts for applications in dentistry.

The method and degree in which the dental implant component surfaces are treated prior to implantation has a critical effect on its success and longevity. As of 2015, the top methods of surface treatments currently employed sand blasting (\sim 5%), SLA (\sim 11%), acid etching (\sim 15%) and spray plasma coating (\sim 40%).(Jemat et al.) While these techniques have been successful in surface preparation, each comes with benefits and shortcomings such as production time, cost and the presence of contaminant by-product such as the embedment of abrasive particles from sand-blasting (**Figure 1C**). The use of a pulsed water jet (PWJ) may open the opportunity to not only fabricate thread geometries and surface prep in the same step, but also to do so in a sterile



Figure 1. Periodontal implants. (A) Components of a common dental implant.(B) Examples of the various implant component designs. (C) Abrasive contamination from sand-blasted surface preparation.

environment (e.g., sterile water source, antibacterial additives) which would lead to a significant drop in manufacturing cost and possibility of compact fabrication machines in the dentist office. For these reasons, we decided to focus this work to generate an extended study to understand how the PWJ can generate optimal surfaces for the response of fibroblast cells (presented herein) with the additional response by osteoblastic cells and human stem cells currently in preparation.

2. EXPERIMENTAL SETUP AND PROCEDURE

2.1 Sample Preparation

30x20x1mm pieces of 99.2%-pure Titanium foil (Alfa Aesar, USA; Cat# 44484) were surface prepped in a PurePulse waterjet booth (Pratt & Whitney Automation, USA). The setup combined a heavy-duty turntable, high pressure pump (NLB Corporation, USA), robotic system with integrated HMI (FANUC, USA) as well as a water collection and filtration system (**Figure 2**). **Table 1** contains both the general operating parameters of the apparatus. **Table 2** contains the specific nozzle transverse speeds which generated the four experimental surface topographies ("E#"). Untreated titanium was kept as the control surface ("CTL").

Table 1. General operating parameters

Parameter	Value
Orifice Diameter (mm)	1.016
Pump Pressure (MPa)	69
FPWJ Frequency (kHz)	40

I	able	2.	Experimental	nozzle	transverse speeds	
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Sample Description	Nozzle Speed	Stand-off Distance
Experimental = "E"	(mm/s)	(mm)
E45	450	3.5
E50	500	2.5
E60	600	2
E80	800	1.5

2.2 Surface Characterization

Visualization of the respective surfaces was achieved with the bright-field function of the alpha300 RSA system (WITec, Germany). Low magnification fields were visualized with an. To visualize focal adhesions, high-magnification 2x2 z-stack tiles were imaged with a 5x ApoPlan objective (NA=0.25, Zeiss, Germany). High magnification fields were visualized with an 20x Plan-Apo objective (NA=0.8, Zeiss). A series of images were taken at different positions in the z-axis ("z-stack") and were merged into a single plane with the depth of field "Stack Focuser" module of FIJI (ImageJ).(Schneider et al.; Schindelin et al.) Microscale roughness was assessed through the range analysis of the brightfield stacks.

Atomic force microscopy (AFM) analysis of surface topography was completed on the alpha300 RSA system (WITec). The Non-Contact (NC)-AFM mode, which resonates a nanometric tip



Figure 2. General view of PurePulse/PWJ experimental setup for Ti surface preparation

within angstroms above the surface as it raster scans, was used to prevent detrimental contact and wearing of the tip as a result of the significant differences in surface topography resulting from the surface erosion. 10 x 10 μ m high-resolution scans (512 x 512) were imaged with the rectangular cantilever of the RFESPA-40 chip (Bruker, USA) with a tip that is characterized by a rotated pyramidal tip with a nominal radius of 8 nm, resonant frequency of 40 kHz and spring constant of 0.9 Nm⁻¹. Source files were imported into Gwyddion and processed for measures of surface area (SA) and roughness (RMS).(Nečas and Klapetek)

Raman and FTIR spectroscopy were carried out to give insight into characteristics of the titanium oxide layer (e.g., crystallinity). Raman spectroscopic analysis was carried out with the Raman module of the alpha300 RSA system. The pairing of a 50x Epiplan-Neofluar objective (NA=0.55, Zeiss) and Xtra 632 nm laser (35 mW, Toptica Photonics, Germany) was used for bulk surface spectra collection which consisted of 6 scans at 10 sec / scan. FTIR spectroscopic analysis was carried out with a Nexus 870 FTIR spectrometer (Thermo Nicolet, USA) equipped with the SAGA accessory (smart aperture grazing angle, 80° with respect to the surface normal; Thermo Nicolet) with an 8-mm-diameter opening. Spectroscopic information was collected in the 400–2000 cm⁻¹ range with a 4 cm⁻¹ resolution (256 scans per spectrum). Infrared data were analyzed by using the OriginPro software (OriginLabs, USA). Spectra were smoothed with the adjacent-averaging method and, after linear baseline subtraction, fitted with Gaussian functions to resolve secondary vibrational components according to previously published literature.(Variola, Nanci, et al.)

2.3 Cell Culture

NIH/3T3 (mouse fibroblast, ATCC, USA) cells were cultured in standard Dulbecco's Modified Eagle Medium (DMEM, Corning, USA) supplemented with 4.5 g/L glucose, L-glutamine, 10% fetal bovine serum (FBS, Gibco, USA), 20 U/ml⁻¹ penicillin and streptomycin (Gibco). Culturing took place in a humidified 5% CO₂ water jacketed incubator at 37°C. Cells were detached from surfaces using appropriate volumes of 0.25% Trypsin (Thermo Fisher, USA) and incubated at 37°C for 5 min. Cells were collected and centrifuged at 190 g for 8 minutes. Successively, the supernatant was discarded, and pellet reconstituted in fresh culture media to desired concentrations specific to the experiment to a total volume of 500 µl per well.

After incubation at different experimental intervals, cells were fixed at room temperature with 4%-PFA for 10 minutes, washed with 1x-PBS and permeabilized with 0.25% Triton-X100 (Sigma-Aldrich) for 10 minutes. Nuclei were stained with NucBlue ReadyProbes (Thermo Fisher) and the actin cytoskeleton was stained via Rhodamine conjugated Phalloidin (Thermo Fisher). Multichannel images were captured with an AxioObserver.Z1 inverted microscope (Zeiss) fitted with the Zeiss Filter Set 49 (Hoechst) and Chroma Filter Set 49005 (Rhodamine). Low-magnification tiles of the surfaces were imaged with a 10x A-Plan (Ph2) Objective (NA=0.25, Zeiss) for assessment of cell proliferation. Mid-magnification tiles of the surfaces were imaged with a 20x Plan-Apo (Ph2, DIC II) Objective (NA=0.8, Zeiss) for assessment of general morphology and cell spreading. Image collections were stitched together with AxioVision Mosaix Software (Zeiss) and focus stacked with Fiji. For the measurement of cell morphology, characteristics and proliferation, multiple custom pipelines were created in CellProfiler.(Carpenter et al.) Three main measurements were scrutinized for the cell structure, namely cell area, perimeter and form factor. Cell area was calculated by counting the scaled number of pixels within the identified cells. Perimeter was calculated by measuring the number of pixels around the boundary of the identified cell periphery. Form factor, calculated as $4^{*}\pi^{*}$ (Area/Perimeter²), is the measure of a cell circularity: a value of 1 is representative of a perfectly circular cell while 0 indicates a linear cell. All data were aggregated, and statistical analysis performed in OriginPro.

3. EXPERIMENTAL RESULTS AND DISCUSSION

3.1 Surface Analyses

Surfaces were first visualized with upright light microscopy (LM) by 5x and 20x objectives in addition to gross morphology by handheld camera (**Figure 3**). Due to the thinness of the 20x objective focal plane versus the roughness of the materials, a sequential 3D stack of focal planes was obtained and an extended depth of focus (EDF) was generated with software to provide a 2D image with all portions in focus (**Figure 3C**). 3D data from these images were processed and microscale roughness was determined, during which it was determined that the E45 surfaces were critically rougher than all other surfaces, seemingly in a non-linear relationship to nozzle-speed (**Figure 4E**).

To better understand the impact of these morphologies at the scale that cellular structures would be identifying and interacting with (nanoscale; 10^{-9} m), we employed non-contact atomic force microscopy (NC-AFM) to for super-resolution visualization of surface topography. NC-AFM represents an in-direct method of cantilever-based nanoscale microscopy. The technique oscillates a microscale cantilever with a nanoscale tip tens of angstroms (Å) above the surface. Interaction with Van der Waals forces will lead to deviations in amplitude or frequency of the cantilever's oscillation which, as a non-destructive method, can be translated to topographical and phase data. 10 x 10 µm fields were imaged (Figure 4A) and processed for determination of nanoscale roughness, range and area enhancement resulting from treatment (Figure 4B-D). Nanoscale roughness was measured with the arithmetic mean (Sa) and surface area enhancement was estimated by the total surface area from the 3D data versus the projected area. For example, in a 10 x 10 μ m field, the projected area is 100 μ m² and a total surface area (due to structural morphology) of 115 μ m² would result in a 15% enhancement. A completely flat surface would have an enhancement of 0%. While E45 presents with the greatest microscale range, E50 was found to have the greater nanoscale roughness and area enhancement (Figure 4E). This may be due to there being a balance between the generation of nanometric structures from the water slug spacing which resulted in elevated nanoscale roughness while preventing a drastic increase in deeper and irregular structures that would result in higher microscale roughness. As expected, E60 presented with higher nanoscale roughness and range, compared to slower speeds, however E80 saw a considerable loss. This may be due to the faster nozzle spacing the slugs far enough that the treatment creates a more uniform and with reduced incidence of deep erosion.

During the surface treatment and erosion of the material, the titanium oxide layer (TiO_2) is removed. While re-passivation occurs relatively quickly, the localized presence of heat effects from friction can lead to the generation of distinct oxide layer characteristics.(Vergara et al.) This TiO_2 layer is known to be critical in the cellular response, with specific mineral forms being able to influence behaviour.(Mazare et al.) To better understand the newly formed oxide layer, we used



Figure 3. Visualization of treated titanium surfaces. (A) Gross visualization from handheld camera with enhanced contrast. (**B**,**C**) Light microscopy at (**B**) 5x and (**C**) 20x magnification.



Figure 4. Analysis of treated surfaces with Atomic Force Microscopy. (A) Non-contact 3Dreconstructions of titanium surfaces. (B-E) Analyses of (B) roughness, (C) area enhancement, (D) nanoscale and (E) microscale ranges.



Figure 5. Spectroscopic analysis of titanium oxide films. (A) Example of an amorphous titanium oxide film containing Raman peaks seen in anatase and rutile forms. (B) Raman analysis of E50 with the blue region representing the field seen in (A). This represents an example of the waveforms seen on all surfaces. (B) Representative Fourier Transform InfraRed (FTIR) spectroscopic analysis of E60 and Control samples.

spectroscopic analysis through Raman and Fourier Transform Infrared (FTIR) spectroscopy to determine the crystallinity and thickness of the oxide layer. Raman analysis found that the fingerprint of all surfaces was found to be representative of an amorphous oxide layer, exemplified in **Figure 5B**, as seen with the presence of both anatase and rutile spectral bands (**Figure 5A**).(Hardcastle et al.) FTIR spectroscopy, which can be employed to determine the thickness of the oxide layer, showed no meaningful differences between control and treated surfaces.(Variola, Nanci, et al.) When taken together, spectroscopic analysis suggests that the use of PWJ does not impact the crystallinity of TiO_2 .

3.2 Biological Analyses

Following the surface analyses, we concluded that the use of E50 surfaces were unnecessary and so only the remaining four surfaces were tested (CTL, E45, E60, E80). NIH-3T3 fibroblastic cells, representing those that exist in oral gums, were cultured on their respective surfaces for 48 hours to determine the short-term response to the titanium, mainly proliferation and cellular morphology. NIH-3T3 cells were found to have their proliferation perturbed on rougher surfaces (E45, E60) while the E80 surfaces showed comparable rates to that seen on control titanium (Figure 6A-D,I). This may be an indication that the cavities produced are incompatible with the size of a normally spread NIH-3T3 (CTRL = \sim 50-70µm) with those on the E45 and E60 surfaces presenting with sized of 25-40 and 35-50 µm, respectively (Figure 6E-H). The presence microscale roughness on E45 that is close to the minimum size of the cells is thought to lead to an inability to extend cellular structures and generate tension across the cytoskeleton for effective spreading. While the CTL surfaces were comparable to that found in E80, it is important to note that the initial surfaces which were composes of cold-rolled unpolished titanium. This CTL titanium presented with an heterogeneous morphology with irregular and stochastic damage from handling, generation and storage which in turn prevents any appropriate control of cell response. By treating these surfaces, at minimum, we are able to generate a comparably homogeneous surface with better predictability on the biological response which may then be translated to downstream dental implants.

4. CONCLUSIONS

In conclusion, our work indicates a cell-specific preference for titanium surfaces with surface morphologies generated with our pulsed water jet technology. While these surfaces consistently presented with amorphous oxide layers, the differences in roughness contributed to the preference of fibroblast cells (representing the gums of the mouth) for smoother surfaces (800 mm/s) with previous work indicating and osteoblast-like cells (representing bone of the tooth/jaw) prefer rougher surfaces (450 mm/s). These results point to the potential generation and surface preparation of periodontal implants in such a manner that it will promote the integration of said implants in bone and thus the success rate. Continuing work will investigate the effects that these surfaces may influence the behaviour of human stem cells, including the potential for their differentiation into new bone cells, which can assist implantation in individuals with health issues (e.g., osteoporosis) and advanced age.



Figure 6. Cellular analyses of cells on treated titanium. (A-H) Fluorescence microscopy of NIH-3T3 cells at the (A-D) global level and (E-H) +50% zoom. Sample orientation is (A,E) CTL, (B,F) E45, (C,G) E60 and (D,H) E80. *Blue* represents the nuclei and *red* represents the actin cytoskeleton of the cell. (I) Proliferation analysis. One-way ANOVA; **=p<0.01.

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7. GLOSSARY

AFM: Atomic force microscopy. A method that employs a cantilever with a nanometric tip for in-direct or direct measuring of material properties (e.g., mechanical, topographical).

Crown: The portion of a dental implant that replaces the area of the tooth.

Implant: The portion of a dental implant that directly interfaces with bone and is often seen as variations of a screw.

Fibroblast: A type of cell that exists in connective tissue. Here-in as NIH-3T3s which represent the oral gum tissue.

Fibrosis: Thickening of connective tissues.

Fibrosis encapsulation: Compartmentalization and isolation of an implant, associated with implant rejection and failure.

HMI: Human-machine interface.

NA: Numerical aperture. An indicator of objective resolution.

Osseointegration: The process during which an implant fuses with bone tissue.

Osteoblast: Bone cell.

SLA: Sandblasted large grit and acid etched treatment.