CELLULAR RESPONSE TO SURFACE WETTABILITY GRADIENT ON MICROTEXTURED SURAFACES

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CELLULAR RESPONSE TO SURFACE WETTABILITY GRADIENT ON MICROTEXTURED SURAFACES

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To the students of the Georgia Institute of Technology

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SUMMARY

Surface chemistry, topography, and energy of titanium implants alter cellular response via variations in protein adsorption, integrin expression, and downstream cell signaling. However, the contribution of hydrophilicity on cell response is difficult to isolate since altering wettability can be a result of changes in surface chemistry or microstructure. Our lab has previously shown that chitosan coating alters both wettability and surface chemistry [18]. This procedure showed that surface wettability regulated osteoblastic maturation in a wettability-dependent manner. Furthermore, the up-regulation of many integrins, proteins, and kinases that are integral in cellular adhesion were affected in a wettability-dependent manner as well. The aim of this study is to examine the effect of wettability on osteoblast maturation and extracellular matrix-related genes without modifying surface microstructure or surface chemistry.

A surface wettability gradient was created on sand-blasted/acid-etched (SLA) surfaces. SLA surfaces were treated with oxygen plasma for 1, 12, 80, or 116 hours to generate an oxygen density gradient. The resulting SLA surfaces are denoted as SLA/aging time (e.g. SLA surfaces aged for 24 hours are designated SLA24). Surfaces were characterized by contact angle. MG63 cells were cultured on SLA to confluence. Cell number, secreted osteocalcin, osteoprotegerin, vascular endothelial growth factor, and alkaline phosphatase levels were measured. Differences in mRNA levels of extracellular matrix-related genes between treatment groups were examined using PCR arrays.

Results indicate that two of the most hydrophobic surfaces, SLA80 and SLA116, express elevated levels of OPG, OCN, and VEGF. However, the most hydrophobic surface,

SLA, displays significantly lower levels for these three biomarkers which indicate that wettability is not the driving factor in these mechanisms. Further testing needs to be conducted to draw a conclusion on these results. From the PCR Array, a set of mRNA associated with the extracellular matrix has been found to be up- and down-regulated on the modSLA surfaces compared to the SLA surfaces. ITGA1, ITGA2, ITGA5, ITGAV, ITGB1, and IL-8 expression was measured for the wettability gradient samples.

In conclusion, results have shown that osteoblast markers are altered by modulation of wettability of rough surfaces. However, due to the significant difference between those surfaces and the SLA surface, which both have more hydrophobic surfaces, we cannot be certain whether the wettability of the surface or some excitation of the surface is due to the plasma treatment. Further testing can allow us to not only validate the results, but also compare them to previous results with similar contact angles. Through this analysis, we expect better understandings of how surface properties affect osteoblast maturation and osseointegration.

CHAPTER 1

INTRODUCTION

Surface Characteristics

Surface characteristics such as topography, chemistry, roughness, and wettability greatly impact implant- bone interactions during osseointegration [1]. These properties do not work independently of one another, as they were recently shown to have synergistically enhanced osseointegration of titanium implants in vivo through fast clotting stabilization followed by a shortened healing time [2]. Properties such as wettability, micron-/submicron-roughness, and chemistry have all been shown to directly influence osteogenesis, osteoblast maturation, and osseointegration.

Depending on localized environments within the body, implants can be subjected to many different types of external environments. In order for the implant to be successful, the surface of the implants must be able to promote interaction with the biological fluids and surrounds. Surface energy on the implant directly impacts the binding of important proteins in serum [8]. Studies have shown that hydrophobic surfaces tend to enhance cellular adhesion, spreading and proliferation [5]. The variation in contact angles between surfaces of different hydrophobicities lead to different bonding interactions between biological species and implants [8]. When comparing wettabilities, Yukoshi and Matsuda observed that cells spread more on a surface that was hydrophobic as opposed to one that was hydrophilic. Cell shape and size were different, with those adhered to the hydrophobic surface being more elongated. Cells number were also found to be reduced on the more hydrophilic surface. Cells cultured on these rougher surfaces have been shown to differentiate more [8].

Our lab has previously shown that roughness and wettability have an important impact on osseointegration while preserving constant surface chemistry [3]. However, wettability and surface chemistry have just recently been decoupled by our lab. With this, the impact of surface wettability can be understood through the control of both surface chemistry and roughness [3].

Microscale roughness has been previously shown to enhance osteoblast differentiation in vitro [8]. Surface topography characteristics range from the millimeter to the nanometer scale. Rough surfaces have been shown to promote osteoblast differentiation and maturation [9]. Previous studies have been able to develop a simple surface modification process that affects surface structures without impacting other important surface properties, such as hydrophobicity [7]. In this same study, changes in osteoblast differentiation were not seen in the absence of microscale roughness, implying that surface roughness and microstructures might have additive or synergistic effects on cellular differentiation. Cells responded on modSLA surfaces by displaying an increase in the production of factors that promote osteogenic differentiation [2].

Z. Schwartz and B.D. Boyan explain that surface chemistry has a direct impact on both serum adsorption and factors found extracellular fluid onto a biomaterial. Not only does this have a direct impact on implant reactions immediately, but it can also affect downstream factors [8].

Surface Treatments

Titanium (Ti) is a common biomaterial used for orthopaedic and dental applications. The material has been shown to have a lower elastic modulus and density in comparison to other metals, as well as a resistance to fatigue through stronger material

properties [4]. In order to observe the effects of surface treatments, gradients are often used to analyze effects. By gradually changing surface chemistry, similar gradual changes in wettability and other physical characteristics can be seen [14]. Wettability gradients are a useful technique to understand the effect of changes in surface energy, such as hydrophobic versus hydrophilic surfaces [14].

Cellular Responses

Even though osteoblast factors are altered, cells do not directly interact with biomaterials [1]. Instead, surface proteins and other cellular components transit functions between cells. In order for osseointegration and osteoblast maturation to occur, a series of events must occur at the implant surface [4]. These events can be briefly summarized as protein adsorption, cellular adherence, proliferation, differentiation, matrix production, and calcification [4]. Studies have shown that these responses are greatly influenced by surface chemistry and composition [5]. Components such as extracellular matrix (ECM), kinases, and transmembrane ECM receptors play a vital role in osteogenesis [1]. However, how these factors change due to surface wettability is not fully understood.

The behavior of adsorbed proteins is related to numerous factors such as charge of the material and surface characteristics [4]. Once a material is implanted into the body, it is immediately coated with proteins, lipids, sugars, and ions [4]. Presence of a hydrophobic surface has been shown to have an effect on production of matrix vesicles and their maturation in the implant environment [8].

On microstructured titanium surfaces, osteoblasts produced elevated levels of many local adhesive factors, such as transforming growth factor beta-1 (TGF- β1) [10][13] as well as prostaglandins E1 and E2 (PGE2) [11]. In the presence of a titanium

biomaterial, alkaline phosphatase specific activity is elevated, hinting to the presence of bone mineralization [8]. Rapid bone growth and successful bone formation do not always go hand in hand [8]. Osteogenesis is known as the promotion of osteoblast migration coupled with the promotion of osteoclast inhibition. Recent studies have shown that cell growth on micro-rough titanium surfaces promotes osteogenesis over cell resorption [12]. Furthermore, research has shown that osteoblasts heighten OPG production when grown on microstructures substrates such as SLA and TCPS [13].

CHAPTER 2

MATERIALS AND METHODS

Titanium Surfaces

Commercially manufactured titanium disks were used in this study (Ø 15mm × 1mm, grade 2, Institut Straumann AG, Basel, Switzerland). Titanium disks were treated with sand-blasting and acid-etching to generate a microtextured surface (SLA, Sa= 2.5 μm). Wettability of original and modified surfaces were examined using contact angle with a 2 ul drop size.

Wettability Gradients with Chitosan

Chitosan coating was performed as described previously [18]. After plasma treatment, surfaces were coated with a 1.5mg/mL solution of chitosan (MW=125,000 to 350,000 g/mol) for 30 minutes. Surfaces were rinsed three times with ultrapure water for 10 minutes, followed by a 24 hour drying in a cell culture hood. The resulting surfaces displayed a more hydrophilic wettability as aging time increased. mRNA was isolated and samples were used for PCR Array analysis of extracellular matrix gene expression. MG63 cells were plated at 10,000 cells/cm^2 density in a 24 well plate. At confluence, cells were incubated with fresh media for 12 hours. At harvest, RNA was extracted using TriZol following the Boyan lab protocol. RNA was quantified and RT-PCR was performed using Qiagen RT^2 First Strand Kit to obtain cDNA. Each group (n=6) was combined and used for the array. Real-time PCR arrays (Human Focal Adhesion PCR Array, PAHS-145C-2) were performed to examine difference in regulation of 84 genes involved in cellular adhesion to extracellular matrix. An array of 84 genes associated with cellular adhesion was analyzed

for up- and down-regulation. Genes were determined to be up- or down-regulated if their ΔC_T values were changed by at least two-fold.

Wettability Gradient

To determine whether the effects of wettability seen in the chitosan experiment were dependent only on wettability, a second set of surfaces were modified using oxygen plasma. Oxygen plasma treatment was conducted on the titanium disks for two minutes to clean the surfaces (PDG-32G, Harrick Plasma NY, USA). This process removes the titanium oxide layer that develops on the outermost surface of the titanium. All treatment procedures were performed in a sterile cell culture hood. Disks were then placed within a 24 well plate and surfaces were aged within the sterile hood. Disks were aged for such time that the same contact angles were obtained as were found in the chitosan experiment (15, 27, 65, 81 degrees). SLA/h is given to each modified SLA sample group, where SLA12 represents the SLA surface aged for 12 hours after oxygen plasma treatment. Cells are then plated at each time point on the titanium disks. Non-modified SLA surfaces and TCPS were used as a control.

Cellular Response

MG63 cells were plated on surfaces at 10,000 cells/cm^2 density in 24 well plates (n=6 surfaces/group). The surfaces were provided by Straumann and were modified with sand-blasting and acid etching prior to sterilization with autoclaving. Surfaces were plated at specific time points associated with the titanium oxide levels that correlated to specific wettabilities (SLA1, SLA12, SLA80, SLA116). Dulbecco's modification of Eagle's medium with 10% fetal bovine serum and 1% penicillin-streptomycin was used to culture the cells. Media was changed 24 hours after plating and every 48 hours thereafter. At

confluence, cells were incubated with fresh media for 24 hours. After 24-hour incubation, conditioned media was collected. Monolayers were washed with PBS, lysed in 0.05% Triton X-100, and sonicated. Total DNA and protein were calculated and data was used to normalize the immunoassay and PCR data. Osteocalcin, OPG, and VEGF, and ALP were measured.

Statistical Analysis

Contact angle measurements on the titanium surfaces were calculated as an average of three measurements per disk for two disks. Nanodrop captured the video of each drop, after which ImageJ software was used to accurately calculate the contact angle of the nanodrop. Mean ± SEM was calculated for each sample group. Data was analyzed using a one-way ANOVA with a Student's t-test and Bonferroni's correction for multiple comparisons between each SLA/h group. Data was statistically significant for $p<0.05$.

The cellular activity experiments were analyzed with 6 individual cultures for each aging point. Total DNA, ALP, OCN, OPG, VEGF and mRNA levels were analyzed using a One Way ANOVA for all titanium surfaces. A Student's t-test and Bonferroni's correction for multiple comparisons between aging groups was used to determine if groups were statistically different ($p<0.05$).

CHAPTER 3

RESULTS

Surface Wettability Gradient

Changes in surface wettability were found to be affected by the aging time post plasma treatment. Previous studies have shown that the surface chemistry and microstructure were preserved between different treatment groups using this process [18]. In order to ensure accurate correlation between previous studies, five different contact angles were desired- 15, 27, 65, 81 degrees. Linear regression analysis was performed to predict the correct aging times associated with the desired contact angles [**Fig.1**]. Procedures were continued until the correct contact angles were achieved, each iteration aiding in the development of a more accurate linear regression of contact angle vs. aging time [**Fig.2a and 2b**].

Four time points were found to correlate with the desired contact angles. Through the novel surface modification technique, results showed that the 15°, 27°, 65°, and 81° contact angles had an aging time of 1, 12, 80, and 116 hours, respectively [**Fig.3**]. These time points were used to age the Ti surfaces to the correct surface wettability, followed by analysis of protein and gene responses associated with changes in surface characteristics.

PCR Array [Figure 4]

-*Cell Cycle*

Cav-1 and Cav-2 encode for important proteins associated with cell cycle checkpoints. The protein has been found in recent research from our lab to be an important

ECM structural protein and receptor for the 1,25 Vitamin D Pathway. When cultured on SLA6 treated with chitosan, the cells expressed heightened levels for both proteins.

-*Cell Signaling*

CRK encodes for a protein that recruits cytoplasmic proteins to tyrosine kinases in the plasma membrane. HRAS encodes an important protein involved in signal transduction pathways due to its ability to bind to GTP and GDP, subsequently rapidly exchanging between the plasma membrane and the Golgi apparatus. , PRKCA is a gene that encodes for a subunit of protein kinase C, an important cellular protein involved in many different cell signaling pathways, such as cell adhesion and cell cycle checkpoints. All three of these genes are up-regulated for cells cultured on SLA6 coated with chitosan.

Conversely, PRKCB encodes for the beta subunit of protein kinase C and is down-regulated on the same SLA6 surface.

-*Cell Motility*

ACTB encodes for the actin protein, while ACTN1 encodes for actinin, an important microfilament protein associated with binding of muscle cells to actin. GSK3B encodes a serine-theronine kinase involved with energy metabolism. PAK1 and PAK2 encode for the p21 protein, an important cell motility checkpoint protein. RAF1, RAF1A, and RAF1B encode for a MAP kinase pathway and are important in cell physiology, such as cell division, apoptosis, differentiation and migration. When cultured on SLA6 treated with chitosan, the cells expressed heightened levels for all above proteins.

-*Focal Adhesion*

A large majority of the genes that are found to be regulated by surface wettability are focal adhesion genes. PARVA, PARVB encode for actin-binding proteins associated

with focal adhesion. PTK2 codes for a cytoplasmic protein associated with focal adhesion between growing cells. VCL codes for vinculin, which is associated with cell-cell junction anchoring. A group of genes coding for Integrin subunits, including ITGA2, ITGA4, ITGA6, ITGAV, ITGA5 are up-regulated on the cells cultured on the more wettable, SLA6 with chitosan coating. These integrin genes are examined further in the following gene response study.

While a large majority of the regulated genes were up-regulated, a few genes associated with focal adhesion were down-regulated on these SLA6 surfaces. RAPGEF1 is not only associated with focal adhesion, but also apoptosis and cell transformation. ROCK1 is a Rho-associated gene that regulates the formation of focal adhesion and stress fibers of fibroblasts.

Gene Response

Integrin alpha 1 (ITGA1) encodes for an important cell-surface receptor involved in cell to cell adhesion. Cells cultured on SLA12, SLA60, and SLA116 treatment surfaces had significantly lower ITGA1 mRNA expression compared to the SLA group [**Figure 5a]**. Compared to the TCPS group, SLA12 showed significantly lower mRNA levels. Furthermore, a significant difference was seen between the most hydrophilic group, SLA1, and the most hydrophobic group, SLA116. No significant difference in mRNA expression was seen between the SLA and TCPS groups.

Integrin alpha 2 (ITGA2) encodes for an important transmembrane receptor associated the recognition of collagen. For ITGA2, the SLA1 and SLA12 showed significantly lower mRNA expression levels than both the TCPS and SLA groups [**Figure 5b]**. The more hydrophobic groups, SLA60 and SLA116, saw no significant differences

between any of the other treatment groups. No significant difference was discovered when comparing the most hydrophobic (SLA116) and most hydrophilic (SLA1) groups. Also, no significant difference was found between the TCPS and SLA groups.

Integrin alpha 5 (ITGA5) encodes for an important cell-surface receptor responsible for the development of fibronectin receptors. When evaluating the mRNA levels for ITGA5, no significant trends were found. The only two groups which showed any significant change in mRNA levels was SLA1 and SLA116 vs. the TCPS group [**Figure 5c]**. Both surfaces were found to have a lower mRNA level than the TCPS control. Those surfaces are the most and least wettable respectively, displaying the inability for wettability to cause any difference in mRNA expression. ITGA5 levels among other treatment groups displayed no significant difference.

Cells on SLA1 disks showed significantly lower ITGAV mRNA levels compared to both the TCPS and SLA groups. No significantly different mRNA levels were seen when comparing the different plasma treated surfaces [**Figure 5d**].

No significant differences were seen from any of the treatment groups for the ITGB1 gene expression analysis, an important gene coding for a protein responsible for cell adhesion and other immunogenic responses [**Figure 5e**].

No significant differences were seen in IL-8 levels when comparing the different surface groups [**Figure 5f**].

Alkaline Phosphatase

Alkaline phosphatase (ALP) was measured for all treatment groups to analyze osteoblast differentiation affected by the different surface wettability. ALP is an early marker for osteogenesis. Results showed that the two most hydrophilic surfaces, SLA1 and

SLA12, had an increase in ALP levels [**Figure 6b**]. These surfaces both displayed heightened levels of ALP, showing that wettability had no direct impact on ALP initiation. TCPS showed no significantly different values from any of the surfaces. No other significant changes in ALP levels were seen for any other treatment groups.

Osteocalcin is a later marker of osteoblast maturation. Cells had increased osteocalcin secretion as wettability decreased, with both SLA80 and SLA116 having significantly higher levels than TCPS, SLA, SLA1 and SLA12 [**Figure 6c**]. Lower surface wettability displayed no significant difference between one another-TCPS, SLA1 and SLA12. The highest surface wettability, SLA, displayed significantly lower osteocalcin levels in comparison to the SLA80 and SLA116, showing that wettability is not the driving factor in osteocalcin release.

Osteoprotegerin levels increased as wettability decreased, with both SLA80 and SLA116 having significantly higher levels than TCPS, SLA, SLA1 and SLA12. Levels on surfaces with the lowest contact angle, SLA1 and SLA12, were not significantly different from TCPS [**Figure 6d**]. The highest surface wettability, SLA, displayed significantly lower osteoprotegerin levels in comparison to the SLA80 and SLA116, showing that wettability is not the driving factor in osteoprotegerin release.

Vascular endothelial growth factor (VEGF) levels were measured for each treatment group to analyze the changes in angiogenesis stimulation in the different surface groups. Results displayed an increase in VEGF mRNA levels as wettability decreased, with both SLA80 and SLA116 having significantly higher levels than TCPS, SLA, SLA1 and SLA12 [**Figure 6e**]. Lower surface wettability displayed no significant difference between one another-TCPS, SLA1 and SLA12. The highest surface wettability, SLA, displayed

significantly lower VEGF mRNA levels in comparison to the SLA80 and SLA116, showing that wettability is not the driving factor in VEGF release.

CHAPTER 4

DISCUSSION

In this study, the cellular responses of MG-63 cells have been compared on titanium surfaces with differing surface wettability. While there are many successful ways to alter surface roughness, wettability, and chemistry, current research has struggled to isolate only one surface modification without altering other properties of the titanium. These modifications are favorable in industry because of they affect osseointegration without altering the mechanical, bulk properties of the material. Our novel wettability gradient has been created without changing the surface chemistry or surface topography. Previous research, such as Baoe Li, et. al., has shown that through anodic oxidation of the titanium surface, surface roughness and hydrophilicity can be increased. Results from this study conclude that these modifications together can lead to accelerated bone formation and improved cellular adhesion and proliferation [15]. However, with the coupling of both surface modifications, it is not evident whether these results are due to roughness, wettability, or a synergy of the two. In some studies, results have shown that a rougher surface will promote attachment and proliferation of MG-63 cells [16]. Contrasting results have shown that these same cells prefer growth and proliferation on smooth surfaces [17]. With varying results in surface roughness results, preserving topography and altering wettability was the most favorable technique to examine this mechanism further.

In order for wettability to be altered while preserving the topography and chemistry of the surface, plasma treatment was conducted and surfaces were aged for differing times to redevelop a passive titanium oxide layer gradient. More hydrophobic surfaces have been found to promote osteoblast development and maturation. In this experiment, wettability

decreases as aging time increases. Previous studies with our group have shown that wettability, paired with surface chemistry changes, affects osteoblast growth and differentiation [18]. Studies have shown that factors such as alkaline phosphatase, osteoprotegerin, osteocalcin, and cell number are all dependent on these surface factors. By isolating surface wettability as the only variable property, the mechanism by which titanium osseointegration occurs can be better understood.

Alkaline phosphatase activity is indicative of osteoblast differentiation. Its presence is necessary for mineralization during bone development. Our results show that alkaline phosphatase levels increase as surface wettability decreases. The cells cultured on the SLA and SLA116 groups display significantly higher alkaline phosphatase levels than the SLA2, SLA12, and SLA80. These results contradict previous research, where results showed that an anodized titanium surface with high hydrophilicity is favorable for biocompatibility. The anodized TiO2 surface displayed a higher ALP activity than a machined surface [19]. However, these results were from a surface that was hydrophilic due to different surface topographies. When all other properties except wettability are preserved, our results show that the more hydrophobic surface promotes alkaline phosphatase activity.

Osteoprotegerin (OPG) is an important protein involved with osteoclastogenesis inhibition. During bone growth, osteoblasts are recruited to the implant surface to promote growth. These osteoblasts up-regulate RANKL expression, which downstream secretes osteoprotegerin. This protein protects bone resorption through the inhibition of osteoclast recruitment to the surface of the implant. While our results show that cells cultured on the two more hydrophobic experimental groups, SLA80 and SLA116, have an increase level

in OPG expression, the most hydrophobic surface (SLA) displays significantly lower OPG expression than both SLA80 and SLA116. Therefore, the results indicate that wettability is not the main factor in OPG expression. Gittens, et al. demonstrated significantly higher OPG levels for nano-modified SLA surfaces compared to the SLA surfaces. Both surfaces have similar macro-scale topographies, however the nano-modified SLA group had a more wettable surface [7]. This conflicts with our experimental group results, which show cells on more hydrophobic surfaces expressing higher OPG levels, but may be explained by the presence of nano-scale features.

The results from this study support the claim that osteocalcin (OCN) regulation is not dependent on surface wettability. Previous studies from our lab have shown that surfaces with similar wettability and topography display difference OCN values, suggesting that a synergistic or alternative mechanism is responsible for this heightened expression [7]. The results from this study show that while experimental surfaces SLA80 and SLA116 with higher wettability display heightened OCN expressions, the SLA group with the highest wettability displays significantly lower OCN levels than both.

Recent studies in the Boyan lab have concluded that VEGF expression was not affected by wettability [18]. The results from this study reinforce the claim that wettability is not the driving factor in VEGF expression. Cells cultured on SLA80 and SLA116 samples displayed elevated VEGF levels; however, there was significantly lower levels for both samples with higher (SLA) and lower (SLA1, SLA12) contact angles. Further testing must be done in order to understand how the changes in surface properties affect the mechanism of VEGF expression.

Interestingly, OCN, OPG and VEGF levels are highest for the SLA80 and SLA116

groups. While these groups are very hydrophobic, the trend does not continue for the highest hydrophobic surface, suggesting the theory that wettability is the driving factor behind these level elevations is false. While all surfaces are cleaned and sterile prior to experimentation, the plasma treatment further cleans this surface and removes the titanium oxide layer. A possible imperfection or contamination on the SLA surface could have caused the samples to interact with the MG-63 cells differently. By repeating this study, our results can be further analyzed more accurately.

CHAPTER 5

CONCLUSION

In conclusion, our group has developed a novel, accurate way to alter surface wettability while preserving surface chemistry and micro-roughness topography. Through this process, any desired contact angle can be obtained through plasma cleaning and aging. These procedures can provide a useful technique for examining the effects on surface wettability on cells without changing other properties in the process.

Results have shown that osteoblast markers are altered by modulation of wettability of rough surfaces. Osteocalcin and osteoprotegerin levels increase significantly compared to the TCPS and SLA surfaces, as well as the more wettability SLA1, and SLA12, on the SLA80 and SLA116 surfaces. However, due to the significant different between those surfaces and the SLA surface, we cannot be certain whether the wettability of the surface or some excitation of the surface due to the plasma treatment. Further testing can not only allow us to validate the results, but also compare them to previous results with similar contact angles. This analysis can provide a better understanding of how surface properties affect osteoblast maturation and osseointegration.

While further testing must be completed before conclusions can be validated, this research further supports the hypothesis that surface roughness and wettability play a vital role in the overall success of titanium implant integration. Titanium implants provide a robust option for dental applications due to its strong bulk mechanical properties and its ability to integrate successfully with the surrounding environment. Further work will continue to create a titanium surface that might provide a more successful option for dental implants in the future for compromised patients.

Chapter 6

FIGURES

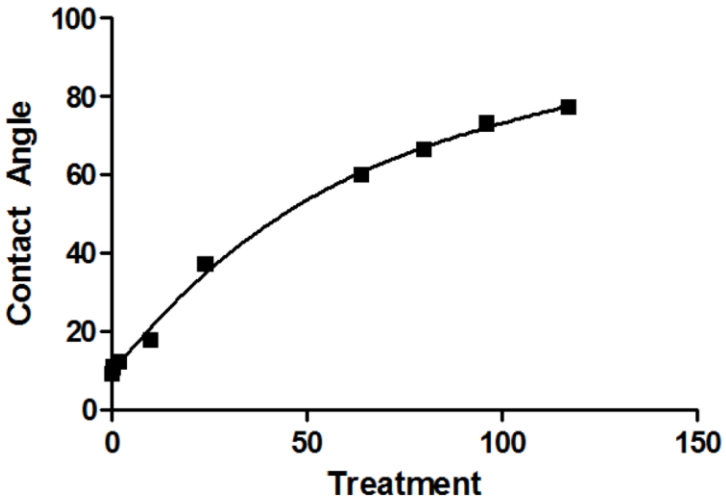

Figure 1. Linear regression of contact angle measurements for titanium disks using nanodrop procedures.

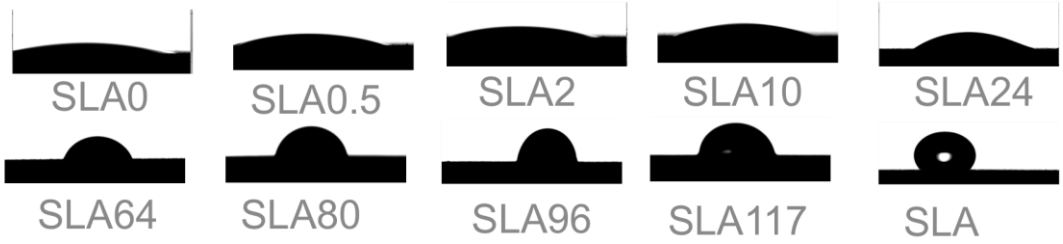

Figure 2a. Nanodrop images of 2uL water droplets on titanium SLA surfaces aged for O, 0.5, 2, 10, 24, 64, 80, 96, and 117 hours. Contact angle measurements quantify surface wettability.

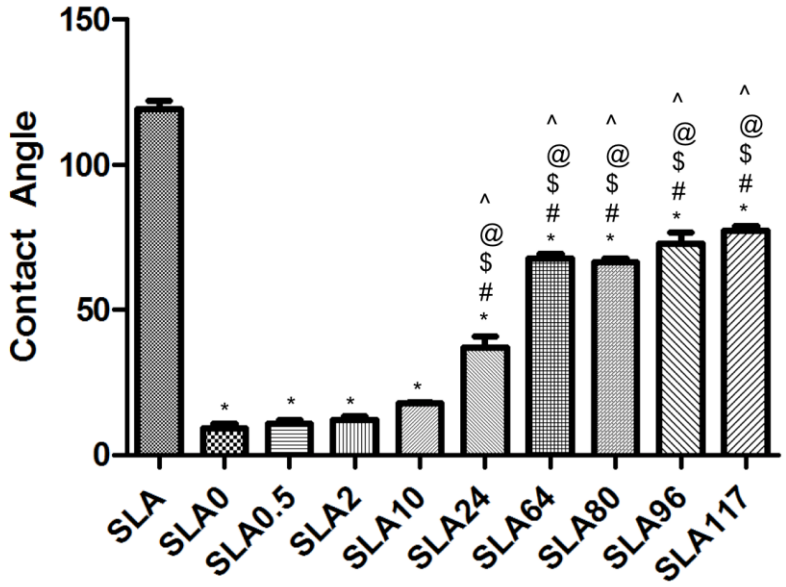

Figure 2b. Comparison of contact angle measurements for titanium SLA surfaces aged for 0. 0.5, 2, 10 24, 64, 80, 96, and 117 hours. Comparisons were considered statistically different for p-values < 0.05. * vs. SLA, # vs. SLA0, $ vs. SLA0.5, @ vs. SLA2, ^ vs. SLA10

Contact Angle (degree)	Aging Time (hours)
15	1
27	12
65	80
81	116

Figure 3. Experimental contact angle measurements and their respective aging time necessary to achieve the contact angle with a 2uL droplet.

Gene	Regulation	Gene	Regulation
ACTB	**4.01**	PARVB	2
ACTN1	2.01	PRKCA	2.02
Cav-1	2.01	PRKCB	2.04
Cav-2	2.03	**PTEN**	**4.06**
CRK	2	PTK2	2
GSK3B	2.01	RAF1	2
HRAS	2	RAF1A	2.02
ITGA2	2.02	**RAF1B**	**4.03**
ITGA4	2	RAPGEF1	2.02
ITGA6	2	ROCK1	2.01
ITGAV	2	RPLP0	2
PAK1	2.01	SCH1	2.01
PAK2	2	VAV2	2.01
PARVA	2.02	VCL	2

Figure 4. Comparison of mRNA levels between SLA and SLA6 coated with chitosan on a PCR Array. Regulation was calculated on a fold increase (black >2.0, bold >4.0) or decrease (red < -2.0) comparing the responses between the two surfaces.

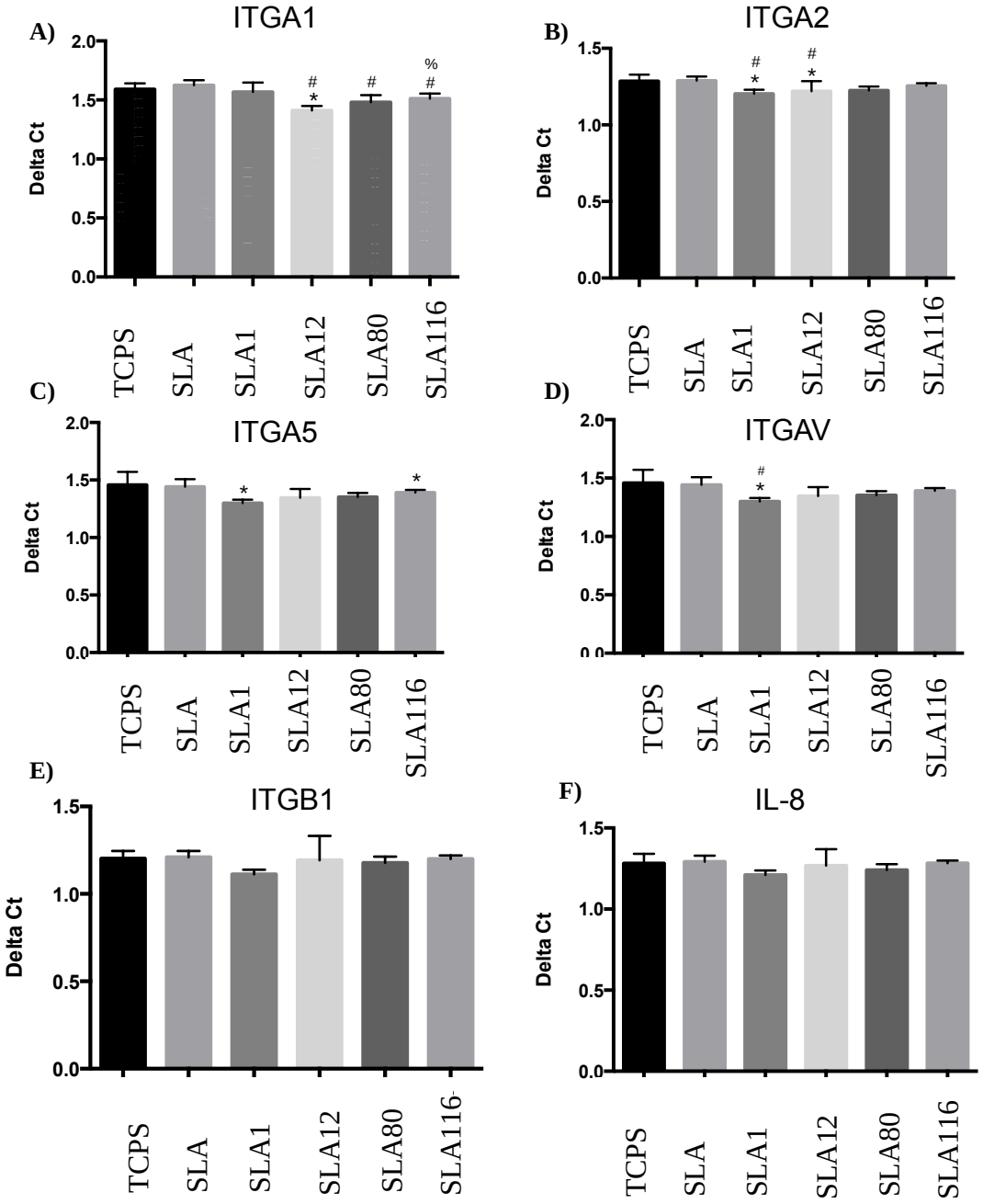

Figure 5. Gene expression for cells cultured on TCPS, SLA, SLA1, SLA12, SLA80 and SLA116. ITGA1, ITGA2, ITGA5, ITGAV, ITGB1, and IL-8 values were measured and normalized to GAPDH. * vs. TCPS, # vs. SLA

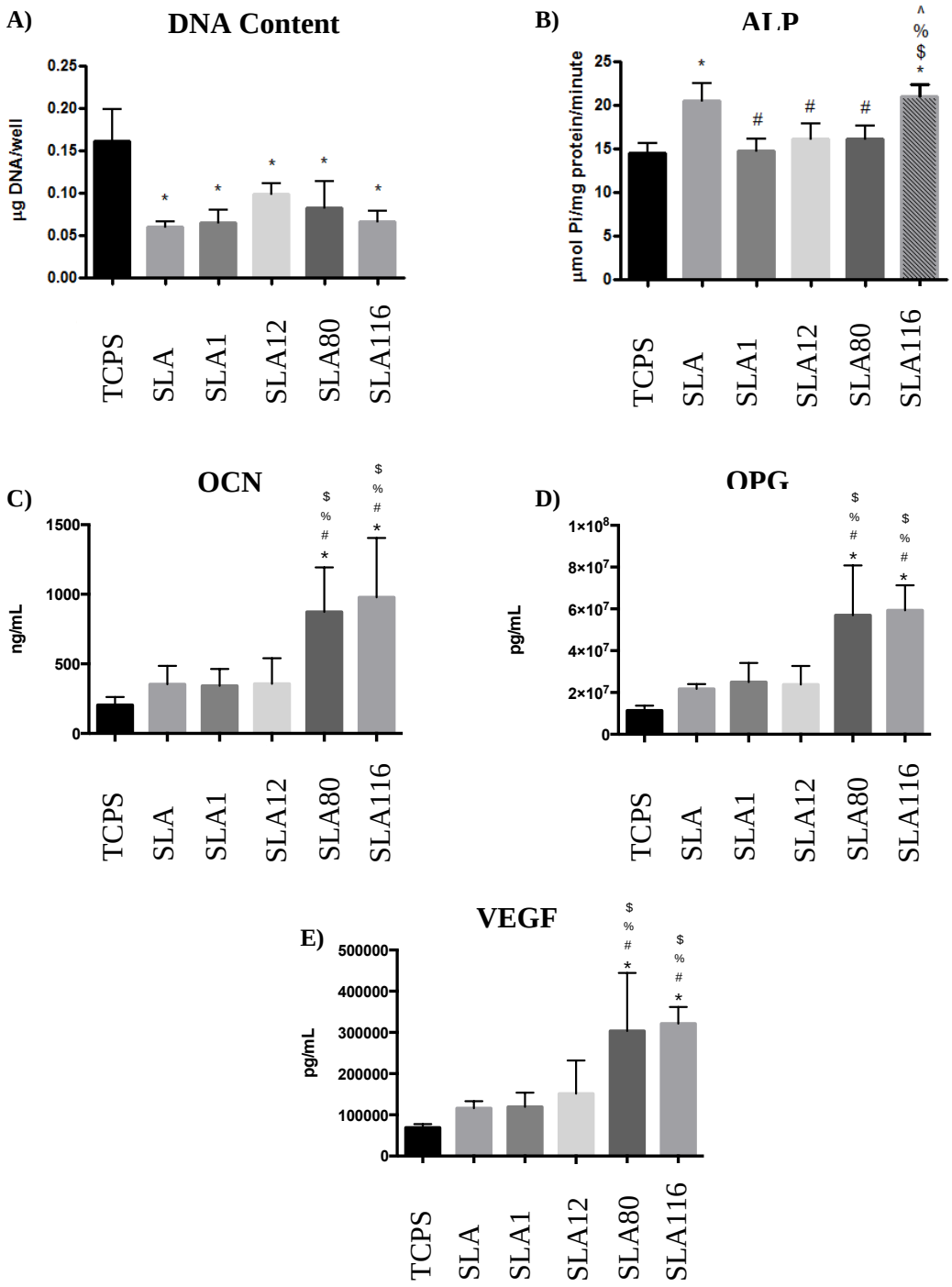

Figure 6. DNA Content and Alkaline Phosphatase, Osteocalcin, Osteoprotegerin, and VEGF expression for cells cultured on TCSP, SLA, SLA1, SLA12, SLA80 and SLA116 (N=6). * vs. TCPS, # vs. SLA, % vs. SLA1, $ vs. SLA12, ^ vs. SLA80

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