Select Nanofabricated Titanium Materials for Enhancing Bone and Skin Growth of Intraosseous Transcutaneous Amputation Prostheses

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Preface

This research is based on the research I completed in the Nanomedicine Laboratory at Brown University from 2006 to 2009. From 2006 to 2007, this research was funded by a fellowship from the Graduate School in the Division of Engineering. From 2007 to 2009, this research was funded by the VA Pre-Doctoral Associated Health Rehabilitation Research Fellowship Program and by the Department of Veterans Affairs, RR & D, A3772C. I am very grateful and humble to these affiliations for their financial support.

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Figure 2.9. AFM micrographs and section analyses revealed distinct topographical differences (both qualitatively and quantitatively) of the Ti surfaces from the vendor and after electron beam evaporation. Root-mean-square roughness (RMS) and average peak-to-valley roughness (RPV) values can be found in Table 2.2.

Figure 2.10. (a) ESCA spectra of elements present in the outermost layer of the Ti before (conventional Ti) and after electron beam evaporation (nanorough Ti). (b-c) Binding energy of the high resolution Ti 2p peaks indicating these surfaces have similar chemistries.

Figure 2.11. XRD spectra of nanorough Ti surfaces after electron beam evaporation and the conventional Ti surfaces as received from the vendor indicated the presence of crystalline TiO₂. Upon further examination of the crystalline phase, slight variations were found demonstrating that the nanorough Ti region possessed more of the anatase TiO₂ phase while the conventional Ti regions possessed more of the rutile TiO₂ phase.

Figure 2.12. Total osteoblast adhesion on nanorough Ti created by electron beam evaporation, conventional Ti surfaces as received from the vendor, and nanopatterned Ti substrates with varying groove widths after four hours.
Values are mean ±SEM; n=3; *p<0.05 compared to the 300 mesh; **p<0.01 compared to the 400 mesh; ***p<0.01 compared to the conventional Ti; #p<0.01 compared to the glass; ##p<0.05 compared to the 400 mesh; ###p<0.1 compared to the 400 mesh.

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Figure 2.14. Fluorescent micrographs indicating osteoblast adhesion on the (a-c) nanopatterned Ti surfaces, (d) nanorough Ti surfaces, (e) and conventional Ti surface after four hours. Scale bar = 50 µm.

Figure 2.15. Fluorescent micrographs indicating osteoblast alignment on the (c) 400 mesh nanopatterned Ti surfaces compared to the (a) 200 mesh, (b) 300 mesh, (d) nanorough Ti, and (e) conventional Ti surfaces. Arrows indicate groove alignment direction on the nanopatterned samples. Scale bar = 50 µm.

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Figure 2.18. One, three, and five day osteoblast densities on the nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces. Values are mean ±SEM; n=3; *p<0.01 compared to the 200 mesh; **p<0.01 compared to the 300 mesh; ***p<0.01 compared to the 400 mesh; #p<0.01 compared to the conventional Ti; ##p<0.01 compared to the etched glass; ###p<0.05 compared to the 400 mesh; +p<0.05 compared to the conventional Ti; ++p<0.05 compared to the 300 mesh; +++p<0.1 compared to the 400 mesh.
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Figure 2.19. Greater osteoblast densities specifically on the nanorough region compared to the conventional region for the varying nanopatterned Ti surfaces after one, three, and five days. Values are mean ±SEM; n=3; *p<0.01 compared to the conventional region on respective days; •p<0.1 compared to the respective regions of 300 mesh; ••p<0.01 compared to the respective regions of 400 mesh; •••p<0.01 compared to the respective regions of 400 mesh; Δp<0.01 compared to the respective regions on 300 mesh.

Figure 2.20. Total intracellular protein content of osteoblasts cultured on the varying nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; *p<0.01 compared to the 200 mesh; **p<0.01 compared to the conventional Ti; ***p<0.05 compared to the conventional Ti; #p<0.01 compared to the respective substrates on day 7; ##p<0.05 compared to the respective substrates on day 14; ###p<0.05 compared to the respective substrates on day 7.

Figure 2.21. Number of osteoblasts at the end of 7, 14, and 21 days on the varying nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces. Values are mean ±SEM; n=4; *p<0.01 compared to the 200 mesh; **p<0.01 compared to the conventional Ti; ***p<0.05 compared to the conventional Ti; #p<0.01 compared to the respective substrates on day 7; ##p<0.05 compared to the respective substrates on day 14; ###p<0.05 compared to the respective substrates on day 7; •p<0.1 compared to the respective substrates on day 7.

Figure 2.22. Total alkaline phosphatase synthesis by osteoblasts cultured on the various nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; *p<0.05 compared to the 400 mesh; **p<0.1 compared to the 300 mesh; ***p<0.01 compared to the conventional Ti; #p<0.1 compared to the 400 mesh; ##p<0.05 compared to the conventional Ti; ###p<0.05 compared to the 300 mesh; +p<0.1 compared to the conventional Ti; ++p<0.01 compared to the respective substrates on day 7; +++p<0.1 compared to the respective
substrates on day 14; • p<0.05 compared to the respective substrates on day 7; •• p<0.05 compared to the respective substrates on day 14.

Figure 2.23. Alkaline phosphatase synthesis by each individual osteoblast cultured on the various nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; * p<0.05 compared to the 400 mesh; ** p<0.01 compared to the conventional Ti; *** p<0.1 compared to the 400 mesh; # p<0.1 compared to the conventional Ti; ## p<0.01 compared to the 400 mesh; ### p<0.05 compared to the 300 mesh; + p<0.1 compared to the respective substrates on day 7; ++ p<0.05 compared to the respective substrates on day 7; +++ p<0.01 compared to the respective substrates on day 7.

Figure 2.24. Calcium deposition by osteoblasts cultured on the varying nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; * p<0.05 compared to the 400 mesh; ** p<0.01 compared to the 400 mesh; *** p<0.01 compared to the 300 mesh; # p<0.1 compared to the conventional Ti; ## p<0.05 compared to the conventional Ti; ### p<0.01 compared to the 300 mesh; + p<0.05 compared to the conventional Ti; ++ p<0.05 compared to the conventional Ti; +++ p<0.01 compared to the respective substrates at day 7; •• p<0.05 compared to the respective substrates at day 7; ••• p<0.01 compared to the respective substrates at day 7; Δ p<0.1 compared to the respective substrates at day 7.

Figure 2.25. Calcein labeling of mineralized deposits on the (a-c) various nanopatterned Ti substrates, (d) nanorough Ti substrates, and (e) conventional Ti substrates at 21 days. Scale bar = 50 µm.

Figure 2.26. SEM micrographs of calcium deposition by osteoblasts on the various nanopatterned Ti substrates confirming their capability to support osteoblast mineralization. As seen in the images, it appears that the presence of the nanopatterns directs the deposition of calcium phosphate by osteoblast into the separate regions.

Figure 2.27. Total collagen synthesis by osteoblasts cultured on the various nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; * p<0.01 compared to the 400 mesh; ** p<0.01 compared to the 300 mesh; *** p<0.01
compared to the 200 mesh; #p<0.01 compared to the conventional Ti; ##p<0.05 compared to the 300 mesh; ###p<0.05 compared to the conventional Ti; +p<0.05 compared to the 400 mesh; ++p<0.01 compared to the respective substrates at day 7; +++p<0.1 compared to the respective substrates at day 7; •p<0.1 compared to the respective substrates at day 14; ••p<0.05 compared to the respective substrates at day 14.

Figure 2.28. Collagen synthesis by each individual osteoblast cultured on the various nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM: n=4; *p<0.05 compared to the 400 mesh; **p<0.01 compared to the conventional Ti; ***p<0.1 compared to the 400 mesh; #p<0.1 compared to the conventional Ti; ##p<0.01 compared to the 400 mesh; ###p<0.05 compared to the 300 mesh; +p<0.1 compared to the respective substrates on day 7; ++p<0.05 compared to the respective substrates on day 7; +++p<0.01 compared to the respective substrates on day 7.

Figure 2.29. Fluorescently stained extracellular collagen synthesis on the various nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces. Qualitatively, directed collagen synthesis was observed on the nanopatterned surfaces, particularly on the 400 mesh substrates (which were observed to have better osteoblast alignment).

Figure 2.30. Graph revealing the relationship between surface energetics and biocompatibility [279]. A material within region A is considered to possess poor biocompatible properties while a material in region B with higher critical surface tension enhances its properties (such as adhesion, proliferation, and function).

Figure 2.31. Surface energy of the conventional and nanorough Ti surfaces. Surface energy was calculated for each sample by measuring the contact angles of three liquids at the sample surface and entering values into the Owens-Wendt equation. Results indicated higher surface energy for the nanorough Ti surfaces compared to the conventional Ti surfaces. Values are mean ±SEM: n=4; *p<0.01 compared to conventional Ti surfaces.

Figure 2.32. Fibronectin adsorption on the conventional, nanorough, and nanopatterned Ti surfaces. Values are mean ±SEM: n=4; *p<0.01 compared to the 400 mesh; **p<0.01 compared to the 300 mesh; ***p<0.01 compared to
the 200 mesh: #p<0.01 compared to conventional Ti surfaces; ##p<0.05 compared to the 400 mesh; ####p<0.05 compared to conventional Ti.

Figure 3.1. Schematic diagram of field-driven ion diffusion during anodization that leads to the formation of an oxide layer on the anode (Ti) surface. (Adapted from [296])

Figure 3.2. Schematic illustration of the formation of nanotubes on the Ti surface during anodization in HF: (a) oxide formation, (b) pit formation, (c) pore and void formation, and (d) fully developed into tubes. (Adapted from [296])

Figure 3.3. Schematic illustration of the anodization process (an electrolytic passivation process used to increase the thickness of the natural oxide layer on metal surfaces) used to create nanotubular surfaces.

Figure 3.4. SEM micrographs of Ti substrates after anodization. The Ti substrates possessed tube-like features approximately 70 to 80 nm in diameter with a 200 nm depth (and, thus, was termed nanotubular Ti). Scale bar = 3 µm (low resolution), 100 nm (high resolution), and 30 nm (high resolution). SEM micrographs of nanorough Ti and conventional Ti can be found in Figure 2.7.

Figure 3.5. AFM micrographs and section analyses revealed both qualitative and quantitative topographical nanofeatures on the nanotubular Ti surfaces. Root-mean-square roughness (RMS) and average peak-to-valley roughness (RPV) values can be found in Table 3.1. AFM micrographs for the nanorough Ti and conventional Ti surfaces can be found in Figure 2.9.

Figure 3.6. (a) ESCA spectra of elements present in the outermost layer of the nanotubular Ti surfaces (Table 3.2). (b) Binding energy of the high resolution Ti 2p peak for the nanotubular Ti surfaces. Binding energies matched those of the conventional and nanorough Ti surfaces (Figure 2.10).

Figure 3.7. XRD spectra of nanotubular Ti surfaces after anodization. Spectra, when compared to Ti and titania (anatase and rutile) standards, indicated the presence of amorphous TiO$_2$. XRD spectra for the nanorough Ti and conventional Ti surfaces can be found in Figure 2.11 (which contained crystalline TiO$_2$). (Adapted from [298])
Figure 3.8. Keratinocyte adhesion on nanotubular Ti created by anodization, nanorough Ti created by electron beam evaporation, and conventional Ti as received from the vendor after four hours. Values are mean ±SEM; n=3; *p<0.01 compared to the conventional Ti surface; **p<0.01 compared to the etched glass; ***p<0.05 compared to the etched glass.

Figure 3.9. Fluorescent micrographs indicating keratinocyte adhesion properties on the (a) nanotubular Ti surfaces, (b) nanorough Ti surfaces, and (c) conventional Ti surfaces after four hours. Scale bar = 100 µm.

Figure 3.10. One, three, and five day keratinocyte densities on the nanotubular Ti, nanorough Ti, and conventional Ti surfaces. Values are mean ±SEM; n=4; *p<0.01 compared to nanotubular Ti surfaces; **p<0.01 compared to conventional Ti surfaces; ***p<0.01 compared to etched glass; #p<0.1 compared to nanotubular Ti surfaces; ##p<0.1 compared to etch glass; ###p<0.05 compared to conventional Ti surfaces.

Figure 3.11. Average surface area of keratinocytes (indicative of cell spreading) on the nanotubular Ti, nanorough Ti, and conventional Ti surfaces after four hours. Values are mean ±SEM; n=3; *p<0.05 compared to conventional Ti surfaces.

Figure 3.12. SEM images of keratinocytes adhered on the (a-d) nanotubular Ti, (e-h) nanorough Ti, and (i-k) unmodified Ti after four hours of culture. Keratinocytes on the nanofabricated Ti substrates (nanotubular and nanorough Ti surface) were flatter and more spread with more defined filopodia. Keratinocytes on the conventional Ti substrates were more round (decreased spreading) with less directed filopodia. Scale bars = (a,i) 10 µm, (e) 20 µm, (b,f,j,k) 2 µm, (c,g) 1 µm, and (d,h) 200 nm.

Figure 3.13. Average surface area of keratinocytes (indicative of cell spreading) on the nanotubular Ti, nanorough Ti, and conventional Ti surface after three and five days. Values are mean ±SEM; n=3; *p<0.01 compared to nanotubular Ti surfaces; **p<0.01 compared to conventional Ti surfaces; ***p<0.05 compared to nanotubular Ti surface.

Figure 3.14. Fluorescent micrographs of the f-actin filaments in the cytoskeleton of the keratinocytes demonstrating cell spreading on the (a) nanotubular Ti, (b) nanorough Ti, and (c) conventional Ti surfaces. Scale bar = 100 µm.
Figure 3.15. Surface energy of the conventional, nanorough Ti, and nanotubular Ti surfaces. Surface energy was calculated for each sample by measuring the contact angles of three liquids at the sample surface and entering values into the Owens-Wendt equation. Results indicated greater surface roughness at the nanoscale correlated to increased surface energy. Values are mean ±SEM; n=4; *p<0.01 compared to conventional Ti surfaces; **p<0.01 compared to nanorough Ti.

Figure 3.16. Fibronec tin adsorption on the conventional, nanorough, and nanotubular Ti surfaces. Values are mean ±SEM; n=4; *p<0.01 compared to conventional Ti surfaces; **p<0.05 compared to nanorough Ti surfaces.

Figure 4.1. Reaction schematic for functionalizing FGF-2 on to nanotubular Ti substrates (created through anodization), electron beam evaporated Ti substrates (created through electron beam evaporation), and conventional Ti substrates (as received from the vendor).

Figure 4.2. Reaction demonstrating how CBQCA transforms from a non-fluorescence molecule into a fluorescence molecule when reacting with amine groups in the presence of cyanide molecules.

Figure 4.3. Fluorescent micrographs of the chemically functioned nanotubular, nanorough, and conventional Ti substrate after step 1 of the process (addition of APTES) with and without CBQCA. Scale bar = 50 µm.

Figure 4.4. Fluorescent micrographs of the chemically functioned nanotubular, nanorough, and conventional Ti substrate after step 2 of the process (addition of maleimide cross-linker) with and without CBQCA. Amine groups present after silanization were completely covered by the SMP moiety thus nothing was found to fluorescence. Scale bar = 50 µm.

Figure 4.5. Fluorescent micrographs of the chemically functioned nanotubular, nanorough, and conventional Ti substrate after step 3 of the process (addition of FGF-2) with and without CBQCA. Scale bar = 50 µm.

Figure 4.6. SEM micrographs of the nanotubular, nanorough, and conventional Ti surfaces after (a) silanization, (b) addition of maleimide cross-linker, and (c) addition of FGF-2. Scale bar = 1 µm (low magnification) and 200 nm (high magnification).
Figure 4.7. Keratinocyte densities on the nanotubular Ti, nanorough Ti, and conventional Ti surfaces as well the functionalized nanotubular Ti, nanorough Ti, and conventional Ti surfaces with FGF-2 after 24 hours. Values are mean ±SEM; n=3; *p<0.01 compared to the nanotubular Ti; **p<0.05 compared to the conventional Ti; ***p<0.1 compared to the nanorough Ti; #p <0.01 compared to the conventional Ti; ##p <0.01 compared to the functionalized conventional Ti; ###p <0.1 compared to the conventional Ti.

Figure 4.8. Fluorescent micrographs indicating keratinocyte proliferation (after 24 hours) properties on the nanotubular Ti surfaces, nanorough Ti surface, and conventional Ti surface as well as the functionalized nanotubular Ti surfaces, nanorough Ti surface, and conventional Ti surface with FGF-2. Scale bar = 50 µm.

Figure 5.1. SEM micrographs of Ti substrates after anodization for 1 minute in 0.5% HF at 20 V. The Ti substrates possessed nanotextured features (and, thus, were termed nanotextured Ti). Scale bar = 2 µm (low resolution) and 200 nm (high resolution). SEM micrographs of nanorough Ti and conventional Ti can be found in Figure 2.7 and nanotubular Ti can be seen in Figure 3.4.

Figure 5.2. AFM micrographs and section analyses revealed topographical nanofeatures on the nanotextured Ti surfaces [214]. Root-mean-square roughness (RMS) and average peak-to-valley roughness (RPV) values can be found in Table 5.1. AFM micrographs for the nanorough Ti and conventional Ti surfaces can be found in Figure 2.9 and in Figure 3.5 for the nanotubular Ti surfaces.

Figure 5.3. (a) ESCA spectra of elements present in the outermost layer of the nanotextured Ti surfaces (Table 5.2). (b) Binding energy of the high resolution Ti 2p peak for the nanotextured Ti surfaces. Binding energies matched those of the conventional Ti, nanorough Ti, and nanotubular Ti surfaces (Figure 2.10 and 3.6).

Figure 5.4. XRD spectra of nanotextured Ti surfaces after anodization. Spectra, when compared to Ti and titania (anatase and rutile) standards, indicated the presence of amorphous TiO₂ (also observed in nanotubular Ti surfaces, Figure 3.7). XRD spectra for the nanorough Ti and conventional Ti
surfaces can be found in Figure 2.11 (contained crystalline TiO₂). (Adapted from [298])

Figure 5.5. Total *S. aureus, S. epidermidis, and P. aeruginosa* colonies on nanorough Ti, conventional Ti, nanotubular Ti, and nanotextured Ti after one hour. Values are mean ±SEM; n=3; *p<0.01 compared to nanorough Ti; **p<0.01 compared to conventional Ti; ***p<0.01 compared to nanotextured Ti; #p<0.1 compared to nanotextured Ti; ##p<0.05 compared to nanotextured Ti for respective bacteria lines.

Figure 5.6. Fluorescent micrographs of (a-d) *S. aureus*, (e-h) *S. epidermidis*, and (i-l) *P. aeruginosa* colonies on conventional, nanorough, nanotextured, and nanotubular Ti surfaces. It was found that bacteria colonies decreased on the (b,f,i) nanorough Ti surfaces compared to all other substrates and increased on the (c,g,k) nanotextured and (d,h,l) nanotubular Ti surfaces compared to the (a,e,i) conventional Ti after one hour.

Figure 5.7. (a) Live and (b) dead colonies of *S. aureus, S. epidermidis, and P. aeruginosa* on nanorough Ti, conventional Ti, nanotubular Ti, and nanotextured Ti surfaces after one hour. Values are mean ±SEM; n=3; *p<0.05 compared to the nanorough Ti; **p<0.01 compared to the nanorough Ti; ***p<0.01 compared to the conventional Ti; #p<0.05 compared to the conventional Ti; ##p<0.01 compared to the nanotextured Ti; ###p<0.05 compared to the nanotextured Ti for the respective bacteria lines.

Figure 5.8. Percentage of live bacteria colonies for *S. aureus, S. epidermidis, and P. aeruginosa* attached on nanorough Ti, conventional Ti, nanotubular Ti, and nanotextured Ti surfaces after one hour. Values are mean ±SEM; n=3; *p<0.1 compared to the nanotextured Ti; **p<0.01 compared to the nanotextured Ti; ***p<0.05 compared to the nanotubular Ti; #p<0.05 compared to the conventional Ti; ##p<0.01 compared to the nanotubular Ti; ###p<0.1 compared to the conventional Ti; +p<0.1 compared to the conventional Ti; ++p<0.1 compared to the nanotubular Ti for respective bacteria lines.

Figure 5.9. Surface energy of the conventional Ti, nanorough Ti, nanotubular Ti, and nanotextured Ti surfaces. Surface energy was calculated for each sample by measuring the contact angles of three liquids at the sample surface and entering values into the Owens-Wendt equation. Results indicated
greater surface roughness at the nanoscale correlating to increased surface energy. Values are mean ±SEM; n=4; *p<0.01 compared to conventional Ti; **p<0.01 compared to nanorough Ti; ***p<0.05 compared to nanotextured Ti.

Figure 5.10. Fibronectin adsorption on the conventional Ti, nanorough Ti, nanotubular Ti, and nanotextured Ti surfaces. Values are mean ±SEM; n=3; *p<0.01 compared to the conventional Ti; **p<0.1 compared to the conventional Ti; ***p<0.05 compared to the nanorough Ti; #p<0.01 compared to the nanorough Ti; ##p<0.1 compared to the nanotextured Ti.

Figure 6.1. Photograph of the Ti6Al4V pins indicating the dimensions and the different regions that will be exposed to bone and skin in the animal model.

Figure 6.2. Schematic illustration of the anodization process used to create nanotubular features on the Ti6Al4V pins.

Figure 6.3. Photographs showing the rats actively using the implanted pin as a leg prosthetic. This particular rat contained a pin that had been modified by anodization (nanotubes).

Figure 6.4. SEM micrographs of Ti6Al4V pins (a) before modification (control), (b) after electron beam evaporation (referred to as nanorough Ti), and (c) after anodization (referred to as nanotubular Ti). After electron beam evaporation, the Ti pins possessed a higher degree of nanometer roughness compared to the smooth control surfaces. The presence of nanotubes was also observed on the Ti surfaces after anodization.

Figure 6.5. X-ray micrographs of the (a) conventional (control), (b) nanorough (electron beam evaporation), and (c) nanotubular (anodization) Ti6Al4V pins implanted in rat femurs at 14, 21, and 28 days.

Figure 6.6. Toluidine blue histology stains of the (a) conventional (control), (b) nanorough (created through electron beam evaporation), and (c) nanotubular (created through anodization) Ti6Al4V pins after 28 days. Clearly, these slides revealed that the nanofabricated surfaces promoted bone growth around the threads of the pins (indicated by the blue stain) compared to the control pins that showed minimal new bone growth (lack of blue stain around the threads).
Figure 6.7. Doxycycline (tetracycline) histology stains of the (a) conventional (control), (b) nanorough (created through electron beam evaporation), and (c) nanotubular (created through anodization) Ti6Al4V pins after 28 days. These slides revealed bone formation was active at the time of animal sacrifice for the nanofabricated surfaces. For the control pins, the bone formation in the last week of the experiment was decidedly less as indicated by the lack of fluorescence from the dye. Magnification = 20x.

Figure 6.8. Hematoxylin and eosin (H&E) stains of the (a) conventional (control), (b) nanorough (created through electron beam evaporation), and (c) nanotubular (created through anodization) Ti6Al4V pins after 28 days. Due to problems encountered during processing, these slides were inconclusive. However, photographs seen in Figure 6.9, revealed improved skin function on the nanorough and nanotubular pins compared to the conventional pins.

Figure 6.9. Photographs taken of the rats over the duration of the experiment (14, 21, and 28 days) to monitor the area at the exit site of the (a) conventional, (b) nanorough, and (c) nanotubular Ti6Al4V pins. These photographs qualitatively revealed better skin growth for the nanofabricated pin surfaces by decreased pus and inflammation and improved skin closure.

Figure 7.1. SEM micrographs of nanopatterned Ti substrates with alternating regions of nanotubular and nanorough features. The dimensions are 22 µm for the nanorough regions and 40 µm for the nanotubular regions.

Figure 7.2. Newly modified ITAP through the use of electron beam evaporation and anodization as a means of improving bone and skin growth. The Ti fixture contains nanopatterns of alternating nanorough and nanotubular regions while the Ti abutment consists of only nanorough Ti features.

Figure A1. SEM micrographs of polyethylene (PE) (a) before and (b) after electron beam evaporation. These images reveal a distinct difference in surface topography with the original PE containing conventional features while the electron beam evaporated PE surfaces contained nanorough features. Scale bar = 1 µm.
Figure A2. Surface energy of the conventional PE and nanorough PE surfaces. Surface energy was calculated for each sample by measuring the contact angles of three liquids at the sample surface and entering values into the Owens-Wendt equation. Results indicated greater surface roughness at the nanoscale correlated to increased surface energy. Data are mean ±SEM; n=4. *p<0.01 compared to conventional PE.

Figure A3. Greater osteoblast adhesion on nanorough PE (created through electron beam evaporation) compared to conventional PE surfaces after four hours. Data are mean ±SEM; n=3; *p<0.01 compared to conventional PE.

Figure B1. SEM images comparing the original Ti samples (those used throughout the preliminary study) and the new Ti samples (item number was the same and no alteration in chemical treatment was found by Alfa Aesar). There was a clear, distinct difference in the topography despite the fact Alfa Aesar claimed they were the same samples.

Figure B2. SEM image demonstrating the flaking that was occurring on the new Ti substrates sent by Alfa Aesar after electron beam evaporation. This was clearly indicating the lack of adhesion of Ti to the original micron rough surface (something that was not seen on the original surfaces). Scale bar = 2 µm.

Figure B3. Tape test demonstrating the strength of Ti coating after electron beam evaporation. Dark regions = Ti coating not adherent to surface (indicating lack of adhesion).
Chapter 1

Introduction

1.1 Limb Prostheses

On January 16, 2007, Andrews Air Force Base near Washington received the 500th amputee from the War on Iraq who lost both legs in a roadside bomb explosion. Of the 22,700 United States troops wounded during the War on Iraq, 2.2% account for the 500 amputations, including toes and fingers [1]. This number rises to 5% when referring to soldiers whose amputation prevents them from returning to duty [1]. More generally speaking, the Army Office of the Surgeon General reported 1,286 amputations (935 major limb amputations and 351 minor limb amputations) from September 2001 to January 2009 [2]. When comparing the number of amputations to other wars of the past century, limb-loss has occurred twice as much in Iraq (excluding Vietnam) [1]. Although the Iraq War is increasing the number of amputees in the United States, the general population also suffers from limb loss. For
example, an average of 133,235 hospital discharges per year in the United States involved limb loss or limb deficiency from 1988 to 1996 [3]. Amputations due to vascular disease accounted for the vast majority of these limb loss discharges (82%) [3]. Trauma-related incidents (16.4%), malignancy (0.9%), and congenital deficiencies (0.8%) were also responsible for these limb loss discharges [3]. Similarly, in Europe, North America, and East Asia, it was reported that from 1995 to 1997, an incidence of 2.8-43.9 per 100,000 individuals suffered specifically from lower limb amputations due to vascular disease (51-93%), trauma (<15%), diabetes (20-80%), and infection (20-80%) [4]. Limb amputation is a potentially devastating and traumatic event in an individual’s life that results in profound physical, psychological, and occupational consequences. When Jerry White, co-founder of Landmine Survivors Network, met with mine victims who had lost their limbs, they said, “Losing our limbs is not the biggest problem. What’s worse is losing our place in society, or being rejected by family and society. Or not being able to find jobs, and then facing discrimination because of our status as people with disabilities. Or being denied rights because of our differences” [5]. Furthermore, the Lower Extremity Assessment Project (LEAP) found that no difference was observed in functional outcome of patients who underwent limb salvage surgery compared to those who suffered an amputation [6-9]. Their mental, physical, and overall health was very poor. Thus, because amputations affect such a wide range of individuals (from soldiers to war
victims to the general population) creating better prosthetic limbs are needed as a means of increasing the quality of life for these individuals.

Currently, prosthetic designs for improving amputee function and mobility rely on the conventional stump-socket attachable device (Figure 1.1). Such conventional socket prosthetics are composed of fiberglass or plastics (such as polypropylene (PP), acrylic, polyethylene (PE), or polyurethane (PU)) and fit into the remaining residual limb (also known as the stump) the way a thumb fits into a thimble. Thus, the motion of the prosthetic is controlled by the motion of the user’s residual limb. However, due to such conventional approaches, there will never be a complete connection between the prosthesis and the remaining bone because of the presence of soft tissue around the stump. This disconnection between the socket and natural bone also prevents proper socket attachment. Poor socket fit applies excessive pressure to the soft tissue of the residual limb which is not suited to tolerate such loads. Therefore, prosthetic efficacy is limited, and the amputee is presented with many problems including:

- the prosthetic feels heavy even though the device is lighter than a real limb,
- short stumps are unable to support the attachment of the prosthetic,
• weather conditions, the amount of activity, and the amputee’s weight can change the size and shape of the stump which alters the original fit of the prosthetic,

• skin-related complications are common (including edemas, cysts, lesions, ulcers, rashes, blistering, calluses, and dermatitis) due to focal points which increase stress caused by non-uniform pressure distributions [10-13],

• bacterial infection in the socket which can spread and disrupt other healthy tissues [10-12, 14],

• pressure necrosis leading to soft tissue breakdown,

• pain, and

• the loss of mechanical integrity is common resulting in increased energy consumption.

On a larger scale, these problems result in failure of the device and disease of the remaining limb, thus preventing ambulation and reducing the amputee’s quality of life. Within the initial stages following amputation, it is common to have several sockets within the first two years due to changes in the residual limb. Once the final prosthesis has been created, they last an average of three to five years. However, during these three years, because the measurement of the stump is not fixed and constantly changing, proper fit of the prosthetic is difficult and requires continued maintenance. Replacement
after three to five years is usually required due to excessive wear and improper fit.

Figure 1.1. Conventional stump-socket attachable prosthesis in which the residual limb (stump) fits into the socket. (Adapted from [15])

For the above reasons, there is a great need to improve the design of the limb prostheses in order to help amputees in their general activities of daily living. One of the newest, more promising limb prosthesis is the intraosseous transcutaneous amputation prostheses (ITAP, Figure 1.2). Inserting this device into an amputee involves a two step surgical procedure. The first stage involves directly anchoring a custom made titanium (Ti) fixture into the shaft of the residual bone, ensuring the implant is securely and strongly attached. The second stage involves penetrating the skin and inserting a Ti abutment that provides the attachment site for the external prosthetic. Benefits of this system compared to the conventional stump-socket prostheses include a full, unrestricted range of movement, improved sensory feedback
since the prosthetic is now directly linked with the skeletal system (also known as osseoperception) [16-18], no fitting problems (the prosthetic is correctly and firmly attached), and reduced skin complications. However, like most devices, ITAPs also suffer from a number of problems that limit their efficacy, including lack of integration with surrounding tissue and infection. These issues are addressed in the next section.

Figure 1.2. (a) Schematic illustration of an intraosseous transcutaneous amputation prostheses (ITAP). (b) Abutment and skin penetration area of ITAP for a trans-femoral amputee. (c) Attachment of a prosthesis to the abutment using an Allen key for a trans-femoral amputee. (Adapted from [19])

1.2 Problems with Current ITAP

Over three million people globally require a prosthesis. More specifically, from 1988 to 1996, an average of 133,235 hospital discharges per year involved limb loss or limb deficiency [3]. For lower limb amputations alone, an incidence of 2.8-43.9 per 100,000 individuals has been observed [4]. Clearly, since prosthetic replacement is one of the most significant solutions
for those who suffer from an amputation, creating a prosthesis that is efficient, comfortable, and long-lasting is crucial. As previously mentioned, conventional stump-socket prostheses are being replaced with ITAP. Although ITAP offer many more advantages than conventional stump-socket prostheses, the patient is still confronted with device complications that lead to limited function and longevity. These complications are present because unlike the stump-socket prosthesis, the surface of the ITAP is in direct contact with living tissue, including bone and skin. Thus, it is essential that interactions between the device and both surrounding tissues (including bone and skin) are favorable. Unfortunately, all materials that are in direct contact with living tissue only function for a finite amount of time due to physiological conditions that change the properties of the device with use. These changes lead to eventual loosening of the bone-anchored implant, improper growth of skin around the abutment, pain for the amputee, and eventual failure of the ITAP. Thus, it is essential to understand the events and mechanisms causing such problems in order to correct such issues and provide the amputee with a device that will allow them to lead a functional, more normal life. The modes of failure at the bone- and skin-metal interface for an ITAP are not fully understood but can be classified into two categories, mechanical and biological.
1.2.1. Mechanical Factors

As with all orthopedic implants, high stress-strain imbalances, implant migration, and wear debris are only a few of the mechanical factors that decrease the longevity of the ITAP. These events often act in conjunction in which high stress-strain imbalances lead to migration of the implant which can often lead to the generation of wear debris, each causing early failure of the ITAP (Figure 1.3).

Figure 1.3. Mechanical factors, including (a) stress-strain imbalances, (b) implant migration, and (c) wear debris create problems leading to early failure of the ITAP at the bone-metal interface. These factors work in conjunction where high stress-strain imbalances lead to migration which often leads to the generation of wear debris. (Adapted from [20])
1.2.1.1. Stress-Strain Imbalances

Bone is constantly undergoing physiological stresses, including compression, tension, torsion, and bending. Experimental research and finite elemental analysis together have revealed that the majority of the load bearing responsibility for the femur and hip is undertaken by cortical bone, the component of bone with the strongest mechanical properties [21]. The addition of the metal fixture into the residual femur of a trans-femoral ITAP, as well as the abutment attached to this fixture, results in stress-strain imbalances between the implant and bone which in turn leads to stress shielding. Stress shielding is the mechanism that protects bone from natural stresses that everyday life exposes to them. Because an implant, such as an ITAP, is stiffer than natural bone, the load from the body is transferred to the implant, thus, affecting the maintenance of bone mass. Instead of stresses flowing from the hip, joints, thighbones, knee joints, low leg bones, feet, and then to the floor, they flow through the components of the ITAP. This means that bone is no longer sufficiently loaded causing the synthesis of less healthy tissue making bone weaker and more susceptible to fracture. On the other hand, the bone directly surrounding the implant, in particular the trabecular bone, is also now bearing some of the load and therefore becomes thicker and stronger. However, the trabecular bone is not equipped or designed to sustain such high physiological loads [21].
As a result of the mismatch in mechanical properties between the metallic implant to both bone and skin, the prosthetic is vulnerable to loosening and fatigue while surrounding bone and tissue is damaged and resorbed [6, 10, 11]. The amount of stress a biomaterial implanted into bone is exposed to depends on the geometry and location of the device, strength of the material used to make the device, and whether or not some additional material, such as bone cement, is used to provide better bonding of the implant to surrounding bone [22, 23].

In addition to the mechanical property mismatch between the Ti implant and bone, there is also mechanical discontinuity between the Ti abutment and skin. This discontinuity results in stress-strain imbalances that cause microtrauma. Any seal that has formed prior to this microtrauma is disrupted leading to chronic injury of the skin [24-29]. In addition, the dominant load in human skin is biaxial tension. However, at the skin-metal interface of the ITAP, device movement and soft tissue inertia during routine physical activity promotes shearing [24]. Shear loading is a highly destructive loading modality that prevents the wound healing process from occurring and thus failure of proper tissue integration to the abutment. As a result, the prosthetic is further vulnerable to loosening and, thus, failure.

Therefore, the design of the prosthetic is critical and must closely match the mechanical properties of the living tissue in order to achieve optimal load
transfer and reduced stress-strain imbalances. As revealed through finite elemental analysis, a few ways this may be achieved are through creating a more fully bonded implant [30], using a material that has a lower Young’s modulus than bone (less stiff) [31], minimizing the shear conditions at the skin-metal interface, and developing safety devices to protect and strengthen the fixation between the abutment and metal fixture.

1.2.1.2. Implant Migration

When there are high stress-strain imbalances at the bone-metal interface and the metal fixture and the abutment, migration of the ITAP may occur. Migration refers to the micromotion (0.1-1 mm) of an implant over time [32]. When this micromotion becomes excessive, the implant shifts to such a high degree that bone in-growth into the surrounding implant does not occur [33]. This leads to the debonding of the implant with juxtaposed bone, resulting in early implant failure. Micromotion is not only observed at the bone-metal interface of an ITAP but also is observed between the abutment and internal fixture implanted into the femur [16]. This micromotion is indicated by black discharge around the skin penetration site. This movement is due to the tightening of the central retaining bolt that holds the abutment in place to a specified torque. The abutment needs to be tightened as a result of general, extended use as well as if the amputee falls and deforms the abutment [16, 34].
Bone cement, such as poly(methyl methacrylate), PMMA, is a filler material that allows for immediate fixation of the implant to surrounding bone, thereby preventing micromotion [35]. Yet, as with any design, disadvantages exist when using bone cement. Problems include excess bone removal in order to make room for bone cement, bone necrosis caused by the high temperature exothermic polymerization process, poor blood circulation due to the monomer vapor created during polymerization, leakage of unreacted monomers into the blood before polymerization, shrinkage of bone cells during polymerization, and mismatched stiffness properties between the bone cement and surrounding tissue [36, 37]. Therefore, when designing a bone implant, a material that enhances early bone formation (and, thus, bonding to the surrounding bone) is desired. This would prevent migration, loosening, and early failure of the device.

1.2.1.3. Wear Debris

Wear debris is another common failure mode for ITAP and is created when two opposing surfaces under load move relative to each other and become lodged in the gap between the implant and bone. Specifically, wear debris for an ITAP is generated at the region where the Ti fixture in bone connects with the Ti abutment that protrudes through the skin (metal-on-metal combination). As mentioned earlier, mechanical failure modes act in conjunction whereby migration of an implant, caused by high stress-strain
imbalances, results in the generation of wear debris at the metal-on-metal interface of the ITAP [38-40]. Wear debris can also be generated from (i) corrosion of the implant caused by the harsh biological environment and associated activities (such as protein adsorption, changes in pH, and changes in temperature), (ii) cracks in the material created by high contact stresses that cause particles to break from the surface, or (iii) abrasion and fatigue.

The presence of wear debris particles result in thinning of the implant components compromising the structural integrity of the implant, inflammation to surrounding tissues, loosening of the implant, and bone cell death. Wear debris particles can also be released into the blood stream to produce additional clinical complications. More importantly, wear debris can cause severe osteolysis, active resorption of surrounding bone [41, 42], leading to implant instability and failure.

The rate of wear production and the amount of wear particles generated are directly related to the failure of the implant [43]. For instance, the smaller the wear rate, the fewer the wear particles generated, the less likely an implant will fail. Metal-on-metal prosthetics (such as the ITAP) exhibit low wear rates (2-20 µm/year), thus resulting in decreased generation of wear particles [44]. However, despite such promise, concerns still exist. For example, although metal-on-metal prosthetics generate fewer particles, the sizes of the particles are on the nanometer scale [45, 46]. As a result, more
wear debris particles are created resulting in a higher surface area and thus having a larger, more damaging effect on cells [47].

Depending on the size of the wear debris particles, certain pathways are activated that negatively affect bone turnover (Figure 1.4). Particles between 0.5-10 µm activate macrophages which then cause the release of cytokines (interleukins, IL-1 and IL-6, and tumor necrosis factors, TNF-α) and prostaglandins (PGE2) [48, 49]. These secretory products mediate bone resorption via osteoclasts (osteolysis). Particles greater than 10 µm activate foreign body giant cell responses that release cytokines (IL-1, TNF-α, TNF-β, platelet derived growth factor, or PDGF) signaling for fibroblasts [48, 49]. These fibroblasts then begin to synthesize collagen (Type III) which leads to formation of fibrous tissue around the implant (as described later in section 1.5) preventing proper osseointegration.
1.2.2. Biological Factors

In addition to mechanical factors, there are a number of biological failure modes at the bone-metal interface as well as at the skin-metal interface of ITAP. Biological factors that contribute to bone complications include the lack of adhesion of bone cells, lack of osseointegration between bone and the implant surface, and infection of the bone. Biological factors that contribute
to skin complications include the lack of adhesion of epithelial cells in the dermis (particularly keratinocytes), epidermal downgrowth, and infection of the skin.

1.2.2.1. Bone-Metal Interface

The lack of osteoblast adhesion [50, 51] (the physiochemical linkage and protein interaction between cells and the implant surface) and lack of osseointegration [52-55] (the bonding between the implant and surrounding bone under load) are biological complications that lead to the short lifespan of orthopedic implants. Osseointegration specifically involves the growth of bone tissue to the surgically implanted device ensuring that the implant is securely and strongly attached. When insufficient bonding between an implant and juxtaposed bone occur, there is no permanent mechanical fixation of the implant to the surrounding bone. This results in loosening and migration of the device as well as regions for possible wear debris to form.

Also, when an orthopedic device is introduced into the body, it is treated as foreign and, thus, the body initiates the natural wound healing process to create a fibrous capsule that blocks off the device from the rest of the body (as described later in section 1.5). This fibrous capsule inhibits sufficient osseointegration of the implant into surrounding tissue by preventing the attachment of bone cells and thus new bone formation. Thus, for an implant
to be effective, the fibrous tissue formation needs to be minimized while new bone formation needs to be maximized.

Another biological factor at the bone-metal interface that leads to failure is nosocomial infection on the implant surface which is a common problem resulting from exposed tissue. Bacteria from the patient’s own skin (mainly *Staphylococcus epidermidis* (*S. epidermidis*)) enter into the wound site during surgical implantation of the device [56] and irreversibly adhere to the surface of the implant, forming an impenetrable biofilm that prevents sufficient osseointegration (Figure 1.5). This biofilm is highly resistant to both host defense mechanisms and antibiotics [57], thereby resulting in early failure and removal of the prostheses.

Figure 1.5. Micrograph of a biofilm on a metal surface. The solid white arrow indicates the biofilm while the open white arrows indicate the metal surface to which the biofilm has attached and covered. (Adapted from [56])
1.2.2.2. Skin-Metal Interface

The abutment of the ITAP is not stabilized by osseointegration since it is not directly anchored to the bone. Instead, stabilization is achieved by the formation of a tight seal between the skin and Ti abutment. This seal is facilitated by epithelial cell adherence (specifically keratinocytes) at the epidermis that leads to strong dermal attachment. However, dermal attachment to the abutment is often poor in ITAP, resulting in epithelial cell (keratinocyte) migration rather than adherence. Research has shown that epidermal cell migration occurs through damaged collagenous tissue (contained in the dermis) but is halted when there is a healthy collagenous matrix [58]. Therefore, disruption of the collagenous matrix (or dermis layer) caused during penetration of the abutment leads to epidermal migration that results in downgrowth along the implant as a means to reestablish continuity of the skin layers (Figure 1.6) [10]. Downgrowth occurs as a direct result of the normal wound healing response to regenerate the protective barrier function of the skin. More specifically, growth factors and the absence of neighboring cells serve as signals for the down regulated proliferation and migration of epithelial cells in the epidermis [59, 60]. Inhibited epithelial cell (keratinocyte) growth eventually leads to extrusion of the implant that leads to failure of the device [27].
Figure 1.6. Schematic representation of epidermal downgrowth formed to re-establish the skin layer. This downgrowth is due to poor dermal attachment and activation of the body’s wound healing response. (Adapted from [10])

This epidermal downgrowth prevents the formation of a tight seal between the skin and implant surface providing a clear route for invasion of bacteria [61, 62]. Infection (caused by the bacteria invasion) results in further breakdown of the skin-metal interface and thus implant failure [27, 28, 63-65]. As bacteria colonize either the implant surface or adjacent damaged tissue sites, biomaterial exit sites become the gateway to infection [66-76], possibly leading to bacteria spreading internally and causing osteomyelitis [76, 77]. According to previous studies, the occurrence of osteomyelitis after insertion of an external fixator is anywhere between 0 to 4% [66-77]. In
addition to osteomyelitis, infection leads to bone implant loosening [72-75] and fracture malunion or nonunion of the ITAP.

1.3 Bone Physiology

To make materials which will better integrate into bone, one needs to understand the composition and natural geometry of bone. Bone is a complex, well organized tissue designed to mainly protect internal organs as well as support physiological stresses and strains. Scale is most important when describing the hierarchical structure of bone and understanding the relationship between these structures and the various levels. The structure of bone can be broken down into three levels: the macrostructure level (such as cortical and trabecular bone), the microstructure level (such as lamellae, osteons, and Haversian systems), and the nanostructure level (such as collagen, noncollagenous proteins, and inorganic minerals). Outlined below are these various levels of bone hierarchy.

1.3.1. Macrostructure and Microstructure of Bone

1.3.1.1. Macrostructure Level Components (cortical and trabecular bone)

At the macrostructure level, bone is divided into two types: the smooth, rigid, white outer layer is called compact or cortical bone and the inner, porous...
region is called cancellous or trabecular bone (Figure 1.7). Cortical bone, largely responsible for the supportive and protective function of the skeleton, accounts for 80% of the total bone mass. It is a highly anisotropic tissue composed of closely packed osteons lying parallel to the long axis of bone. Due to the minimal gaps and spaces between the osteons, cortical bone is characterized by less than 30% porosity with a pore size up to 1 mm in diameter. On the other hand, trabecular bone accounts for 20% of the total bone mass with nearly ten times the surface area of cortical bone. Trabecular bone is an organized isotropic tissue composed of a three-dimensional network of rods and plates known as trabeculae. Due to the large spaces between the individual trabeculae, trabecular bone is characterized by 50-90% porosity with a pore size up to several millimeters in diameter.

1.3.1.2. Microstructure Level Components (lamellae, osteons, and Haversian systems)

At the microstructure level, cortical bone consists of two structures, woven and lamellar bone. Woven bone is a weak, immature, disorganized form that is composed of a small number of randomly oriented, coarse collagen fibers. This disorientation causes the mechanical properties of woven bone to exhibit isotropic characteristics. Woven bone is found more at the newly formed regions of healing fractures, the metaphyseal regions of growing bone, and the diseased regions. Woven bone is remodeled into strong, mature, highly
organized bone known as lamellae composed of many stress-oriented, parallel collagen fibers. This specific orientation causes the mechanical properties of lamellar bone to exhibit anisotropic features with the greatest strength parallel to the longitudinal axis of collagen fibers. Lamellar bone is formed into concentric ring-shaped matrices called osteons that surround a central canal known as the Haversian canal containing blood vessels and nerves (Figure 1.7 and Figure 1.8).
Figure 1.7. (a) Schematic diagram of smooth, rigid cortical (compact) bone composed of closely packed osteons and porous trabecular (spongy) bone composed of a network of rods and plates. (b) Close-up region of an osteon that surrounds the Haversian canal containing blood vessels and nerves. (c) Micrograph of cross-section view of an osteon. (Adapted from [78])
1.3.2. Nanostructure of Bone

Regardless of whether bone is woven or lamellar, natural bone is a composite material composed of two phases: the organic phase (comprised of proteins) and the inorganic phase (mainly consisting of inorganic minerals). The matrix of the human femur is composed of approximately 70% inorganic phase, 20% organic phase, and 10% water [79-81]. Without the presence of these building blocks, the remaining structure of bone would not form.

1.3.2.1. Organic phase

The main component of the organic phase of bone is Type I collagen, which makes up about 90% of the organic matrix, while the remaining 10% of the
matrix consists of noncollagenous proteins (such as bone inductive proteins) and other macromolecules (such as growth factors and cytokines) [79-82].

Type I collagen found in bone is synthesized by osteoblasts (bone-forming cells) and is secreted as a triple helical procollagen into the extracellular matrix, where collagen molecules are stabilized by the cross-linking of reactive aldehydes among the collagen chains. The 12 types of collagen found in the body consist of three polypeptide chains composed of approximately 1400 amino acids. Type I collagen (molecular weight 139 kDa) possesses two identical α1(I) chains and one unique α2(I) chain (Figure 1.9). The combination of these chains produces a triple helical structure 300 nm long and 1.5 nm in diameter (Figure 1.9 and 1.10). Several collagen molecules spontaneously align along the helical axis and group as a bundle to form collagen fibrils that provide structure to bone but not rigidity (Figure 1.9 and 1.10) [83-85]. The collagen molecules have a periodicity of 67 nm which gives collagen fibrils their characteristic banded structure. Gaps (called hole-zones) exist between the ends of the molecules and between the sides of parallel molecules (Figure 1.10) [81, 86-88].
Figure 1.9. Schematic representation of Type I collagen in bone. Type I collagen possesses two identical α1(I) chains and one unique α2(I) chain whose combination produces a triple helical structure 300 nm long and 1.5 nm thick with a periodicity of 67 nm. Together, these molecules align along the helical axis to form collagen fibrils. (Adapted and redrawn from [85])
Numerous noncollagenous proteins are found in the organic matrix of bone including growth factors and cytokines (insulin-like growth factors (IGF) and osteogenic proteins), bone inductive proteins (osteonectin, osteopontin, and osteocalcin), and extracellular matrix compounds (bone sialoprotein, bone proteoglycans, and other phosphoproteins and proteolipids) [81]. These noncollagenous proteins are responsible for regulating the size and orientation of the minerals in the inorganic phase, serve as the reservoir for calcium and phosphate ions, and inhibit and regulate bone synthesis and resorption. Thus, even though noncollagenous proteins contribute very little
to the overall weight of bone, their contribution to the biological function of bone is extremely important.

1.3.2.2. Inorganic Phase

The main component of the inorganic phase of bone is crystalline calcium hydroxyapatite (HA), Ca$_{10}$(PO$_4$)$_6$(OH)$_2$. These plate-like crystals are found in the hole zones amongst the collagen molecules (Figure 1.10) and are 2·5 nm thick and 20·80 nm long [79, 81]. The sizes of the crystals are directly related to the maturity of the bone. In other words, a new bone matrix contains HA crystals that are less crystalline and smaller than those of a developed, mature bone matrix. Unlike collagen in the organic phase, HA crystals contribute to the rigidity of bone. Due to their location, the growth of the HA crystals are limited, thus, forcing them to be discrete and discontinuous. They do however grow with a specific crystalline orientation, the c axes of the crystals are roughly parallel to the long axes of the collagen fibrils [90]. Thus, the inorganic minerals are deposited on and along the aligned collagen fibrils. Such alignment (of the collagen fibrils and HA crystals) leads to the overall anisotropy of bone in which the mechanical properties vary depending on the direction of loading, either across or along the axis (Table 1.1).
1.3.3. Mechanical Properties of Bone

Cortical bone is more dense, mechanically stronger, and stiffer (Young’s modulus of 17 GPa) than trabecular bone (Young’s modulus of 1 GPa) [79, 80, 91, 92]. Properties of cortical and trabecular bone can be seen in Table 1.1. Because of the anisotropic behavior observed not only in cortical bone but also in the constituent components of bone (Type I collagen and HA crystals), the mechanical properties of bone vary depending on the direction of loading, as seen in Table 1.1. Sex, age, race, diet, health, and anatomical location are variables that can change the properties of bone, including mass, density, and mechanical properties. For example, bone mass can decrease by 50% in trabecular bone and 25% in cortical bone after a person turns 40 [79]. Such a phenomena is due to a decrease in the remodeling process of bone where formation of new bone becomes constant while bone resorption increases [79]. Thus, as a person ages, their bones become less stiff, less strong, and more brittle explaining why the elderly are more prone to fractures.
Table 1.1. Density and mechanical properties of healthy human bone, including the femur, tibia, humerus, radius, hip, and spine [79, 80, 91, 92].

<table>
<thead>
<tr>
<th></th>
<th>Trabecular Bone</th>
<th>Cortical Bone (Longitude)</th>
<th>Cortical Bone (Transverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/cm$^3$)</td>
<td>0.05-0.7</td>
<td>0.7-1.8</td>
<td>0.7-1.8</td>
</tr>
<tr>
<td>Elastic Modulus (GPa)</td>
<td>0.1-0.5</td>
<td>17-30</td>
<td>7-13</td>
</tr>
<tr>
<td>Ultimate Tensile Strength (MPa)</td>
<td>2-20</td>
<td>130-150</td>
<td>50-60</td>
</tr>
<tr>
<td>Compressive Strength (MPa)</td>
<td>0.2-4</td>
<td>190</td>
<td>130</td>
</tr>
</tbody>
</table>

1.3.4. Bone Cells

The anisotropic properties of bone are due to the coordinated functions of bone-forming cells (osteoblasts), bone-resorbing cells (osteoclasts), and bone-mechanotransduction cells (osteocytes), as described below. Figure 1.11 is an electron micrograph of an osteon present in cortical bone that highlights the presence of osteoblasts, osteoclasts, and osteocytes.
Figure 1.11. Electron micrograph of an osteon present in cortical bone which houses osteoblasts (OB), osteoclasts (OC), and osteocytes (O) that are involved in the bone remodeling process. (Adapted from [93])

1.3.4.1. Osteoblasts

Osteoblasts, located in an osteon of cortical bone (Figure 1.7 and 1.8), are cells responsible for bone formation. They are large, randomly oriented, cuboidal cells with an average diameter of 10 to 50 µm and typically differentiate from the same mesenchymal osteoprogenitor cell line as do osteocytes and preosteoblasts [94]. Osteoblasts secrete large quantities of osteoid that consists of aligned collagen fibers. The collagen fibrils within the osteoid seed crystals of calcium salts (HA) that mineralize the bone matrix.

A schematic of the time course of osteoblast function and synthesis of extracellular matrix proteins on a newly implanted biomaterial is shown in Figure 1.12 [81, 95]. First, after initial adhesion to the surface of the
implant, osteoblasts actively proliferate and express genes for Type I collagen, vitronectin, and fibronectin. Fibronectin (molecular weight, 273 kDa) and vitronectin (75 kDa) are glycoproteins present at high concentrations in the extracellular matrix of bone. Fibronectin and vitronectin are both highly hydrophilic proteins. At the end of proliferation, the extracellular matrix development and maturation process begins, and osteoblasts start to differentiate from non-calcium to calcium depositing cells. In addition, alkaline phosphatase activity (a dephosphorylation enzyme active in an alkaline environment that is an indicator of bone turnover and bone cell function) and mRNA expression for proteins (such as osteopontin and collagenase) are increased. As the mineralization process begins and mineral nodules form, osteoblasts synthesize and deposit bone sialoprotein (a component of mineralized bone), osteocalcin (a calcium-binding protein), and other matrix proteins that eventually form HA crystals.
Figure 1.12. Time course of osteoblast functions after adhesion on a newly implanted biomaterial. (Adapted from [81, 95])

1.3.4.2. Osteocytes

When osteoblasts are entrapped within the mineralized bone matrix they have synthesized, they stop making osteoid and become an osteocyte. Osteocytes are located around the central lumen of an osteon and in between lamellae (Figure 1.7 and 1.8). Similar to osteoblasts, osteocytes are cuboidal in shape with no specific orientation. Osteocytes contribute to new bone synthesis by secreting growth factors, such as IGF and tissue growth factor-β (TGF-β), that further control osteoblast functions by promoting their
differentiation from immature, non-calcium secreting osteoblasts into mature, calcium secreting osteoblasts [79]. Osteocytes are also thought to be the ‘communication cells’ of bone because they possess extensive long branches with which they establish contacts and interactions with adjacent osteocytes through small channels called canaliculi [79]. Through these cell processes, osteocytes are believed to be sensitive to physiological stress and strain signals experienced by bone and, thus, direct functions of osteoblasts and osteoclasts.

1.3.4.3. Osteoclasts

Osteoclasts, located in the regions of bone resorptive surfaces in pits, called Howship’s lacunae, are cells responsible for bone resorption by removing mineralized matrix and decomposing the organic matrix (Type I collagen). They have an average diameter of 100 μm, up to 100 nuclei per cell, and typically differentiate from hematopoietic pluripotent cells of the bone marrow [94]. Osteoclasts, when not on the surface of bone, are considered to be inactive and do not possess a ruffled border. When osteoclasts sweep across disrupted bone surfaces to dissolve bone, they become active and form ruffled cell membrane edges to increase their total surface area of attachment onto the resorptive surfaces. They then secrete tartrate-resistant acid phosphatase (TRAP) and form Howship’s lacunae (resorption pits). The presence of TRAP results in the release of hydrogen ions through the carbonic
anhydrase system that lower the pH of the local environment, which increases the solubility of HA crystals. Finally, the organic component of bone matrix is then removed through acidic proteolytic digestion and bone is resorbed.

1.3.5. Bone Remodeling

Bone remodeling is a dynamic, lifelong process that allows bone to change and respond to different kinds of stress produced by physical activity or mechanical loads. Through the remodeling process, bone has the ability to regenerate when diseased or damaged and also remodel when loading conditions change. The process keeps bone mass constant by removing old or injured bone and adding new bone. Bone remodeling takes place in a bone modeling unit (BMU) or osteon (Figure 1.7 and 1.8) and involves both osteoblasts and osteoclasts. At any one moment, 20% of the cancellous bone surface is undergoing remodeling [96]. Figure 1.13 illustrates how cells cooperate in the specific, highly organized bone remodeling process [81, 97]. First, osteocytes sense when mechanical stress and forces alter local bone structure and, as a result, secrete a paracrine signal (such as IGF-I and TGF-β) which activates osteoclasts to resorb the old bone [96]. Once the bone has been resorbed, Howship’s lacunae or resorption cavities are formed. This resorption of bone by osteoclasts can take place over a course of ten days [98]. The termination of bone resorption and initiation of bone formation in the
resorption lacunae occurs through a coupling mechanism that is activated by growth and proteinases (such as TGF-β, IGF-I, IGF-II, and plasminogen activators) [96]. After osteoblasts have repaired the surface and filled the cavities with new bone, the new bone is then calcified, restored, and the remodeling process is complete. Bone formation can take up to three months to complete [98].

Figure 1.13. Schematic representation of coordinated bone cell functions that maintain homeostasis during bone remodeling. Osteoclasts and osteoblasts act simultaneously towards remodeling bone by replacing old bone tissue with new bone tissue. Bone remodeling begins with the decomposition of bone by osteoclasts which create resorption pits. Osteoblasts are then recruited and start forming new bone that is later calcified and restored. (Adapted from [99])
After an individual turns 40, bone remodeling speeds up, becomes unbalanced, and bone mass is no longer kept constant. In other words, new bone formation is unable to keep up with the rate of bone resorption, leading to an overall decrease in bone density [100]. Osteoporosis is a major bone disease caused by increased bone resorption and decreased bone formation (Figure 1.14). Individuals with osteoporosis exhibit weaker, more fragile bones that more easily fracture.

1.4 Skin Physiology

Improving skin growth is also important in the development of ITAP. Similar to bone, one needs to understand the composition and natural geometry of skin in order to make materials which will better integrate with skin. As the largest organ of the body, skin covers an area of about 1 to 2 m², ranges in thickness from 1.5-4.0 mm and weighs 4-5 kg [78]. Skin serves as a physical barrier for muscles, bones, ligaments, and internal organs to the
outside environment. There are three structural levels of skin: the epidermis, the dermis, and the subcutaneous fatty tissue (Figure 1.15). The epidermis provides skin with an elastic rigidity while the dermis provides viscoelasticity to the skin [24].

Figure 1.15. Schematic three-dimensional view of the skin, demonstrating the two main layers, the epidermis and dermis. (Adapted from [78])
1.4.1. Epidermis

The epidermis is a stratified squamous epithelium that forms the thin, outer layer of skin which serves as the physical and chemical barrier to the interior body and the hostile exterior environment. The thickness of the epidermis ranges from 0.01 to 2 mm depending on the location in the body [101]. The epidermis is composed of four layers: the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale (Figure 1.16). The epidermis has no vascularization and each layer is composed of varying epithelial cell types that will be described in the next section. The cells are closely packed and produced by cell division of the basal layer. In fact, at any one moment, 30% of the cells in skin are preparing for division [101].

Figure 1.16. (a) Light microscope micrograph and (b) schematic diagram indicating the epidermal layers of the human skin (can also be seen in Figure 1.18). Each layer is composed of varying cell types described later (Figure 1.18). Scale bar = (a) 100 µm. (Adapted from [78, 102])
The stratum corneum is the outermost layer of the epidermis and is 10-40 µm thick. The stratum corneum is known as the cornified part of the epidermis because it consists of 25 to 30 rows of differentiated, flat, dead cells that lack nuclei and are filled with keratin. Keratin is an elaborately coiled fibrous protein that helps keep the skin hydrated by preventing water loss and gives skin considerable mechanical strength and flexibility that protects against abrasion and penetration. Keratin, like fibronectin and vitronectin in the extracellular matrix of bone, has a strong hydrophilic character. Thus, the presence of keratin in the stratum corneum allows this layer to form a barrier that shields the interior body from elements in the hostile external environment, including light and heat waves, chemicals, and microorganisms that cause infection. In addition to keratin, the stratum corneum contains a lipid-filled extracellular space that helps prevent water loss [103]. The formation and molecular organization of this extracellular space is complex. More specifically, the extracellular space contains 39-48 nm thick regions interconnected by thinner (9-33 nm) linker regions [104] (Figure 1.17) indicating that skin, like bone, consists of nanostructures.
Figure 1.17. (a) Low magnification micrograph of the stratum corneum epidermal layer. (b) Enlargement of the lower part of (a) indicating the extracellular matrix regions are around 44 nm thick. These extracellular regions are connected by thinner linker regions that range from 9-33 nm in thickness as indicated in (c) and (d) by the solid black arrows. Scale bar = (a,b) 500 nm and (c,d) 200 nm. (Adapted from [104]).
The 3 µm thick stratum granulosum, also known as the granular layer, is the second layer of the epidermis. It consists of three to five layers of differentiated, flattened cells whose nuclei and organelles have begun to disintegrate. These cells develop into darkly stained granules of keratohyalin and lamellae known to make skin more resistant to destruction. Keratohyalin granules are the precursors of keratin and the lamellated granules secrete a glycolipid into the extracellular matrix that is responsible for decreasing water loss across the epidermis, as discussed above.

The stratum spinosum, also known as the spinous layer, is the third layer of the epidermis and is 50-150 µm thick. It consists of eight to ten rows of closely packed, differentiated polyhedral (many-sided) cells. More specifically, these cells form a web-like system of intermediate filaments that mainly consist of tension-resisting bundles of pre-keratin filaments; thus, the deposition of keratin in cells begins in this layer.

The stratum basale, the deepest layer of the epidermis, attaches to the underlying dermis and has a thickness ranging from 5-10 µm. It consists of a continuous, single layer of undifferentiated, cuboidal cells that are considered to be the stem cells of the epidermis. This is the only layer of the epidermis where cells have the capacity for DNA synthesis and mitosis.
1.4.2. Dermis

Making up the bulk of the skin, the dermis is found directly beneath the epidermis. It provides the structural support as well as nourishes the epidermis (which lacks vasculature). The dermis is also responsible for cushioning the body from stress and strain. The thickness of the dermis layer ranges from 0.3 to 3 mm. The dermis is comprised of two layers: the outer portion known as the papillary layer and the inner portion known as the reticular layer.

The papillary layer accounts for one fifth the total thickness of the dermis and consists of small, fingerlike projections called papillae which greatly increase the surface area of the dermis (Figure 1.15). These papillae extend toward the epidermis, creating ridges into the overlying epidermis and strengthening the connection between the epidermis and dermis. The papillae also contain tactile receptors that provide the sensation of touch.

The reticular layer varies in thickness which contributes to the differences in the overall thickness of skin. The reticular layer provides skin strength, stretchability, and elasticity. This region is attached to underlying organs, including bone and muscle, by the subcutaneous layer known as the hypodermis. The hypodermis is also responsible for insulating the body from the cold and stores energy in the form of fat.
1.4.3. Structural Organization of the Epidermis and Dermis

1.4.3.1. Epidermis Components

The epidermis contains no blood vessels and consists of four layers composed of three types of specialized epithelial cells: keratinocytes (most abundant throughout the epidermis), melanocytes, and Langerhans (Figure 1.18). Because there are no blood vessels, cells in the stratum basal are nourished by the diffusion from blood capillaries of the dermis. Keratinocytes, which comprise about 90% of the cells in the epidermis, arise from the deepest part of the epidermis (the basal layer) as immature, undifferentiated cells. As they mature through the layers of the epidermis, they grow and flatten while simultaneously producing and filling with keratin, which, as previously mentioned, helps waterproof and protect the skin in addition to underlying tissues and organs. Keratinocytes also secrete a material made of lipids, cholesterol, saturated fatty acids, and ceramides into the intercellular spaces of the epidermis layers that increase the cohesion between the cells which contributes to the protective barrier properties of skin. Eventually, keratinocytes in the outer most layer of the epidermis (corneum layer) are merely cellular skeletons filled with keratin and a degenerated nucleus (and other organelles). In addition, enzymes disrupt the cohesion between the cells causing separation from the surface one by one.
Keratinocytes are connected to one another in the basal layer by anchoring junctions called desmosomes. Desmosomes, major components of the extracellular space of the viable epidermis, are also found at the interface between the stratum corneum and stratum granulosum layers of the epidermis (Figure 1.18). Similar to collagen and HA crystals in bone, desmosomes are one component that provides the nanostructure of skin. More specifically, desmosomes express a transverse periodicity of 5 nm and have an extracellular core of around 32.6 nm thick and a plasma membrane around 4 nm thick [104] (Figure 1.19).

Melanocytes, found in the stratum basale and spinosum layers of the epidermis, comprise 8% of the cells in the epidermis. Melanocytes produce melanin, a brown-black pigment, which is responsible for the diversity in human skin color and for absorbing ultraviolet light. Melanocytes have long, slender spider-like projections that extend between and transfer granules of melanin to keratinocytes. After entering into the keratinocytes, the granules cluster together to form a protective veil over the nucleus that shields the genetic material from being damaged by ultraviolet light.

Langerhan cells originate from bone marrow and migrate to the epidermis, particularly to the stratum spinosum. These cells are responsible for protecting the body against infections by interacting with white blood cells in the immune system and acting as phagocytes that ingest foreign substances.
Figure 1.18. Cellular components of the epidermal layer. (a) Micrograph of the four major epidermal layers. (b) Diagram indicating the layers and the distributions of different cell types corresponding to each layer. Keratinocytes are the dominant cell throughout all layers of the epidermis while melanocytes and Langerhan cells are less numerous. (Adapted from [78])
Figure 1.19. (a) High magnification micrograph of desmosomes located throughout the epidermis. (b) Enlargement of the middle left part of (a). Desmosomes have a plasma membrane around 4 nm thick and an extracellular core around 33 nm thick. As indicated by the solid black arrow in the right image, desmosome periodicity is 5 nm. Scale bar = 50 nm. (Adapted from [104])

1.4.3.2. Dermis Components

The dermis contains only a few cell types, including fibroblasts, macrophages, and adipocytes, and is a soft tissue composed of blood vessels (that provide nourishment and waste removal from the epidermis) and connective tissue containing collagen (Type III in the upper layers and Type I in the lower layers [105]) and elastic fibers. In fact, skin is known to be comprised of 75% collagen [85]. As described in section 1.3.2, collagen is a triple helical protein that possesses nanosize features. Thus, similar to bone cells, skin cells are accustomed to interacting with nanophase materials due to the nanostructured components found in both the epidermis and dermis. The
combination of collagen and elastic fibers explains why skin is strong, flexible, and expandable. More specifically, the papillary layer consists of areolar connective tissue containing a thin arrangement of collagen and elastic fibers. On the other hand, the reticular layer consists of dense, irregular connective tissue containing interlacing bundles of collagen and course elastic fibers. Space between the fibers is filled with adipose tissue, hair follicles, nerves, sweat glands, and lymph vessels. The hypodermis that binds the skin to underlying structures is composed of connective tissue, nerves, blood vessels, and fat cells.

1.4.4. Growth Process of Skin

Because of the many layers and components present in skin, the growth of skin is a very meticulous process. Cells in the bottom basal layer are constantly undergoing mitosis. After division, one of the daughter cells remains in the basal layer in an undifferentiated state where it continues to generate new cells. The other daughter cells migrate upward toward the stratum corneum and differentiate into keratinocytes. As the keratinocytes migrate, they are unable to divide but begin the process towards terminal differentiation. During the keratinocytes journey to the surface of the skin, its physiology, chemical composition, morphology, and orientation change resulting in the production of a dead, flattened, enucleated keratinocyte,
which eventually is sloughed off and replaced by other inner keratinocytes differentiating upward. This process can be seen in Figure 1.20.

This process of epidermal growth and differentiation can be subdivided into four parts corresponding to each layer. The cells (keratinocytes) produced in the basal layer possess an intracellular cytoskeleton composed of a relatively dispersed, but extensive, network of keratin filaments made of a 1:1 ratio of two distinct keratin proteins, K5 (58 kDa) and K14 (50 kDa) [106, 107]. In addition, the cells possess desmosomes, calcium-activated membrane junctions that interconnect the cells into a three-dimensional lattice [108]. Production of new cells in the basal layer pushes the newly differentiated keratinocytes upward to the spinous layer. This layer determines the differentiation rate of the keratinocytes [109]. Cells in the spinous layer are postmitotic but still metabolically active and spend most of their time synthesizing two new keratin proteins, K1 (67 kDa) and K10 (56.5 kDa) [106, 110]. Rather than possessing a cytoskeleton composed of dispersed keratin filaments, the cells in the spinous layer possess cytoskeletal filaments that aggregate into thin bundles [110]. Cells in the spinous layer are also known to make glutamine and lysine-rich envelope proteins (such as involucrin) that deposit on the inner surface of the plasma membrane of each cell [111]. Finally, cells in the spinous layer create membrane-coating granules that fuse with the plasma membrane and release lipids into the intercellular spaces of the granular and corneum layers [112]. As the migration continues
and the cells reach the granular layer, they stop generating keratin and envelope proteins. They begin producing their final proteins, including loricrin and filaggrin (a basic protein responsible for creating large, macrofibrillar cables from the thin filament bundles produced in the spinous layer) [113, 114]. This process of increased filament packing enables keratin filaments to survive the destructive phase of the cells that occurs next. At this point in the cycle, the cells have become permeable allowing for an influx of calcium that activates epidermal transglutaminase, an enzyme which catalyzes the formation of ε-(γ-glutamyl) lysine isopeptide bonds known to biochemically cross-link the enveloped proteins into a cage [111]. The activation of epidermal transglutaminase, along with the release of other lytic enzymes, results in the loss of metabolic reactions that leads to the deterioration of the nuclei, cytoplasm, and other organelles (the cell is now called a corneocyte). Metabolic termination is partly due to no capillaries present throughout the layers of the epidermis, thus preventing them from receiving nutrients. By the time the cells reach the stratum corneum, they are terminally differentiated, flat keratinocytes with a skeleton composed only of the macrofibrillar cables of keratin filaments that are sealed together by lipids. The desmosomes connecting the cells together have also been dissolved by proteolytic enzymes. The remnants of the keratinocytes are then shed from the epidermis and are replaced with underlying cells that undergo the same progression. This process is known as keratinization and is
completed within four to six weeks (Figure 1.20). Each day, millions of these dead cells rub off of the skin’s surface, thus giving humans a totally new epidermis every 25 to 45 days.

Figure 1.20. Schematic representation of epidermal growth. In the basal layer, cells divide into two identical daughter cells where one remains in the basal layer to divide again while the other migrates upward and differentiates into a keratinocyte. During this migration, the cell undergoes a number of morphological and biochemical changes (i.e. become more flat). By the time the cell reaches the corneum layer, the nucleus, cytoplasm, and other organelles deteriorate (now called a corneocyte). These cells are then sloughed off and replaced continuously by cells below that have undergone the same process (known as keratinization). (Adapted from [115])
A common question regarding this complex process of epidermal growth and differentiation is what the underlying molecular mechanism is. Unfortunately, the trigger responsible for keratinocyte terminal differentiation has yet to be identified [106]. However, research has contributed methods aimed at optimizing terminal differentiation in vitro that has been able to make advances to provide clues on which factors cause terminal differentiation. To do this, reproducing the epidermis in vitro must first be obtained. In doing so, it was found that one factor needed to initiate an array of differentiation processes (including assembly of desmosomes and activation of epidermal transglutaminase) is calcium [111, 116-118]. Another group of regulators that has been linked to reproducing the diminishing features of terminal differentiation in the human epidermis are retinoids [119, 120]. For example, when retinoids were removed from serum, cultured epidermal cells indicated both morphological and biochemical characteristics of those seen in terminal differentiation (including reduced motility and expression of K1 and K10) [120]. Calcium and retinoids alone are not sufficient enough for obtaining in vitro conditions comparable to the human epidermis because when tested in vitro, few spinous and granular cells were generated [120]. It was found, however, that when epidermal cells were cultured in the presence of high concentrations of calcium and low concentrations of retinol on a floating raft of collagen and fibroblasts (which provides nutrients via diffusion similar to the dermis in natural skin) at the
air-liquid interface, a near optimal balance between growth and differentiation was achieved [121-123].

While creating this in vitro culture system, a number of extracellular regulators were found to control the proper balance between epidermal growth and differentiation. Epidermal growth factor (EGF) [124], TGF-α [125, 126], low concentrations of retinoic acid [120, 127], keratinocyte growth factors (KGF) [128], and two cytokines, IL-6 [129] and IL-1α [130, 131], are the positive growth regulators discovered. EGF and TGF-α are the more prominent factors that activate the tyrosine kinase EGF receptors located primarily on cells in the basal layer [132] and lead to differentiation. TGF-α (along with retinoids) have also been shown to enhance keratinocyte migration which corresponds to increased cell growth [120, 126].

There are also negative growth factors that are responsible for causing the keratinocytes to eventually withdraw from their cell cycle and terminally differentiate (keratinization). Movement of a cell from the basal layer may contribute (but is not enough) to activate the cascade of biochemical changes that terminates keratinocyte differentiation. Other factors, including TGF-β [122, 133, 134], retinoids [127], and calcium [113, 120], slow or inhibit DNA synthesis and cell division and re-tailor the architecture of the keratinizing layers above. Expression of TGF-β specifically appears to be dominant in the
differentiating layers above the stratum basal [135], thus, probably causing terminal differentiation.

1.5 The Tissue-Implant Interface

An ITAP device is deemed successful when the processes (bone remodeling and skin growth) are complete, and there is formation and maintenance of healthy new tissue (bone and skin) juxtaposed to the device. The difference between successful tissue integration and failure of an implanted material depends on various cellular or molecular reactions of the body at the tissue-implant interface. Specifically, the events are mediated by how the biomaterial surface interacts with different mechanisms of the normal wound healing response as well as interactions with various proteins in the body.

1.5.1. Wound Healing Response of Bone to Biomaterials

Whether injected, inserted, or surgically implanted, placing a biomaterial into the body inevitably causes damage to surrounding tissues or organs. Insertion of a biomaterial initiates a series of host responses by the body and mechanisms are activated to maintain homeostasis and heal the wound. The sequence of events involves acute inflammatory responses, chronic inflammatory responses, granulation tissue development, foreign body reaction, and possible fibrous capsule development [136-138] (Figure 1.21). This process allows the body to either (i) incorporate the biomaterial into the
body by recruiting osteoblasts and keratinocytes that will form new bone and skin leading to successful integration, or (ii) the biomaterial will be treated as a foreign object, recruit fibroblasts, and wall off the implant with soft fibrous tissue preventing successful integration. Therefore, to keep this wound healing response to a minimum and prevent capsule formation, excess tissue damage should be avoided during implantation and the recruitment of osteoblasts and keratinocytes should be rapid to quickly form new bone and skin, respectively.

Figure 1.21. Host response to an implanted biomaterial. Successful integration involves the recruitment of osteoblasts and, thus, new bone formation around the implant. Poor integration involves recruitment of fibroblasts and the creation of soft fibrous tissue around the implant. (Adapted from [136-138])

The duration of acute inflammation is relatively short, lasting from minutes to days depending on the extent of injury. During acute inflammation, white blood cells (leukocytes), such as polymorphonuclear granulocytes (PMN) or neutrophils, monocytes, and platelets, are recruited to the injury site by
chemical mediators. If the response persists, monocytes differentiate into macrophages where they begin to phagocytosis foreign material. The biomaterials have an initial adsorbed layer of proteins to which macrophages attach through a process called opsonization. Because the size of the device and the cells themselves are so different, the cells are unable to internalize the implant and remove it. This leads to frustrated phagocytosis, the extracellular release of all cellular products to degrade the foreign object. These macrophages then secrete derivative agents (superoxides and free radicals) that severely damage the juxtaposed tissue and implant. This prolonged effect leads to chronic inflammation which also has a short duration.

The chronic inflammation phase is therefore characterized by the presence of monocytes and macrophages. In addition, lymphocytes and plasma cells are recruited to the site of injury and begin antibody production, formation of blood vessels and formation of connective tissue, and the macrophages continue to increase in number in order to try and remove foreign objects. The macrophages begin to produce and secrete biologically active molecules, including fibroblast growth factors (FGF) and TGF-β, into the surrounding area. The presence of these chemicals in the environment surrounding the implant recruits additional cells that initiate the wound healing response and reform the damaged tissue.
During wound healing, a pink, soft granular substance, known as granulation tissue, is formed. Granulation tissue can be present within three to five days following implantation of the biomaterial. Granulation tissue is characterized by the proliferation of fibroblasts and the formation of new small blood vessels (neovascularization) by endothelial cells [136-138]. Fibroblasts are active in synthesizing collagen (Type III) that then forms the soft, fibrous tissue capsule around the implant. The device becomes surrounded by granulomas in which frustrated macrophages fuse into foreign body giant cells as a means to accomplish phagocytosis. However, the device soon becomes encapsulated in fibrous tissue. The more extensive the tissue damage, the more granulation tissue that forms, and thus the amount of fibrous tissue. The presence of foreign body giant cells and granulation tissue characterizes the foreign body reaction stage.

The end stage healing response after implantation is fibrosis or fibrous encapsulation (known as scar tissue) about 50-200 µm thick [139]. The production of this fibrous capsule is dependent on the area, form, and topography of the implanted device in addition to the extent of injury caused during implantation. Formation of this scar tissue is the last defense step of the immune system against a foreign material in the body. This fibrous layer can increase due to excessive micromotion of the implant which damages the newly-formed fibrin layer and vasculature. This results in the reoccurrence of the wound healing process to repair the new damage around the scar
tissue [140]. The presence of the fibrous capsule inhibits bonding of the biomaterial to the surrounding bone and skin, thus, preventing mechanical stability of the device due to the stress-strain imbalances that will be described later. Therefore, for new orthopedic implant surfaces, the goal is to promote the attachment, proliferation, and growth of bone and skin cells (osteoblasts and keratinocytes) to generate new bone and skin tissue quickly to prevent the formation of scar tissue. Figure 1.22 is a schematic illustration that summarizes the body’s response to a biomaterial.

Figure 1.22. Step by step schematic diagram of the wound healing process of the body that creates a fibrotic tissue around a biomaterial which inhibits bonding of the implant to surrounding tissue. (Adapted from [139])
1.5.2. Protein Interactions with Biomaterials

Initial interactions between proteins and the biomaterial surface are of the utmost importance in promoting new bone and skin growth and, thus, the success of ITAP. This is due to the fact that implant surface properties control and drive initial protein adsorption that will then interact with select cell membrane receptors and mediate cell function (Figure 1.23). Protein adsorption, and thus cell adhesion, depends on both the surface properties of the biomaterial and the properties of the protein. Surface properties that have been shown to affect protein adsorption include surface chemistry, surface charge, surface topography, and surface energy [141-149]. Because the adsorption of specific proteins enhances subsequent cell adhesion on materials, it is important to understand the mechanisms and pathways behind the process.
Figure 1.23. Schematic diagram of cell interactions with specific proteins in the extracellular matrix. The integrin being expressed by the cell allows for recognition of specific proteins depending on the adhesive domain exposed (such as the RGD, arginine-glycine-aspartic acid (Arg-Gly-Asp), peptide sequence), thus allowing for cellular adhesion. Biomaterial surface properties (chemistry, charge, topography, and surface energy) affect interactions of adsorbed proteins and thus cell attachment. (Adapted from [81, 150])

The first type of protein-mediated cell attachment is influenced by the conformation of the protein (i.e. specific peptide sequence) and the type of integrin-binding site expressed by the cell. Integrins are transmembrane heterodimeric glycoproteins consisting of two subunits, an α and β subunit, that allow cells to interact with each other as well as with extracellular matrix proteins [151]. Research has identified at least 8 β subunits and 14 α subunits that are concentrated at loci called focal adhesion sites [152]. These focal adhesion sites interact with intracellular cytoskeletal molecules that
have been shown to control, direct, and modulate cell function in response to extracellular signals [81, 153]. There are numerous combinations of the β and α subunits, each with the potential to bind to one or more peptide sequences expressed by proteins. In addition to integrin expression, protein conformation is important to direct cell attachment. A protein’s conformation exposes a particular adhesive domain (or peptide sequence) that is specific for anchorage dependent cells. Exposure of this adhesive domain allows the proteins to attach to their respective cell membrane receptors (or the integrins) (Figure 1.23). For example, integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$ bind to the RGD (arginine-glycine-aspartic acid, Arg-Gly-Asp) epitope, the cell adhesive region of vitronectin, fibronectin, and Type I collagen [154].

The second mechanism of protein-mediated attachment occurs through binding sites of cell membrane heparin sulfate proteoglycans. Heparin binding sites are separate from integrins and contain positively charged basic amino acids that interact with negatively charged carboxylate and sulfate groups of the glycosaminoglycans found in the cell membrane [155]. Specifically, heparin binding sites have been shown to have an effect on osteoblast adhesion. For example, when the heparin binding sites were blocked with platelet factor IV, 45-100% of osteoblast adhesion to fibronectin was blocked [156, 157].
The influence proteins have on cellular adhesion (and, thus, implant success) has been proven through many studies. Fibronectin and vitronectin are highly hydrophilic proteins (as previously stated) that have specifically been researched because these proteins seem to be more pertinent for mediating cellular adhesion, particularly of osteoblasts [141, 142, 147, 158]. Fibronectin promotes cell adhesion, migration, differentiation, and morphology [81] while vitronectin has also been shown to play an important role in cell adhesion and migration, the immune response, homeostasis, and blood coagulation [81]. In the presence of media supplemented with fibronectin, vitronectin, and other proteins found in serum, cells were seen to attach and spread on Ti surfaces, and when these proteins were absent from the media, cells failed to significantly attach and spread on the surface [142]. When only one of the proteins was added to the media, similar cell spreading was observed suggesting that fibronectin and vitronectin are both important for mediating the adhesion of osteoblasts. A similar study revealed that cell attachment and spreading of osteoblasts on Ti surfaces was reduced by 80% when vitronectin was removed from the media containing serum, [159]. In the same manner, when osteoblasts were exposed to fibronectin-depleted media, cell attachment and spreading was reduced but not to the same extent as vitronectin-depleted media. Therefore, it can be concluded from this study along with others [160] that vitronectin is the main protein in serum that adsorbs to the biomaterial surface and regulates osteoblast adhesion.
1.6 An Emerging Field: Nanophase Materials

Nanotechnology was first introduced in 1959 by Richard Feynman who proposed developing molecular machines through atomic precision. Nanotechnology was first published in a scientific journal in 1974 by Norio Taniguchi’s who defined the term and applied the concept to ion-sputtering machining [161, 162]. Since then, nanotechnology has revolutionized traditional science and engineering disciplines. Nanotechnology, specifically, is the engineering of materials at the atomic, molecular, and supramolecular levels that leads to significant modification in material properties. Nanotechnology has been applied to many fields to improve cancer cell detection [163], drug delivery [164, 165], tissue engineering [166], and imaging [167]. Nanotechnology has specifically been beneficial to the medical field due to the enhancement of cytocompatibility, mechanical, and electrical properties of nanomaterials compared to conventional materials. Although experts define nanomaterials differently, the most commonly accepted definition is a material with constituent structural units (such as grain sizes, particle sizes, widths, heights, etc.) less than 100 nm in at least one direction. With a decrease in the dimensions of a nanomaterial compared to a conventional, micron scale material, changes in physiochemical properties occur due to the nanomaterial’s small size (surface area and size
distribution), chemical composition (purity, crystallinity), structure (surface reactivity, surface groups), solubility, shape, and particle aggregation.

The motivation for using nanotechnology in biomedical applications is that tissues in the human body, including bone and skin, contain nanometer features and dimensions as previously described. Nanometer features in bone include the HA crystals and Type I collagen fibers. Nanometer features in skin include the desmosomes, the extracellular core of the cornified layer present in the epidermis, and Type I and III collagen present in the dermis. Thus, after examining the fundamental structure and components of bone and skin, it is clear that osteoblasts and keratinocytes are accustomed to interacting with nanophase materials naturally. Therefore, since the surface of the biomaterial, in particular an ITAP, will be interacting with surrounding tissues (bone and skin) and associated cells (osteoblasts and keratinocytes), a biologically inspired device containing nanometer features is crucial in promoting cell functions as well as guiding tissue regeneration. Current conventional, micron size implants do not mimic this nanostructured environment of natural tissue, and this discrepancy may explain many of the complications mentioned the previous section. As a result, stress-strain imbalances, implant migration, generation of wear debris, epidermal downgrowth, and infection experienced in current conventional materials may become minimized leading to the greater overall success of nanostructured ITAP.
1.6.1. Nanomaterials

Ceramics, polymers, metals, organic materials, and composites can all be engineered to contain nanophase features that are biomimetic natural tissue. Several factors and properties must be taken into account when deciding on a material to use for an orthopedic application. First, the material must exhibit superb biocompatibility properties. Next, a material is needed with a yield stress that is greater than the stress applied from the forces of the joint as referenced earlier. Finally, the material rigidity and elasticity must be considered in order to prevent large amounts of undesirable stress shielding. Examining stress-strain curves is one way to compare material properties (Figure 1.24). Stress-strain curves reveal that ceramics (in general) have the highest elastic modulus but are brittle, while metals have a lower elastic modulus but are extremely ductile. Hence, metals are prime candidates for load bearing applications such as ITAP components. From the graph, polymers appear to be better candidates for skin applications due to their similarity in elastic properties. However, the abutment component of an ITAP is connected to the Ti fixture implanted in bone and, thus, undergoes constant load. Therefore, polymers would not be the desired material choice for the abutment but rather metals would be a more fitting choice. Clearly, choosing a material for a specific application involves best fitting the mechanical properties to the requirements of that application.
Figure 1.24. Stress-strain curves examining the different materials used in orthopedic applications. Mechanical properties must be considered when deciding the most desirable material for a specification application. The (•) represents the ultimate stress and strain that produces material failure by fracture. (Adapted from [168])

Although minimal (if any) research has been completed on the growth of skin in contact with nanophase materials, extensive research has been completed towards improving bone formation on nanophase materials. Since both bone and skin contain organized nanometer structures as previously shown, it can be hypothesized skin, as well as bone growth, would benefit from nanotechnology.

1.6.1.1. Ceramics

Ceramics are non-metallic inorganic materials that possess excellent cytocompatibility properties, high compressive strength, and osteoconductive properties. They are some of the most chemically stable biomaterials that can be easily modified to improve strength, hardness, wear resistance, and thermal expansion specific to a particular application. However, ceramics are
not mechanically tough or flexible which prevent them from being the main choice material in load bearing applications, such as ITAP. Thus, ceramics are primarily used as bone fillers and spacers, bone bonding agents, dental implants, and coatings on more durable implant materials, such as metals.

The first published scientific article showing increased bone cell adhesion on a nanophase material was completed on ceramic materials. That study revealed that alumina with grain sizes between 49-67 nm and titania with grain sizes between 32-56 nm (each prepared by compacting nanophase powders of various grain sizes using a tool-steel die via a uniaxial pressing cycle) promoted osteoblast adhesion by 50% and 35%, respectively, compared to their conventional, micron sized counterparts [169, 170]. Further research on nanoceramics, including alumina, titania, and HA (a popular bone graft substitute, coating, and filler), consisting of grains, fibers, pores, or particles with sizes less than 100 nm revealed similar trends of enhanced osteoblast proliferation and long term functions (as measured by intracellular and extracellular matrix protein synthesis, such as collagen as well as alkaline phosphatase, and calcium containing mineral deposition) [171-182]. In vivo studies have also demonstrated enhanced new bone formation on metals coated with nanophase HA compared to metals coated with conventional HA [183, 184]. As shown in Figure 1.25, histology of rat calvaria (after two weeks) revealed increased bone formation on tantalum (Ta) surfaces coated
with nanophase HA compared to both Ta surface coated with micron size HA and uncoated Ta surfaces.

Figure 1.25. Histology of rat calvaria after tantalum (Ta) scaffolds coated with either nanophase or conventional HA were implanted for two weeks. Results revealed increased new bone infiltration (indicated by the pink stain) on the Ta surfaces coated with nano HA compared to either the Ta surface coated with micron HA or the uncoated Ta surfaces. (Adapted from [183]).

Furthermore, since osteoblasts and osteoclasts have to work in conjunction for the proper formation and maintenance of new bone juxtaposed to an implant, examining osteoclast behavior is important. Research has revealed that osteoclast function (such as synthesis of TRAP and formation of
resorption pits) was improved on nanophase ceramics compared to conventional ceramics [182, 185]. Specifically, osteoclast functions, including synthesis of TRAP and formation of resorption pits, increased two-fold on nanophase HA compared to conventional HA [185].

Finally, although enhanced bone cell function observed on the these nanoceramics was promising for improving the efficacy of implants, preventing certain wound healing responses, such as prolonged interactions of inflammatory cells (i.e. macrophages) and fibrous tissue capsule formation, also needed to be studied. Results showed that fibroblast adhesion decreased on nano compared to micron ceramics [172, 182] as well as decreased macrophage density (after 24 hours) on nano compared to micron ceramics [186]. Also, with infection as another problem that leads to failure of orthopedic implants, including ITAP, research has examined bacteria adhesion on nanoceramics [187]. It was found that the adhesion of *S. epidermidis* on nanophase ZnO and TiO$_2$ decreased by 60% and 69%, respectively [187].

1.6.1.2. Polymers

Polymers are synthetic materials with excellent biocompatibility, biodegradability properties, and mechanical properties. The mechanical strength of polymers are comparable to that of soft tissues in the body [188] and can be easily controlled through precise management of the polymer
chemical composition, crystallinity, molecular weight, molecular weight distribution, and porosity [189-191]. Polymers have other unique properties including viscosity, malleability, and moldability. They also have excellent friction properties. However, polymers can suffer from fatigue-related cracking and poor setting and fixation due to impact-induced breakage. In addition, the degradation products produced during polymerization can elicit an unwanted host response. Nanophase polymers (such as poly(lactic-co-glycolic acid), PLGA, and PU synthesized through chemical etching in NaOH and HNO₃, respectively, for varying time periods) have improved cellular adhesion and proliferation for a range of cells including endothelial, smooth muscle cells, chondrocytes, and osteoblasts [192-201]. Fibroblast density was also observed on nanophase polymers (including PLGA, PU, polycaprolactone, PCL, synthesized through chemical etching in NaOH and HNO₃, respectively, for varying time periods) and, similar to nanophase ceramics, decreased on nanophase polymers compared to conventional polymers, thus inhibiting fibrous capsule formation around the implant [200-202].

1.6.1.3. Metals

Metals and their alloys are the most commonly used material in orthopedic applications, including ITAP. Metals have superb mechanical and biocompatibility properties (making them great candidates for load bearing
applications) and have been used for fracture repair, joint replacement surgery, and ITAP. Three metals (and their alloys) commonly used in orthopedic applications are Ti, stainless steel, and cobalt chromium. Compared to stainless steel and cobalt chromium, Ti is most commonly chosen (especially for ITAP) due to its lower elastic modulus, yield strength, ultimate tensile stress, and corrosion resistant properties due to the presence of an adherent oxide passive film layer (Table 1.2). However, it is important to note that although desirable, Ti (along with stainless steel and cobalt chromium) properties (such as density, elastic modulus, and tensile strength) are still higher than the properties of natural bone (Table 1.1 in section 1.3.3).

Table 1.2. Density and mechanical properties of metal alloys. (Adapted from [203])

<table>
<thead>
<tr>
<th></th>
<th>Stainless Steel (316L Annealed)</th>
<th>CoCrMo (F75 Cast)</th>
<th>Ti6Al4V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/cm³)</td>
<td>8</td>
<td>8.3</td>
<td>4.42</td>
</tr>
<tr>
<td>Elastic Modulus (GPa)</td>
<td>193</td>
<td>220</td>
<td>100</td>
</tr>
<tr>
<td>Ultimate Tensile Strength (MPa)</td>
<td>485</td>
<td>655</td>
<td>860</td>
</tr>
<tr>
<td>Yield Strength (MPa)</td>
<td>172</td>
<td>450</td>
<td>795</td>
</tr>
</tbody>
</table>
Titanium is also thought to possess a unique hydrated Ti peroxo matrix attached to the oxide layer that makes it more desirable than the stainless steel and cobalt chromium for orthopedic applications (Figure 1.26). Macrophages and PMNs (the first inflammatory cells to arrive at the site of implantation) produce and secrete oxidative metabolites (such as proteolytic enzymes, cytokines, superoxide, and hydrogen peroxide) that interact with the oxide layer to form the peroxo matrix [17, 204, 205]. As a result, it is this peroxo matrix that is thought to be in direct contact with the living tissue, not the actual oxide layer (Figure 1.26). This layer has been shown to possess anti-inflammatory properties that further enhances the biocompatibility properties of Ti for in vivo applications [206]. For example, in the presence of a peroxo matrix, bone necrosis is not induced, which prevents the leakage of mediators that lead to enhanced inflammation [206, 207]. Thus, the matrix on the Ti surfaces produces an anti-inflammatory effect that inhibits the advancement of the wound healing response which leads to failure of the device [206]. The solubility and stability of the peroxo complex is too low for the other metals, thus making Ti the preferred material in orthopedic applications [17].
Figure 1.26. An artist’s view of the Ti implant interface. Living tissue is in direct contact with a thin layer of Ti peroxy compounds that covers the Ti oxide and produces anti-inflammatory properties that reduce the onset of wound healing. (Adapted from [17, 204])

As with nanoceramics and nanopolymers, research has been completed to indicate higher osteoblast adhesion and proliferation, enhanced synthesis of extracellular matrix proteins (such as collagen, alkaline phosphatase, etc.), and improved deposition of calcium on nanophase metals, such as Ti, Ti6Al4V, and CoCrMo (created by powder processing and anodization described in the following sections) compared to conventional Ti [208-215].
1.6.2. Nanofabrication Techniques

Since nanophase materials are an exciting new opportunity in the field of orthopedics, methods used to create such topography are important. Some methods have already been explained in the above sections but a more detailed list is described here. Nanophase ceramics are commonly created through powder consolidation followed by sintering. Grain size can be controlled by altering the sintering time and temperature [170, 171, 175, 182, 185]. In addition, nanophase ceramics, in particular HA, can be engineered through wet chemistry techniques in which the grain size is controlled by changing the time and temperature of HA precipitation [171, 182, 185]. When coated on a material (such as a metal), ion beam sputtering [216] and pulsed laser deposition [217] are used to create ceramic coatings containing nanosize grains. Both of these nanofabrication techniques involve bombardment of an argon ion beam on a pressed or sintered ceramic target, depending on which process is used. The nanosize features can be precisely controlled by the rate of deposition during the coating process.

Nanophase polymers can either be designed with an ordered or non-ordered topography. Chemical etching [193-195], phase separation, and colloidal lithography are methods used to create non-ordered nanorough topographies [218]. Electron beam lithography and photolithography are techniques used to create ordered topographies with nanometer features. Polymers are also
easily engineered to contain three-dimensional features. This is of importance since most tissues in the body are organized into three-dimensional structures that function as organs and organ systems. Various techniques used to create these three-dimensional matrices including particulate leaching, textile technologies, three-dimensional printing techniques, freeze drying and cross-linking, and melt molding [138, 219-222].

Nanophase metals are commonly engineered through mechanical methods (machining, grinding, sand blasting), chemical methods (acid etching such as H₂O₂ [223], passivation, chemical vapor deposition), electrochemical methods (electropolishing, anodization), coatings (plasma and ion spraying, electron beam evaporation), and powder processing which consolidates nanometal powders without the use of heat [210, 224-230]. Most of these techniques can alter not only surface roughness but also chemistry and crystal structure. Thus, determining if bone cell function on nanophase metals is a direct result of roughness, chemistry, or crystallinity becomes difficult.

1.6.3. Reasons for Improved Cellular Behavior on Nanophase Materials

As mentioned and shown throughout this dissertation, because tissues in the human body (including bone and skin) exist in the nanometer regime, cell behavior is enhanced on materials possessing nanofeatures. However,
understanding the reasons behind improved osteoblast and keratinocyte cell behavior on nanophase materials versus micron size materials is essential for engineering better ITAP. Importantly, numerous studies have already indicated why.

1.6.3.1. Protein Interactions at the Tissue-Implant Interface

As stated earlier, initial optimal protein adsorption events with the implant surface is of paramount importance in mediating cell adhesion and function and, thus, determining the success of the implant. In fact, research has revealed that nanophase materials, whether ceramics, polymers, or metals, enhance the adsorption of proteins necessary for cell adhesion [170, 171, 231]. For example, three-dimensional nanofibrous poly(L-lactic) acid, PLLA, scaffolds (fabricated through phase separation techniques) with 50-500 nm fiber diameter selectively adsorbed more fibronectin and vitronectin (proteins that stimulate osteoblast adhesion) that lead to increased osteoblast functions compared to micron size PLLA scaffolds [232]. In addition to increased protein adsorption, studies revealed that when adsorbed to nanophase materials, proteins displayed altered conformation or bioactivity [233]. For example, it was observed that when vitronectin was adsorbed to nanophase ceramics, the exposure of the peptide sequence, RGD (Arg-Gly-Asp), responsible for bone cell adhesion, was enhanced.
Changes in the surface properties of nanophase materials, including grain boundaries, surface roughness, wettability and surface energetics, will alter protein interactions (including adsorption, conformation, and bioactivity) and subsequently enhance bone cell adhesion and function. For example, since proteins are charged molecules, altered surface energetics due to increased grain boundaries on nanophase materials improve protein interactions [234]. Specifically, adsorption of vitronectin and fibronectin (very hydrophilic proteins as previously stated) increased on nanophase ceramics with greater wettability (more hydrophilic surfaces) [160, 233]. Furthermore, increased surface roughness is one of the most important mechanisms by which protein adsorption is enhanced and effectively stimulates the formation of new bone and skin (Figure 1.27). For example, when comparing the concentration and bioactivity of adsorbed vitronectin on alumina varying only in surface roughness, the nanophase alumina promoted higher vitronectin adsorption and bioactivity that resulted in enhanced osteoblast adhesion compared to conventional alumina [235].
Nanophase materials, compared to conventional materials, possess increased surface roughness to promote protein adsorption since the surface features are closer to the size of the protein itself (nanoscale). This in turn enhances new bone formation which improves the overall success rates of ITAP. Although specific for bone, the same concepts apply for the enhancement of skin growth. (Adapted from [236])

1.6.3.2. Grain Boundaries

Compared to conventional materials, the size of the grains on the surface of nanophase materials are in the nanometer regimen and possess greater number of grain boundaries [237]. Since surface properties (such as surface energy, area, topography, and charge) depend on the grain size of a material, they will be altered on nanophase surfaces compared to conventional surfaces. For example, nanophase materials possess higher surface area, increased electron delocalization resulting in less acidic OH- groups, and higher numbers of atoms at the surface [238] (Figure 1.28). These special surface properties of nanophase materials influence select protein
interactions that control and enhance subsequent cell function and tissue growth.

Figure 1.28. Changes in surface properties of nanophase materials that influence protein interactions for controlling cell function and subsequent tissue growth. (a) Higher number of atoms at the surface of nanophase compared to conventional materials. (b) Nanophase materials possess higher surface area with increased portions of surface defects and grain boundaries and altered electron delocalization resulting in less acidic OH\(^-\) groups. (Adapted from [239])
High electron delocalization on nanophase materials due to increased numbers of grain boundaries creates more electronegative surfaces compared to conventional materials (Figure 1.28). This in turn increases electrostatic attractions between the surface and proteins, which in turn results in increased protein adsorption and thus improves cellular attachment. Nanophase materials possess increased grain boundaries, surface roughness, surface energetics, and wettability closer to the actual size of the protein itself, thus resulting in enhanced select protein adsorption and conformation and eventually new tissue growth around the biomaterial. These properties that influence protein interaction are further examined in the next sections.

1.6.3.3. Surface Energetics and Wettability

Surface atoms are exposed to a different local environment than the atoms present in the bulk of the material, thus, resulting in a difference of the energy associated with the atoms. The excess energy associated with surface atoms is called surface free energy. Surface energy of a material can be determined by examining the wettability of the surface. Wettability is the ability of a liquid to maintain contact with a solid surface resulting from intermolecular interactions between the liquid and solid. Contact angles are used to determine whether or not a surface is wettable (also termed hydrophilic) or nonwettable (also termed hydrophobic) which is then used to determine the surface energy.
Research has revealed a direct relationship between surface energetics and wettability to cellular adhesion [240, 241]. For example, the more ions present on a material, the higher the surface energy. When smooth muscle cells were placed on a polystyrene surface possessing five times as many ions as the control material (created through ion implantation), their adhesion increased two-fold [240]. Furthermore, twice as many osteoblasts adhered on HA whose surface energy increased 5 times compared to calcium apatite (44 mJ/m$^2$ and 9 mJ/m$^2$, respectively) [241].

Because nanophase materials have been identified for improving functions of a number of cell lines in addition to improving select protein interactions, examining the role of surface energy and wettability on cell behavior and protein adsorption has been examined. First, examining surface energy on the cell function has been extensively studied. For example, when endothelial and bone cells were placed onto nanorough Ti surfaces (created through electron beam evaporation) possessing 20-40 nm surface features and displayed a higher surface energy compared to the conventional Ti surfaces, they adhered twice as much [242]. In addition, endothelial cell adhesion was also shown to increase on nano PLGA (created using a template of polystyrene nanobeads with a 190-960 nm diameter) that had increased surface energy compare to its counterpart, micron PLGA [243]. Interestingly, surface energy of nanophase materials has also been linked to the inhibition of competitive cell function. For example, the presence of astrocytes results
in glial scar tissue formation that prevents axon regeneration in neural applications. It has been shown that nano zinc oxide particles (60 nm diameter) possess higher surface energy than conventional zinc oxide particles and in turn decrease astrocyte attachment [244]. The same trend was seen when examining astrocytes on carbon nanofibers (60-200 nm fiber diameter) compared to conventional carbon fibers. More specifically, carbon nanofibers possessing increased surface energy compared to their micron size counterparts and showed a decrease in astrocyte adhesion and proliferation [245].

Second, in addition to examining surface energy on cell behavior, the effect that wettability has on cell response has been studied. When aqueous contact angles were examined on nanophase alumina that possessed grain sizes ranging from 167 to 24 m, the contact angles were three times smaller on 24 nm grain size alumina surfaces, indicating an increase in wettability or hydrophilicity [185]. In addition, when titania nanosized particles were embedded in PLGA, the surfaces became more hydrophilic (more wettable) than those containing micron size titania particles and resulted in an increase in osteoblast adhesion [246].

Third, since proteins are charged molecules, alterations in surface energetics and wettability will influence protein interactions. Understanding such behavior is important since, as stated numerous times, select protein
interactions affect cell functions. For example, the adsorption of vitronectin
(which stimulates osteoblast adhesion) increased on ceramic surfaces that
possessed greater wettability (more hydrophilic surfaces) and resulted in
improved osteoblast attachment and spreading [160]. Furthermore,
enhanced fibronectin (a key protein for mediating anchorage-dependent
osteoblast adhesion) adsorption was observed on carbon nanotubes embedded
in poly(carbonate) urethane (PCU) that were found to have increased surface
energy and were more wettable (more hydrophilic) compared to PCU
containing no carbon nanotubes [234].

1.6.3.4. Surface Roughness

Another property researchers have examined to improve the efficacy of
orthopedic implants by improving the rate of osseointegration of bone and
preventing downgrowth of the dermis is altering the surface roughness of the
material. From these studies, a direct relationship has been established that
increasing surface roughness correlates to enhanced osteoblast function (an
even osteoclast function) [142, 149, 225, 226, 247-250]. More specifically,
surfaces with increased roughness, <10 µm (created by machining, acid
etching, grit blasting, and heat treating), enhanced osteoblast adhesion,
proliferation, and production of collagen, alkaline phosphatase, and calcium
deposition [142, 149, 225, 226, 248, 250]. However, since the body consists of
constituent nanostructures, examining osteoblast response on surfaces with
nanometer topography was completed on carbon nanofiber based materials and polymer casts of these carbon nanofibers [251]. It was found that osteoblast adhesion increased on both the carbon nanofiber and polymer casts of carbon nanofibers compared to their conventional counterparts. Thus, nanometer surface roughness, not chemistry, was the contributing factor that increased osteoblast adhesion.

Furthermore, not only have polymers been investigated, but ceramics of the same crystallinity and chemistry varying only in degree of nanometer surface features have demonstrated the importance of nanometer roughness on osteoblast function. Specifically, a 23 nm grain size alumina surface was found to have approximately 50% more surface area for osteoblast adhesion than that of a 177 nm grain size alumina surface [170] (Figure 1.29). Similarly, titania surfaces possessing 32 nm grain sizes had 35% more surface area that titania surfaces possessing 2.12 µm grain sizes [170] (Figure 1.29). When these micrographs are compared to micrographs revealing the topography of natural bone (Figure 1.30), it can be seen that the roughness of nanophase ceramics closely resembles the roughness of natural bone.
Figure 1.29. Representative topography of nanophase and conventional alumina and titania. Micrographs of (a) nanophase alumina with 23 nm grain sizes, (b) conventional alumina with 177 nm grain sizes, (c) nanophase titania with 32 nm grain sizes, and (d) conventional titania with 2.12 µm grain sizes. (Adapted from [170])
Data revealed that compared to conventional counterparts, osteoblast adhesion and function (such as alkaline phosphatase activity, calcium deposition, and collagen synthesis) drastically improved on nanophase alumina, titania, and HA [170, 171, 182, 233, 253]. Not only was there an increase observed in osteoblast functions on these nanophase ceramics, osteoclasts also increased the number of resorption pits and secretion of tartrate-resistant acid phosphatase [182, 185, 254]. In addition to increasing osteoblast and osteoclast function, these nanophase ceramics simultaneously decreased fibroblast (cells that create the fibrous tissue around an implanted material preventing proper bone integration) functions including adhesion, proliferation, and morphology [182, 233, 254]. Fibroblast proliferation not only decreased on nanophase ceramics, but their proliferation also reduced on nanophase polymers, in particular a polystyrene/poly(4-bromostyrene) blend.
with islands 95 nm high [255]. Although not reported, this change in fibroblast response could limit formation of the fibrous capsule thus improving implant success rate. Such success has already been demonstrated in vivo which indicated Ta scaffolds coated with nano HA implanted into rat calvaria increased new bone growth (Figure 1.25).

In addition to polymers and ceramics, nanophase metals, such as Ti, Ti6Al4V, CoCrMo, (synthesized through powder processing and polishing) also increase nanometer surface features (less than 1 µm) to increase of cell behavior [209-212]. Osteoblasts are not the only cell line whose behavior is altered in the presence of nanometer surface features. Smooth muscle cell function in the presence of nanophase polymers (specifically PLGA, PU, and PCL developed by acid etching) increased compared to conventional polymers [192, 193]. Lastly, endothelial cells on nitinol (NiTi) with nanometer surface roughness compared to NiTi with a submicron roughness (created through powder processing) was improved [256], thus adding validity to this idea that nanoroughness influences a wide range of cell functions on a wide range of materials that may not have even been researched yet.

1.7 Objective

The objective of this research was to design an ITAP that both enhances bone function on Ti rods implanted into the residual bone as well as improve skin growth around the abutment in order to create a tight skin seal while
simultaneously reducing bacterial infection. It is well established that cells behave differently on different materials. With that in mind, this research was particularly focused on the idea of designing an ITAP where one part of the device (the bone-anchored metallic rod) has a distinct surface topography which best promotes bone cell growth while the other part of the prosthetic (the abutment) is engineered to have a different surface topography which enhances skin cell growth with each section inhibiting bacteria growth. In particular, these surface topographies will contain nanoscale features and select nanopatterns due to the advantages and promise such materials have been show to have in improving bone and skin tissue regeneration. The specific objectives of this dissertation included the:

- Design and creation of novel nanopatterned Ti surfaces with varying degrees of nanopattern widths for enhancing anisotropic bone tissue growth,
- Investigation of osteoblast adhesion behavior on these nanopatterned Ti surfaces,
- Investigation of osteoblast long term behavior on these nanopatterned Ti surfaces (e.g. proliferation, total protein synthesis, alkaline phosphatase activity, calcium deposition, and collagen synthesis),
- Design and creation of various nanotextured Ti surfaces, including nanorough and nanotubular, for enhancing skin tissue growth,
• Investigation of keratinocyte adhesion, proliferation, and spreading behavior on these various nanotextured Ti surfaces,

• Functionalization of the nanotextured Ti surfaces with growth factors specific for enhancing skin growth,

• Determination of the role of surface roughness, surface wettability, and surface energetics on both osteoblast and keratinocyte functions,

• Design of polymers with Ti coatings containing nanoscale features to demonstrate the versatility of these nanofabrication methods,

• Investigation of osteoblast adhesion on these polymer coated nanometer materials,

• Investigation of bacterial adhesion on these nanofabricated materials, and the

• Translation of the above in vitro results on these nanofabricated materials in vivo.
Chapter 2

Enhancing Bone Growth Through The Use of Nanopatterned Titanium Surfaces

2.1 Introduction

As mentioned throughout the introduction of this dissertation, research focusing on improving the integration and tissue growth at the bone-metal interface of orthopedic devices has been directed towards the development and use of novel nanophase materials. To reiterate, a material with constituent structural units (such as grain size, particle size, width, height) less than 100 nm in at least one direction is considered a nanophase material. Nanophase materials, compared to conventional phase materials, possess surface characteristics (such as topography, wettability, charge, and surface
free energy) that are more reactive allowing for optimal interactions with select proteins (such as fibronectin and vitronectin) and subsequently with pertinent bone cell types (such as osteoblasts). Numerous studies have clearly documented that the material properties of nanophase implants compared to conventional implants of the same chemistry lead to biologically improved responses from bone resulting in increased tissue regeneration. Such behavior has been traced back to the fact that nature itself exists in the nanometer regime, especially tissues in the human body [257]. As previously stated, human bone is assembled from nanosized organic and mineral phases into larger architectures. Specifically, Type I collagen and hydroxyapatite crystals compositionally and structurally are nanomaterials (Figure 1.9 and 1.10). However, in trying to duplicate the nanostructure of bone, it is important to note that the synthesis and deposition of these components in certain human bone spontaneously align in parallel sheets (leading to the overall anisotropy of bone). Figure 2.1 shows an atomic force micrograph (AFM) of cortical bone revealing numerous collagen fibrils aligned in parallel sheets [258]. Since bone cells are thereby accustomed to interacting with structures containing nanometer features arranged in a specific array, research has examined the amplitude of surface roughness as well as the organization and orientation of such surface features on osteoblast behavior.
Components of bone are not the only structures in the human body known to have unique organizational patterns. For example, the endothelium is composed of elongated vascular endothelial cells that align in the direction of blood flow. In addition to the organization of structural components, nanoscale topographical cues, independent of chemistry, generated by the extracellular matrix can be a complex meshwork of pores, ridges, and/or fibers in the nanometer regime [259]. These organized cues directly affect the ability of cells to orient, migrate, and produce directed cytoskeletal features, each of which play an important role in tissue development and organization. Therefore, interest in the effect of organized nanometer surface features on cellular response has arisen.
Observations have shown that organized, nanostructured topographical surfaces (such as patterns, grooves, pores, ridges, orthogonal and cylindrical imprints, wells, and nodes) dictate and enhance cellular (such as osteoblasts and vascular endothelial cells) morphology and motility that allows for better stability and adhesion [259, 260]. For example, endothelial cells examined on highly parallel nanopatterned gold and silicon surfaces (60-150 nm pattern size) created using orthogonal biofunctionalization imprint lithography revealed confluent layers of endothelial cells adherent in the direction of the nanopatterns [261]. In addition, their filopodia extended in the direction of the nanopatterned features [261]. Endothelial cells have also been studied on linearly, aligned, grooved nanopatterned titanium (Ti) surfaces (750 nm-100 µm pattern size) created using plasma-based dry etching techniques. Results demonstrated enhanced endothelial coverage in addition to endothelial alignment similar to the natural endothelium [262].

Fibroblasts have also been studied on parallel cylindrical nanocolumns (center-to-center spacing of 230 nm) synthesized on poly(methyl methacrylate), PMMA, by colloidal lithography [263]. It was observed that the fibroblasts had a higher number of filopodia and were more spread and round when compared to a flat control sample with the no patterns. Anchorage dependent cells (such as fibroblast) need to spread in order to enter the G1 and G2 phases of the cell cycle [264], thus, suggesting that nanocolumn patterned surfaces can enhance fibroblast growth. In addition,
fibroblasts were examined on PMMA surfaces containing orderly, aligned column-shaped perforations (center-to-center spacing ranging from 70-500 nm) synthesized by nanoimprinting [265]. It was found that fibroblast adhesion and morphology varied depending on the dimensions of the pattern perforations with the larger perforations possessing more rounded fibroblasts.

Smooth muscle cells are another cell line that has been commonly used to examine the effect of nanopatterns on cellular behavior [266, 267]. Nanoimprint lithography was used to create linearly, aligned patterned surfaces (350 nm-10 μm pattern size) on tissue-culture polystyrene (PS) [266]. Smooth muscle cells attached and proliferated well on these nanoimprinted surfaces, while elongation and alignment was more pronounced compared to micron patterned surfaces. Furthermore, smooth muscle cell response was also examined on linearly, aligned patterned surfaces (350 nm pattern size) on PMMA and polydimethylsiloxane (PDMS) created by nanoimprint lithography [267]. It was found that significant elongation and alignment in the smooth muscle cell cytoskeleton and nuclei occurred on the nanopatterned surface while the polarization of microtubule organizing centers showed a preference towards the axis of cell alignment.

Furthermore, mesenchymal stem cells (MSC) were examined on micron patterned silicon chips containing parallel strips with 5 μm width spacings
between them [268]. The MSC vividly aligned along the grooves and exhibited an elongated shape in the direction of the micron patterns (Figure 2.2). MSCs have also been examined on an array of nanopatterns (pits 300 nm center-to-center spacings) produced by lithography [269]. It was observed that the MSC were highly elongated and aligned, while markers for differentiation were enhanced compared to surfaces with random nanotopographies.

![Figure 2.2](image)

Figure 2.2. (a) Light microscopy overview and (b) scanning electron microscope (SEM) image of mesenchymal stem cells (MSC) on micron patterned silicon substrates indicating the alignment of cells on the grooves. (Adapted from [268])

Studies examining osteoblast behavior on patterned substrates created using imprint lithography have also resulted in osteoblast alignment and elongation. It has been demonstrated that osteoblasts are influenced by parallel, grooved surfaces [149, 270-272] and do not adhere and proliferate as
much on less organized surfaces compared to polished, well-organized surfaces [273]. Osteoblast functions (deposition of calcium as well as synthesis of alkaline phosphatase and collagen) and morphology have also been examined on microscale patterns (20 µm width patterns) of carbon nanofibers (CNF) on poly(carbonate) urethane (PCU) synthesized through lithography techniques [274]. It was found that selective osteoblast adhesion and alignment occurred on the CNF patterns placed on PCU. In addition, this directed osteoblast adhesion translated into enhanced calcium phosphate mineral deposition along the linear patterns. Furthermore, since spatial control of cell distribution is known to enhance osteoblast adhesion, proliferation, and differentiation, researchers have examined the effect protein adsorption on these patterned surfaces has on osteoblast functions [160, 275, 276]. It was found that in the presence of select proteins, including fibronectin and vitronectin, osteoblasts aligned with the direction of these surface patterns, while with the depletion of the proteins, the cells arranged themselves randomly (Figure 2.3). Not only does the presence of aligned patterns enhance osteoblast adhesion and proliferation, these surface patterns could also direct the secretion of collagen and deposition of hydroxyapatite into the aligned structures to mimic natural bone. Clearly, understanding which features evoke spatial reactions in cells to enhance their differentiation may allow for the design of better biomaterials.
Figure 2.3. Osteoblasts grown on patterned surfaces (50 µm and 100 µm wide regions) with serum (containing vitronectin and fibronectin) and without serum after two hours. Data indicated the importance spatial control of osteoblast distribution on cell proliferation and differentiation as well as the importance of protein interactions on mediating cell behavior. Scale bar = 250 µm. (Adapted from [160])

Therefore, in this study, Ti substrates containing periodic arrays of parallel aligned grooves with spacings ranging from 22 to 80 µm were fabricated using electron beam evaporation. These nanopatterns were designed to have alternating regions of conventional, micron scale features and nanoscale features. As previously explained, research has been completed comparing cell responses, including osteoblasts, to nanophase versus conventional phase materials. In most cases, osteoblasts are only exposed to one type of roughness at a time. By creating a material in which osteoblasts were exposed to various topographical features at the same time, more conclusive evidence that cells truly respond to regions with higher nanometer surface roughness can be elucidated. Furthermore, since the objective for designing orthopedic devices is now aimed at closely mimicking the structure of bone,
fabricating surfaces not only with the desired roughness but also with proper orientation is ideal. Thus, another reason for creating nanopatterned Ti surfaces in this study was to examine osteoblast function in the presence of an array of parallel, aligned grooves since natural bone possesses components that are linearly aligned.

Thus, the specific objectives of this chapter included the:

- Design and fabrication of Ti substrates with aligned nanopatterns that alternated in roughness using electron beam evaporation,
- Characterization of the fabricated nanopatterned Ti substrates (using scanning electron microscopy, atomic force microscopy, electron spectroscopy for chemical analysis, and X-ray diffraction),
- Elucidation of osteoblast adhesion, proliferation, and long term studies (such as total protein synthesis, alkaline phosphatase activity, calcium deposition, and collagen synthesis) on the nanopatterned Ti substrates,
- Examination of osteoblast morphology on these nanopatterned Ti substrates,
- Determination of protein adsorption studies on the nanopatterned Ti substrates which may explain the results above, and
- Understanding the mechanism for osteoblast behavior observed on these nanopatterned Ti substrates and the role that surface roughness,
surface wettability, and surface energetics may have played in mediating such responses.

2.2 Materials and Methods

2.2.1. Sample Preparation

Experiments conducted throughout this chapter involved a series of samples, including (i) conventional Ti substrates as received from the vendor, (ii) nanorough Ti substrates created through electron beam evaporation, and (iii) three types of nanopatterned Ti substrates (of alternating roughnesses) created by electron beam evaporation. The base material for all sample types were Ti foils (100 x 100 x 1 mm; 99.2% pure; Alfa Aesar, Ward Hill, MA, USA) that were cut into 10 x 10 mm squares using a shear cutter. After cutting into the desired dimensions, all substrates were ultrasonically cleaned with a diluted cleaning solution (Branson, Dabury, CT, USA) for 20 minutes followed by sonication in acetone, 70% ethanol, and deionized water (DI) for 10 minutes. Substrates were then dried in an oven (VWR, Bridgeport, NJ, USA) at 40ºC for 15 minutes, or until dry.

2.2.1.1. Titanium Substrates as Received from the Vendor

The Ti foils as received from the vendor (described above) represented the current materials used as orthopedic implants. No surface modifications or
treatments were carried out, and the preparation for cell experiments only involved the cleaning process mentioned above followed by sterilization in a steam autoclave at 120ºC and 17 psi for 30 minutes.

2.2.1.2. Electron Beam Evaporated Titanium Substrates

A Temescal Electron Beam Evaporator (Reston, VA, USA) was used to fabricate Ti substrates containing nanometer features. Electron beam evaporation (Figure 2.4) concentrates a large amount of heat produced by high energy electron beam bombardment on the source material to be deposited, in this case 99.995% pure Ti pellets (Kamis, Mahopac Falls, NY, USA). The electron beam is generated by an electron gun that uses the thermoionic emission of electrons produced by an incandescent filament under high vacuum. A magnet focuses and bends the electron trajectory so that the beam is accelerated towards a graphite crucible (Lesker, Clairton, PA, USA) containing the source material. As the beam rotates and hits the surface of the source material, heating and vaporization occur (atoms transform into the gaseous phase). The vapor flow then condenses and precipitates into a solid form onto the substrate surface located at the top of the vacuum chamber. In this study, Ti was deposited onto the Ti substrates at a rate of 3.5 Å/s and at a thickness of 500 nm. Following deposition, the Ti samples were rinsed thoroughly with DI, air dried, and sterilized in a steam autoclave at 120ºC and 17 psi for 30 minutes.
2.2.1.3. Nanopatterned Ti Substrates

Parallel bar copper gilder grids (Figure 2.5 (a)); Electron Microscopy Sciences, Hatfield, PA, USA) attached to the surface of the commercially pure Ti substrates received from the vendor (described above) were used to create
arrays of parallel, aligned grooves. The mesh consisted of dividers called bars that when placed on the Ti substrate masked/hid the Ti surface from alteration (thus, maintaining the conventional surface configuration) (Figure 2.5 (b)). In between the dividers was empty space called holes that when placed on the Ti substrate exposed the Ti surface (Figure 2.5 (b)). The size of the linear nanopattern on the Ti surface was easily varied by using meshes whose bar and hole dimensions differed. This study in particular examined three different kinds of nanopatterned Ti substrates by using three different types of copper meshes. More specifically, a 200 mesh, a 300 mesh, and a 400 mesh were each used with a 3.05 mm diameter and a thickness of 18 µm. The 200 mesh had a bar width of 45 µm and a hole width of 80 µm, the 300 mesh had a bar width of 35 µm and a hole width of 48 µm, and the 400 mesh had a bar width of 40 µm and a hole width of 22 µm (Table 2.1). Four meshes of each type were placed on one Ti substrate which was then placed in the electron beam evaporator. All electron beam parameters mentioned above (section 2.2.1.2) were used to create the nanopatterned Ti substrates. Once electron beam evaporation was completed, the meshes were removed and the substrates were rinsed thoroughly in DI water. Due to the design of the meshes, the nanopatterned Ti substrates after electron beam evaporation possessed alternating regions of varying roughnesses. In particular, the regions of the Ti surface that were covered by the bar of the mesh maintained the original surface of the Ti as received from vendor while the regions of the
Ti surface that remained exposed from the holes of the mesh were transformed into a nanoscale topography during electron beam evaporation. After rinsing with DI water, all nanopatterned Ti substrates were air dried and sterilized in a steam autoclave at 120°C and 17 psi for 30 minutes.

Figure 2.5. (a) Parallel bar copper gilder grids attached onto the surface of the Ti substrates to create arrays of parallel aligned grooves. Four meshes were placed on one Ti substrate. (b) Magnified view of the mesh specifically indicating the bars that masked the Ti surface and the holes that exposed the Ti surface during electron beam evaporation. The covered region maintained the roughness of the conventional Ti substrates while the exposed region possessed the features of the nanorough Ti substrates.

Table 2.1. Dimensions of the three types of meshes used to create the three types of nanopatterned Ti substrates used throughout this study.

<table>
<thead>
<tr>
<th>Mesh</th>
<th>Hole (µm)</th>
<th>Bar (µm)</th>
<th>Diameter (mm)</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>80</td>
<td>45</td>
<td>3.05</td>
<td>18</td>
</tr>
<tr>
<td>300</td>
<td>48</td>
<td>35</td>
<td>3.05</td>
<td>18</td>
</tr>
<tr>
<td>400</td>
<td>22</td>
<td>40</td>
<td>3.05</td>
<td>18</td>
</tr>
</tbody>
</table>
2.2.1.4. Reference Material

Borosilicate glass coverslips (reference material) obtained from Fisher Scientific (Agawam, MA, USA) were degreased in acetone for 10 minutes, sonicated in acetone for 10 minutes, and rinsed thoroughly in DI water. Afterwards, the same process was repeated with 70% ethanol. Finally, the coverslips were etched in 1 M NaOH for one hour followed by thoroughly rinsing in DI water. They were then dried in an oven at 40°C for 30 minutes, or until dry, and sterilized in a steam autoclave at 120°C and 17 psi for 30 minutes.

2.2.2. Sample Characterization

Conventional (as received from the vendor), nanorough (created through electron beam evaporation), and nanopatterned Ti substrates (created through electron beam evaporation) were characterized for roughness, crystallinity, chemistry, wettability, and surface energy.

2.2.2.1. Scanning Electron Microscopy (SEM)

For qualitative surface roughness analysis, SEM was completed on conventional (as received from the vendor), nanorough (created through electron beam evaporation), and nanopatterned Ti substrates (created through electron beam evaporation). Images were taken using a LEO 1530VP FE-4800 SEM (Zeiss, Peabody, MA, USA). Digital images were
created using secondary electrons collected with an in-lens detector at an accelerating voltage of 3 kV for the desired magnifications.

2.2.2.2. Atomic Force Microscopy (AFM)

For quantitative surface roughness analysis, AFM was performed on the conventional and nanorough Ti substrates using an Asylum Research AFM (Santa Barbara, CA, USA). Each sample was analyzed in ambient air under non-contact mode using a silicone ultrasharp cantilever (probe tip radius of 10 nm; MikroMasch, Wilsonville, OR, USA). The analyzed field was 1 x 1 µm at a scan rate of 1 Hz. Image analysis software was used to generate micrographs and to quantitatively compare the root-mean-square roughness (RMS) and peak-to-valley roughness (RPV) of the conventional and nanorough Ti substrates. Measurements were run in triplicate per substrate type. RMS is the estimated standard deviation of the bidimensional Gaussian-type distribution of heights around the mean value of the collected points. It is determined using the standard definition [277]:

$$R_q = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (z_i - \bar{z})^2}, \text{ where } \bar{z} = \text{mean } z \text{ height} \quad \text{[Eq 2.1].}$$

RPV is the difference between the highest peak and the lowest valley of the collected points. It is determined using the standard definition [277]:
2.2.2.3. Electron Spectroscopy for Chemical Analysis (ESCA)

For chemical analysis, ESCA was performed using a Perkin Elmer 5500 Multitechnique Surface Analyzer System (Waltham, MA, USA). An aluminum K-alpha monochromatized X-ray source was used to stimulate photoemission of the inner shell electrons of the Ti surface. The energy from these electrons was then recorded and analyzed for the conventional and nanorough Ti substrates. Wide scans of the sample were used to generate low-resolution spectra to identify and quantify the percentage of different elements on the surface (approximately up to 100 Å deep). Narrow scans were used to generate high-resolution spectra carbon C1s to examine the binding energy of the functional groups associated with the materials.

2.2.2.4. X-ray Diffraction (XRD)

Phase analysis was carried out by XRD using copper K-alpha radiation from a Siemens Diffractometer D5000 Kristalloflex (Bruker AXS, Chicago, IL, USA). Spectra were taken using a power supply of 30 mA and 40 kV while the diffraction angles (2θ) ranged from 20° to 70°/min. Diffraction signal intensity was recorded and processed using DiffracPlus:TexEval software (Bruker AXS). Resulting XRD spectra were compared with Ti (JCPS No.
050682) and titania (anatase and rutile; JCPS No. 211272 and 211276, respectively) standards. XRD was performed on conventional and nanorough Ti substrates.

2.2.2.5. Adhesion Strength

To determine the adhesion strength of the coating created during electron beam evaporation, a peel test was completed following the ASTM standard B 571 – 97, Standard Practice for Qualitative Adhesion Testing of Metallic Coatings. Three different types of adhesive-backed tape, including standard scotch tape, duct tape, and double-sided tape, were used on the three different substrates. To ensure that the results were reproducible, substrates were tightly sealed to a solid surface using super glue (the angle of tape pull was 90° to the surface), the width of the tape was the same for all tape types, the coating thickness was equal, and the rate of pull was the same. Failure at the coating interface, demonstrated by the appearance of Ti on the surface of the tape after removal, was evidence of inadequate coating adhesion.

2.2.3. Cytocompatibility Assays

2.2.3.1. Cell Culture

Commercially available human osteoblasts (bone-forming cells, CRL-11372, American Type Culture Collection, population numbers 4-9) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM: Hyclone, Logan, UT/USA)
supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin-
streptomycin (P/S; HyClone) under standard cell culture conditions (5% CO₂/95% humidified air environment at 37ºC).

2.2.3.2. Cell Adhesion

Osteoblasts were enzymatically lifted from the tissue culture polystyrene culture dish using 1 mL of a Trypsin-EDTA 1x solution (Sigma Aldrich, Milwaukee, WI, USA). Osteoblasts were then suspended in DMEM supplemented with 10% FBS and 1% P/S and were seeded at a density of 3500 cells/cm² onto the conventional, nanorough, and nanopatterned Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air environment at 37ºC) for four hours. After this time period, substrates were rinsed in phosphate buffered saline (PBS) to remove any non-adherent cells. Adherent cells on the substrates were fixed with 10% formalin based acetate buffer (Fisher Scientific) for 10 minutes and then stained with 4',6-diamidino-2-phenylindole, DAPI dilactate (Sigma Aldrich). The cell nuclei were then visualized and counted in situ using a Zeiss Axiovert 200M fluorescence microscope. Images were captured using an Orca ER camera. Adhesion experiments were run in duplicate and repeated three different times per substrate type. Cell density (cells/cm²) was determined by averaging the number of adherent cells in five random fields per substrate.
2.2.3.3. Cell Morphology

Additional qualitative experiments were completed to examine the direction and elongation of osteoblasts on the nanopatterned Ti substrates. Osteoblasts were enzymatically lifted from the tissue culture polystyrene culture dish using 1 mL of a Trypsin-EDTA 1x solution (Sigma Aldrich). Osteoblasts were then suspended in DMEM supplemented with 10% FBS and 1% P/S and were seeded at a density of 3500 cells/cm² onto the conventional, nanorough, and nanopatterned Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C) for four hours. After this time period, substrates were rinsed in phosphate buffered saline (PBS) to remove any non-adherent cells. Adherent cells on the substrates were fixed with 10% formalin based acetate buffer (Fisher Scientific) for 10 minutes followed by 0.1% Triton-X-100 (Sigma Aldrich) for 10 minutes. The f-actin filaments in the cytoskeleton of the cells were then stained with rhodamine phalloidin (Invitrogen, Carlsbad, CA, USA) and visualized in five random fields per substrate using a Zeiss Axiovert 200M fluorescence microscope. Images were captured using an Orca ER camera. Morphology experiments after four hours was run in duplicate and repeated three different times per substrate type. Surface area of each cell (µm²) was calculated using Image J software and then averaged over each of the five random fields per substrate.
Osteoblast morphology on the substrates of interest was also observed using a LEO 1530VP SEM. For this purpose, osteoblasts were enzymatically lifted from the tissue culture polystyrene culture dish using 1 mL of a Trypsin-EDTA 1x solution. Osteoblasts were then suspended in DMEM supplemented with 10% FBS and 1% P/S and were seeded at a density of 3500 cells/cm\(^2\) onto the conventional, nanorough, and nanopatterned Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO\(_2\)/95% humidified air environment at 37°C) for four hours. At the end of this time period, the cells were rinsed three times in PBS to remove any non-adherent cells. Adherent cells on the substrates were fixed with 10% formalin based acetate buffer for 10 minutes, rinsed three times in PBS, and then dehydrated through a series of ethanol solutions (10, 30, 50, 70, 90, 100, 100, and 100%) and subsequently dried via critical point drying (Ladd Research Critical Point Dryer, Williston, VA, USA). The specimens were immersed in liquid CO\(_2\) until there was a complete exchange of liquid CO\(_2\) for the ethanol in the specimens. The specimens were then heated above 34°C under 7.6 MPa, where all liquid CO\(_2\) was converted to gaseous CO\(_2\) until the specimens were dry. Before imaging, all the specimens were sputter-coated with a thin layer of gold-palladium using an ISI PS-2 Coating Unit in a 100mTorr vacuum argon environment for three minutes and 10 mA of current. SEM images were created using secondary electrons collected with the in-lens detector at a 3 kV acceleration voltage at desired magnifications.
2.2.3.4. Cell Proliferation

Osteoblast proliferation studies were conducted similar to the procedure for osteoblast adhesion studies. Osteoblasts were enzymatically lifted from the tissue culture polystyrene culture dish using 1 mL of a Trypsin-EDTA 1x solution (Sigma Aldrich). Osteoblasts were then suspended in DMEM supplemented with 10% FBS and 1% P/S and were seeded at a density of 1500 cells/cm$^2$ onto the conventional, nanorough, and nanopatterned Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO$_2$/95% humidified air environment at 37°C) for one, three, and five days. The media was changed every other day until the prescribed culturing time period elapsed. After this time period, substrates were rinsed in phosphate buffered saline (PBS) to remove any non-adherent cells. Adherent cells on the substrates were fixed with 10% formalin based acetate buffer (Fisher Scientific) for 10 minutes and then stained with DAPI dilactate (Sigma Aldrich). The cell nuclei were then visualized and counted in situ using a Zeiss Axiovert 200M fluorescence microscope. Images were captured using an Orca ER camera. Proliferation experiments were run in duplicate and repeated three different times per substrate type. Cell density (cells/cm$^2$) was determined by averaging the number of adherent cells in five random fields per substrate.
2.2.3.5. Cell Differentiation

Long term osteoblast functions were investigated by examining total intracellular protein synthesis, alkaline phosphatase activity, calcium deposition, and collagen synthesis. Osteoblasts were enzymatically lifted from the tissue culture polystyrene culture dish using 1 mL of a Trypsin-EDTA 1x solution (Sigma Aldrich). Osteoblasts were then suspended in DMEM supplemented with 10% FBS, 1% P/S, 50 µg/mL L-ascorbate acid (Sigma Aldrich), and 10 mM β-glycerophosphate (Sigma Aldrich) and were seeded at a density of 100,000 cells/cm² onto the conventional, nanorough, and nanopatterned Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C) for 7, 14, and 21 days. The media was changed every other day until the prescribed culturing time period elapsed. At the end of the prescribed time periods, the cells were rinsed in Tris-buffered saline (TBS) comprised of 42 mM Tris-HCl, 8 mM Tris Base, and 0.15 M NaCl (Sigma Aldrich). They were then lysed using distilled water and five freeze-thaw cycles which removed intracellular and membrane bound proteins. However, the calcium deposits remained on the substrate.

2.2.3.5.1. Total Intracellular Protein

Total intracellular protein content in the cell lysates was measured spectrophotometrically using a commercially available BCA™ Protein Assay
Reagent kit (Pierce Biotechnology, Rockford, IL, USA) and following manufacturer’s instructions. For this purpose, 150 µL of aliquots of each protein-containing, distilled water supernatant were mixed with 150 µL of working reagent (25:24:1 of sodium carbonate, sodium bicarbonate, and sodium tartrate: bicinchoninic acid: cupric sulfate) in a standard 96-well flat bottom culture plate. This mixture was then covered and incubated for two hours at 37ºC. At the end of this period, the plate was cooled to room temperature, and light absorbance was measured at 562 nm on a spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) and analyzed with computer software (SoftMax Pro 5; Molecular Devices). Total intracellular protein synthesized by osteoblasts cultured on conventional, nanorough, and nanopatterned Ti substrates was determined from a standard curve of absorbance versus known concentrations of albumin run in parallel with experimental samples. Total intracellular protein synthesis was run in duplicate and repeated four different times.

2.2.3.5.2. Alkaline Phosphatase Activity

Alkaline phosphatase activity is an early indicator for osteoblast differentiation (Figure 1.12). Thus, alkaline phosphatase activity in the cell lysates was measured spectrophotometrically using a commercially available Alkaline/Acid Phosphatase kit (R·R·A·pS·V·A, Upstate Cell Signaling Solutions, Millipore, Billerica, MA, USA) and following manufacturer’s
instructions. For this purpose, 25 µL aliquots of the distilled water supernatants were mixed with 5 µL NiCl₂ (40 mM in DI water), 5 µL bovine serum albumin (BSA, 5 mg/mL), 5 µL phosphopeptide stock solution, and 40 µL p-nitrophenyl phosphate (pNPP) Ser/Thr buffer in a standard 96-well flat bottom culture plate. The plate was then incubated for 15 minutes at 37°C. After this prescribe time period, 25 µL aliquots of the combined solution were transferred into three new standard 96-well flat bottom culture plate. This was followed by the addition of a 100 µL Malachite Green Solution in order to detect alkaline phosphatase activity. After incubating for 15 minutes at room temperature, light absorbance was measured at 650 nm on a spectrophotometer and analyzed with computer software. Alkaline phosphatase activity synthesized by osteoblasts cultured on conventional, nanorough, and nanopatterned Ti substrates was determined from a standard curve of absorbance versus known concentrations of phosphate run in parallel with experimental samples. One unit of alkaline phosphatase activity was equivalent to one nmol pNPP hydrolyzed per minute. The alkaline phosphatase activity was normalized by substrate surface area. Thus, the final results were reported as nanomoles of converted pNPP/min/mg protein/square centimeter. Alkaline phosphatase activity was run in duplicate and repeated four different times.
2.2.3.5.3. Calcium Deposition

Calcium deposition is one of the most important indicators of osteoblast differentiation and is an end stage indicator of osteoblast function (as described in section 1.3.4). To determine calcium deposition in this study, a commercially available calcium reagent kit (Pointe Scientific, Canton, MI, USA) was used following manufacturer's instructions. After the cells were lysed and removed for the purpose described in the total intracellular protein content section, the extracellular matrix on all conventional, nanorough, and nanopatterned Ti substrates was immersed and treated with 0.6 M HCl for 24 hours at 37ºC. After the prescribed time period, 20 µL of the dissolved calcium present in the acidic supernatant was measured by reacting with 200 µL o-cresolphthalein complexone reagent in a standard 96-well flat bottom culture plate for one minute at room temperature to form a purple color solution. Light absorbance was measured at 570 nm on a spectrophotometer and analyzed with computer software. Total calcium concentration (mg/dL) by osteoblasts cultured on conventional, nanorough, and nanopatterned Ti substrates was determined from a standard curve of absorbance versus known concentrations of calcium (Sigma Aldrich) run in parallel with experimental samples. Calcium concentration values were normalized by substrate surface area. Thus, the final results were reported as µg of calcium/mg protein/square centimeter. Calcium deposition was run in duplicate and repeated four different times.
In addition, qualitative examination of calcium mineralization on the conventional, nanorough, and nanopatterned Ti substrates was examined under fluorescence as well as under SEM. To examine fluorescently stained calcium, osteoblasts were first enzymatically lifted from the tissue culture polystyrene culture dish using 1 mL of a Trypsin-EDTA 1x solution (Sigma Aldrich). Osteoblasts were then suspended in DMEM supplemented with 10% FBS, 1% P/S, 50 µg/mL L-ascorbate acid (Sigma Aldrich), and 10 mM β-glycerophosphate (Sigma Aldrich) and were seeded at a density of 20,000 cells/cm² onto the conventional, nanorough, and nanopatterned Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C) for 14 days. The media was changed every other day until the prescribed culturing time period elapsed. At the end of the prescribed time period, the cells were incubated with calcinein (Sigma Aldrich) at 5 µg/mL in DMEM supplemented with 10% FBS, 1% P/S, 50 µg/mL L-ascorbate acid, and 10 mM β-glycerophosphate for 24 hours under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C). At the end of this prescribed period, substrates were rinsed three times in PBS and then fixed with 3.7% acetate buffer (Sigma Aldrich) for 15 minutes. All substrates were washed twice in PBS and treated with 1% denatured BSA in PBS (prepared by heating at 80°C for 5 minutes) for 10 minutes. After rinsing three times with PBS, the stained
substrates were visualized in situ using a Zeiss Axiovert 200M fluorescence microscope. Images were captured using an Orca ER camera.

To examine calcium deposition on the conventional, nanorough, and nanopatterned Ti substrates using SEM, osteoblasts were first enzymatically lifted from the tissue culture polystyrene culture dish using 1 mL of a Trypsin-EDTA 1x solution. Osteoblasts were then suspended in DMEM supplemented with 10% FBS, 1% P/S, 50 µg/mL L-ascorbate acid, and 10 mM β-glycerophosphate and were seeded at a density of 20,000 cells/cm² onto the conventional, nanorough, and nanopatterned Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C) for 21 days. The media was changed every other day until the prescribed culturing time period elapsed. At the end of the prescribed time period, the cells were rinsed in Tris-buffered saline (TBS) comprised of 42 mM Tris-HCl, 8 mM Tris Base, and 0.15 M NaCl (Sigma Aldrich). They were then lysed using distilled water and three freeze-thaw cycles which removed intracellular and membrane bound proteins. The remaining mineral deposits on the substrates were then visualized by SEM using secondary electrons collected with the in-lens detector at a 5 kV acceleration voltage at desired magnifications.
2.2.3.5.4. Collagen Synthesis

Collagen content in the cell lysates was measured spectrophotometrically by drying 100 µL aliquots of the distilled water supernatants in a standard 96-well flat bottom culture plate. Once dry, the wells were rinsed three times with DI water for one minute per wash followed by the addition of 0.1% Direct Red stain (Sigma Aldrich), and incubated for one hour at room temperature. The wells were then washed five times with 0.01 M HCl for ten seconds per wash to remove unbound stain. The collagen bound stain was then desorbed by washing with 0.1 M NaOH for five minutes. The eluted stain was then mixed several times into a multichannel pipette and was placed into a new standard 96-well flat bottom culture plate. Light absorbance was measured at 540 nm on a spectrophotometer and analyzed with computer software. Collagen content by osteoblasts cultured on conventional, nanorough, and nanopatterned Ti substrates was determined from a standard curve of absorbance versus known concentrations of collagen Type I run in parallel with experimental samples. Total collagen content was normalized by substrate area and expressed as mg/cm².

In addition, qualitative examination of extracellular collagen on the conventional, nanorough, and nanopatterned Ti substrates was examined under fluorescence through an enzyme-linked immunosorbent assay (ELISA) described in more detail in the following section. The purpose of this
The experiment was to determine if directing osteoblast orientation on nanopatterned Ti would result in aligned collagen synthesis. First, osteoblasts were enzymatically lifted from the tissue culture polystyrene culture dish using 1 mL of a Trypsin·EDTA 1x solution (Sigma Aldrich). Osteoblasts were then suspended in DMEM supplemented with 10% FBS, 1% P/S, 50 µg/mL L-ascorbate acid (Sigma Aldrich), and 10 mM β-glycerophosphate (Sigma Aldrich) and were seeded at a density of 5,000 cells/cm² onto the conventional, nanorough, and nanopatterned Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C) for five days. The media was changed every other day until the prescribed culturing time period elapsed. At the end of the prescribed time period, the cells were rinsed three times in PBS, fixed in cold acetone for 15 minutes, rinsed three times in PBS, and treated with 1mg/mL BSA for 30 minutes at 23°C. Once complete, the substrates were then incubated at 23°C for two hours with rabbit antiserum to Type I collagen (ab34710, Abcam, Cambridge, MA, USA) using a 1:10,000 dilution in PBS. After rinsing three times in PBS, the substrates were then incubated at 23°C for one hour with Cy2-conjugated goat anti-rabbit IgG (1:400 dilution with an ABTS kit (Vector Laboratories, Burlingame, CA, USA). Following an additional three rinses with PBS, cells were evaluated in situ using a Zeiss Axiovert 200M fluorescence microscope. Images were
captured using an Orca ER camera. This procedure was adapted from Qiao and colleagues [278].

2.2.4. Mechanisms for Osteoblast Response

2.2.4.1. Surface Energetics and Wettability

Material surface energy and wettability were investigated with a drop shape analysis system (EasyDrop; Kruss, Hamburg, Germany). The contact angle of 3 μL sessile droplets was measured at two locations on each of four samples for the conventional and nanorough Ti substrates. To determine surface energy, three different liquid solvents (distilled water, glycerol, and polyethylene glycol 200) were used. Measurements were taken five seconds after placing the droplet on the sample surface under ambient conditions. Drop shape analysis software (DSA1; Kruss) was used to calculate the surface energy by entering surface tension and contact angle (θ) data into the Owens-Wendt equation:

\[ 1 + \gamma_1 \cos \theta = 2(\sqrt{\gamma_{ds} \gamma_{dl}} + \sqrt{\gamma_{ps} \gamma_{pl}}) \]  

[Eq 2.3]

where \( \gamma_{ds} \) and \( \gamma_{ps} \) are the respective dispersion and polar terms of the solid surface tension, \( \gamma_s \); \( \gamma_{dl} \) and \( \gamma_{pl} \) are the respective dispersion and polar terms of the liquid surface tension, \( \gamma_l \). Other theories were investigated (Fowkes and Zisman) but results showed the same trend of surface energy per substrate type as those obtained with the Owens–Wendt model.
2.2.4.2. Fibronectin Adsorption (ELISA)

An enzyme-linked immunosorbent assay (ELISA) is a well established procedure for measuring the amounts of protein (in this study fibronectin) adsorption to implant surfaces, here to the conventional, nanorough, and nanopatterned Ti substrates (Figure 2.6). Substrates were placed in a standard 24-well culture plate and immersed in 1 mL of Dulbecco’s Modified Eagle’s Medium (DMEM; Hyclone) supplemented with and without 10% FBS and 1% P/S for 24 hours at 37°C in 5% CO₂/95% humidified air. After rinsing in PBS, BSA (2 wt% in PBS, Sigma Aldrich) was then used to block all areas that did not react with fibronectin. Fibronectin was then directly linked with primary rabbit anti-bovine fibronectin (AB2047, Millipore) for one hour at 37°C in 5% CO₂/95% humidified air. After rinsing three times with 0.05% Tween 20 for five minutes with each rinse, the samples were further incubated for another one hour at 37°C in 5% CO₂/95% humidified air with a secondary goat anti-rabbit conjugated with horseradish peroxidase (HRP) used to react with dye molecules (Bio-Rad, Hercules, CA, USA). After rinsing three times with 0.05% Tween 20 for five minutes with each rinse, the amount of fibronectin adsorbed to the surfaces was measured with an ABTS substrate kit (Vector Laboratories) that reacted only with the HRP. Light absorbance was measured at 405 nm on a spectrophotometer and analyzed with computer software. The average absorbance was subtracted by the average absorbance obtained from the negative controls soaked in
DMEM with no FBS or P/S. ELISA was performed in duplicate and repeated three different times per substrate.

Figure 2.6. ELISA reaction with adsorbed fibronectin on Ti substrates.

2.2.5. Statistics

Data were represented by the mean value with the standard error of the mean (SEM) noted. A one-tailed, paired standard student t-test was used to check statistical significance between means, and p<0.1 was considered statistically significant.
2.3 Results and Discussion

2.3.1. Sample Characterization

2.3.1.1. Scanning Electron Microscopy (SEM)

Results of the present study provided evidence of a strong adhesion of the outer nanostructured Ti surface on all the Ti surfaces created through electron beam evaporation using the peel test. This was determined due to the fact that none of the Ti was removed from these surfaces using the scotch, duct, or double-sided tape.

Low and high scanning electron micrographs revealed distinct differences in the topography of the commercially pure Ti as received from the vendor and the electron beam evaporated Ti (Figure 2.7). The electron beam evaporated Ti substrates displayed a high degree of nanometer surface roughness. Thus, throughout the remainder of this dissertation, these Ti substrates will be referred to as nanorough Ti. On the other hand, the Ti received from the vendor did possess some nanoscale features but overall exhibited a smoother surface with more features on the micron scale. As a result, these Ti substrates will be referred to as conventional Ti throughout the remainder of this dissertation.
As expected, these topographies were identical on the nanopatterned Ti substrates after electron beam evaporation (Figure 2.8). It was vividly clear that the presence of the meshes created linearly aligned grooves of alternating nanometer and micrometer topographies. The region exposed by the hole in the mesh during electron beam evaporation possessed increased nanometer surface roughness compared to the region masked by the bar of the mesh which possessed micrometer surface roughness. The 200 mesh had the largest hole width (80 µm, Table 2.1) meaning that these nanopatterned Ti substrates contained the greatest area of nanorough topography. The 400 mesh nanopatterned Ti substrates had the smallest hole width (22 µm, Table 2.1) resulting in the least amount of nanometer surface area coverage. More specifically, upon further calculation, the total hole area (or total nanorough surface area) was 2.961 mm$^2$ for the 200 mesh, 2.700 mm$^2$ for the 300 mesh, and 1.830 mm$^2$ for the 400 mesh. Therefore, there was an 8.79% difference in total nanorough surface area between the 200 and 300 mesh nanopattern substrates, a 32.23% difference between the 300 and 400 mesh nanopatterned substrates, and a 38.19% difference between the 200 and 400 mesh nanopatterned substrates. Clearly, the 200 and 300 mesh nanopatterned surfaces were more similar in their dimensions and nanorough surface area while a more significant difference was present when compared to the 400 mesh nanopatterned surfaces.
Figure 2.7. SEM micrographs of Ti substrates (a) after electron beam evaporation and (b) as received from the vendor. The Ti substrates possessed a higher degree of nanometer roughness after electron beam evaporation (and, thus, was termed nanorough Ti) while the Ti substrates obtained directly from the vendor possessed more microscale features (and, thus, was termed conventional Ti). Scale bar = 20 µm (low resolution) and 200 nm (high resolution).
(a) 200 Mesh Patterned Ti Substrate
(b) 300 Mesh Patterned Ti Substrate
400 Mesh (1 K)

Bar = Conventional Region (50 K)

Interface Between Hole and Bar (30 K)

Hole = Nanorough Region (50 K)

As Received from the Vendor Region

Electron Beam Evaporated Region

(c) 400 Mesh Patterned Ti Substrate
Figure 2.8. SEM micrographs of the arrays of parallel, aligned grooves on Ti substrates after electron beam evaporation. The hole and bar components of the different meshes varied in width size (Table 2.1). The (a) 200 mesh with its 80 µm hole contained the greatest area of nanometer surface roughness while the (c) 400 mesh with its 22 µm hole contained the least area of nanometer surface roughness. As expected, these alternating topographical regions respectively matched those of Ti substrates as received from the vendor and the Ti substrates after electron beam evaporation containing no meshes.

2.3.1.2. Atomic Force Microscopy (AFM)

The qualitative evidence from SEM characterization revealed the presence of nanometer and micrometer surface roughness on the Ti substrates received from the vendor, Ti substrates after electron beam evaporation, and the nanopatterned Ti substrates after electron beam evaporation. Such observations were further confirmed by quantitative data from AFM (Figure 2.9). Root-mean-square roughness (RMS) was larger for the surfaces containing the nanorough topographies created by electron beam evaporation (RMS = 11 nm) compared to the surfaces containing conventional, micron size topographies (RMS = 6 nm). Average peak-to-valley roughness (RPV) was also found to have the largest nanometer rough topographies (RPV = 30-40 nm). Table 2.2 summarizes the values found for RMS and RPV. Thus, from the thorough analysis of both SEM and AFM, it can be concluded that during the cell studies (described in the next section), osteoblasts will be exposed to surfaces with unique and different surface topographies varying in their degree of nanoroughness.
Table 2.2. Qualitative data obtained from AFM indicating the substrates created by electron beam evaporation contained more surface features in the nanometer regime than those received directly from vendor. RMS represents root-mean-square roughness while RPV represents peak-to-valley roughness.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RMS (nm)</th>
<th>RPV (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron Beam Evaporated Ti (nanorough topography)</td>
<td>11</td>
<td>30-40</td>
</tr>
<tr>
<td>As Received Ti (conventional topography)</td>
<td>6</td>
<td>10-20</td>
</tr>
</tbody>
</table>
Figure 2.9. AFM micrographs and section analyses revealed distinct topographical differences (both qualitatively and quantitatively) of the Ti surfaces from the vendor and after electron beam evaporation. Root-mean-square roughness (RMS) and average peak-to-valley roughness (RPV) values can be found in Table 2.2.
2.3.1.3. Electron Spectroscopy for Chemical Analysis (ESCA)

Because it is well-known that alterations in chemistry and/or crystal structures can influence cell function, each of these must be investigated in order to determine which elements may explain the response of osteoblasts observed throughout this study.

ESCA was performed on the electron beam evaporated nanorough Ti surface and on the as received conventional Ti surfaces in order to determine if there was a difference in the surface oxide compositions. One high resolution ESCA spot was taken on each surface and revealed only one identifiable peak, Ti 2p (Figure 2.10). Therefore, the oxide layer present on the electron beam evaporated nanorough Ti surfaces and on the as received conventional Ti surfaces (Figure 2.10) was the same. ESCA further indicated that the outermost layers consisted of O and Ti (Table 2.3). Thus, it can be concluded that the chemistries of the nanorough and conventional Ti surfaces were the same.

Table 2.3. Atomic percentage of selective elements in the outermost layers of Ti before (conventional Ti) and after electron beam evaporation (nanorough Ti) as examined by ESCA.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>O (%)</th>
<th>Ti (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanorough Ti</td>
<td>49.34</td>
<td>50.66</td>
</tr>
<tr>
<td>Conventional Ti</td>
<td>51.23</td>
<td>48.77</td>
</tr>
</tbody>
</table>
Figure 2.10. (a) ESCA spectra of elements present in the outermost layer of the Ti before (conventional Ti) and after electron beam evaporation (nanorough Ti). (b-c) Binding energy of the high resolution Ti 2p peaks indicating these surfaces have similar chemistries.
2.3.1.4. X-ray Diffraction (XRD)

Finally, this study determined whether or not the phases present on the surfaces of the various Ti substrates were similar. XRD patterns provided evidence that Ti was present on both the nanorough Ti surfaces and the as received conventional Ti (Figure 2.11). No traces of anatase TiO$_2$ or rutile TiO$_2$ phase was found on either surface type. As a result, it can be concluded that the osteoblast response (as described in the next sections) on the varying regions is only due to a variation in topography and not chemistry and crystallinity.

![XRD spectra of nanorough Ti surfaces after electron beam evaporation and the conventional Ti surfaces as received from the vendor indicated the presence of crystalline TiO$_2$. Upon further examination of the crystalline phase, slight variations were found demonstrating that the nanorough Ti region possessed more of the anatase TiO$_2$ phase while the conventional Ti regions possessed more of the rutile TiO$_2$ phase.](image-url)
2.3.2. Osteoblast Response

2.3.2.1. Adhesion

Figure 2.12 shows the results of total osteoblast adhesion on conventional, nanorough, and nanopatterned Ti substrates. First, data from this graph confirmed the trend that numerous studies have previously shown – osteoblasts adhered better to nanophase metals compared to their conventional counterparts. More specifically, osteoblast adhesion was significantly greater (p<0.01) on the nanorough Ti surfaces prepared by electron beam evaporation compared to the conventional Ti surfaces as received from the vendor. More conclusive evidence revealed this direct relationship between increasing surface nanoroughness and enhanced cellular adhesion which was also strongly observed on the nanopatterned Ti substrates. A substrate with patterned features containing alternating regions of conventional and nanorough topographies provides the ability to expose an identical cell population under the same environmental conditions to a material with varying roughnesses. In addition to examining total cells adherent to the nanopatterned Ti substrates, as in Figure 2.12, cells attaching to each individual region was also analyzed (Figure 2.13). Data revealed that when simultaneously exposed to surfaces of varying topography, cells adhered significantly more to regions possessing the most
nanoscale features (Figure 2.13), thus further confirming that osteoblasts respond differently to varying surface roughness.

Secondly, Figure 2.12 showed that among the nanopatterned Ti surfaces, total osteoblast adhesion varied. More specifically, osteoblast adhered significantly more to the 200 and the 300 mesh (nanopatterns with the largest nanorough surface area) nanopatterned Ti surfaces (p<0.05 and p<0.1, respectively) compared to the 200 mesh (nanopatterns with the least nanorough surface area). There was no significance observed in total osteoblast adhesion when comparing the 200 and 300 meshes. Therefore, from these results, there appears to be a dimensional threshold to which the osteoblasts adhere better since the 200 and 300 mesh nanopattern Ti surfaces were similar in dimensions and total nanorough surface area compared to the 400 mesh nanopattern Ti surfaces. When further examining osteoblasts adherent only to the nanorough region of these nanopatterned surfaces, it was observed that more osteoblasts significantly adhered to the nanorough region of the 200 mesh compared to both the 300 and 400 mesh. Similarly, when comparing the osteoblasts adherent to the nanorough regions of the 300 and 400 mesh nanopatterned Ti surfaces, osteoblasts were significantly greater on the nanorough region with the 300 mesh. Interestingly, roughly the same number of osteoblasts adhered to the conventional region on each mesh as no significant difference was observed.
Fluorescently stained osteoblast nuclei on the various substrates are shown in Figure 2.14.

Third, comparing the 300 and 400 mesh nanopatterned Ti surfaces to the complete nanorough Ti surfaces (no patterns) revealed that osteoblasts adhered significantly more to the nanorough Ti surface (Figure 2.12). No significant difference was noted between the 200 mesh and nanorough Ti surface. Fourth, there was no statistical difference observed between the nanopatterned Ti surfaces and the conventional Ti surfaces.
Figure 2.12. Total osteoblast adhesion on nanorough Ti created by electron beam evaporation, conventional Ti surfaces as received from the vendor, and nanopatterned Ti substrates with varying groove widths after four hours. Values are mean ±SEM; n=3; *p<0.05 compared to the 300 mesh; **p<0.01 compared to the 400 mesh; ***p<0.01 compared to the conventional Ti; #p<0.01 compared to the glass; ###p<0.05 compared to the 400 mesh; ####p<0.1 compared to the 400 mesh.

Figure 2.13. Osteoblast specifically attached to the nanorough region and conventional rough region of the varying nanopatterned Ti surface (created through electron beam evaporation) after four hours. Values are mean ±SEM; n=3; *p<0.01 compared to the conventional region on respective days; •p<0.05 compared to the respective regions of 300 mesh; ••p<0.01 compared to the respective regions of 400 mesh.
Figure 2.14. Fluorescent micrographs indicating osteoblast adhesion on the (a-c) nanopatterned Ti surfaces, (d) nanorough Ti surfaces, (e) and convention Ti surface after four hours. Scale bar = 50 µm.
2.3.2.2. Morphology

Osteoblast morphology after four hours was also examined specifically to determine if the presence of the linear nanopatterns affected cell alignment. Interestingly, although osteoblasts adhered better on the 200 mesh nanopatterned Ti surfaces (nanopatterns with largest groove widths), cell alignment was different when compared to the 400 mesh nanopatterned Ti surfaces. More specifically, osteoblast alignment increased with decreasing feature size. Qualitatively, the observation of cell growth on the parallel grooved surfaces using f-actin labeling showed distinct orientation of cells along the grooves after four hours on the 400 mesh nanopatterned Ti surfaces compared to the 200 and 300 mesh as well as the non-patterned nanorough and conventional Ti surfaces (Figure 2.15). The f-actin filaments of the cell were more elongated and aligned to the direction of the nanopattern on the 400 mesh Ti surfaces (the nanopatterned with the smallest width dimension). Although there were cells that did align with the direction of the groove, compared to the 400 mesh, it was not as well-defined on the 200 and 300 mesh nanopatterned Ti surfaces which contained the larger groove widths. However, when compared to the non-patterned nanorough and conventional Ti surfaces, cell elongation and alignment was qualitatively observed to increase on the 200 and 300 mesh nanopattern Ti surfaces. Osteoblast alignment after four hours was also qualitatively examined under SEM, as seen in Figure 2.16, and further confirmed that osteoblast orientation was
affected by the presence of nanopatterns. Surface area of osteoblasts on the various nanopatterned Ti surfaces was examined to provide more quantitative and revealed similar trends (Figure 2.17). More specifically, compared to the nanorough and conventional Ti substrates, osteoblasts present on the nanopatterned Ti substrates revealed significantly less spreading thus indicative of more elongated cells due to the presence of the linear patterns (Figure 2.17). Upon further examining the surface area of osteoblasts on the nanopatterned Ti substrates, cells on the 400 mesh nanopattern Ti substrates had the smallest surface area (Figure 2.17). Thus, although the 400 mesh nanopattern Ti substrates did not promote the most osteoblast adhesion, these nanopatterns affected the orientation of the osteoblast more when compared to the 200 or 300 mesh nanopatterned Ti substrates that possessed the largest nanopattern grooves.

Results from osteoblast adhesion and morphology demonstrated that the presence of linear nanopatterns did affect cell orientation. More specifically, when the grooves were wider than the cells, effects on orientation were not very extensive. These results are in correlation with other researchers who revealed that as the groove size is reduced to the width of the cell or less, orientation of the cell becomes more extensive [149]. As mentioned earlier, osteoblasts are on average 10-40 µm in diameter, thereby explaining the better alignment behavior on the nanopatterned surfaces with 22 µm width grooves compared to the other surfaces containing 40 and 48 µm width
grooves (Table 2.1). This data therefore suggested that mimicking both the nanometer size and linear orientation of natural bone can affect overall osteoblast behavior.
(a) 200 mesh (80:45 µm)

(b) 300 mesh (48:35 µm)

(c) 400 mesh (22:40 µm)
Figure 2.15. Fluorescent micrographs indicating osteoblast alignment on the (c) 400 mesh nanopatterned Ti surfaces compared to the (a) 200 mesh, (b) 300 mesh, (d) nanorough Ti, and (e) conventional Ti surfaces. Arrows indicate groove alignment direction on the nanopatterned samples. Scale bar = 50 µm.
Figure 2.16. SEM micrographs revealing osteoblast alignment in the presence of the nanopatterned features on the Ti surfaces.
Figure 2.17. Average surface area of osteoblasts on the various substrates. Osteoblasts on the 400 mesh were the least spread indicating that these nanopattern dimensions affected the elongation of osteoblasts the most. Values are mean ±SEM; n=3; *p<0.01 compared to the 400 mesh; **p<0.1 compared to the 400 mesh; ***p<0.01 compared to the respective regions of 400 mesh; ###p<0.01 compared to the 400 mesh; #p<0.1 compared to 200 mesh; ##p<0.1 compared to 300 mesh.

2.3.2.3. Proliferation

Furthermore, the results and trends revealed from the osteoblast adhesion data were also seen during the osteoblast proliferation experiments (Figure 2.12). First, total osteoblast proliferation was significantly greater for the nanorough Ti substrates compared to the conventional Ti substrates for each time period examined in this study (Figure 2.18). Similarly, the division of cells for the varying regions on the nanopatterned substrates revealed that
osteoblasts proliferated significantly more (p<0.01) on the nanorough region compared to the conventional regions (Figure 2.19).

Second, over the course of one, three, and five days, a significant difference was noted in the total osteoblast proliferation on the nanopatterned Ti substrates with the 200 mesh (Figure 2.18) compared to the nanopatterned substrates made with the 400 mesh. By day five, a statistical difference was also noted between the 200 and 300 mesh nanopatterned Ti substrates, with the 200 mesh nanopatterned surfaces having more osteoblasts. Also by day five, the 300 mesh nanopatterned Ti surfaces possessed significantly more osteoblasts than the 400 mesh. Thus, it can be concluded that varying the width dimension of the nanopatterns can affect the osteoblast proliferation rate, and in this study, the larger width dimensions with the largest nanorough surface area increased osteoblast proliferation rate the best.

When examining cells specifically attached to the nanorough region on these patterned Ti substrates, osteoblasts grew significantly more on the nanorough regions of the 200 mesh compared to the 300 and 400 mesh after one, three, and five days (Figure 2.19). Furthermore, statistically greater osteoblast proliferation on the 300 mesh compared to the 400 mesh nanopatterned Ti surfaces was observed after one, three, and five days. Similar to the adhesion data, excluding day three, the number of osteoblasts adherent to the conventional region on each nanopatterned surface was comparable with no statistical significance observed. Thus, observations
from the adhesion and proliferation data revealed that osteoblasts responded differently to surfaces not only containing different surfaces roughnesses but also to different groove widths.

Third, except for the 200 mesh on day three, comparing the nanopatterned Ti surfaces compared to the non-patterned nanorough Ti surfaces revealed that osteoblasts adhered significantly more to the non-patterned nanorough Ti surface after one, three, and five days (Figure 2.18). There was no significance found between the 200 mesh nanopatterned Ti surfaces and nanorough Ti surfaces on day three. Fourth, when comparing the nanopatterned Ti surfaces to the conventional Ti surfaces, it was found that the 200 mesh nanopatterned Ti surface better promoted osteoblast proliferation on day one and five (Figure 2.18). Osteoblast proliferation rate was significantly greater on the 300 mesh nanopatterned Ti surfaces compared to the conventional Ti surface after day five. There was no statistical difference observed between the 400 mesh nanopatterned Ti surfaces and the conventional Ti surfaces over the prescribed time period. Thus, enhanced osteoblast behavior was observed in the presence of nanopatterns with specific dimensions (largest width providing the most nanorough rough surface area) compared to conventional Ti surfaces.
Figure 2.18. One, three, and five day osteoblast densities on the nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces. Values are mean ±SEM; n=3; *p<0.01 compared to the 200 mesh; **p<0.01 compared to the 300 mesh; ***p<0.01 compared to the 400 mesh; #p<0.01 compared to the conventional Ti; ##p<0.01 compared to the etched glass; ###p<0.05 compared to the 400 mesh; +p<0.05 compared to the conventional Ti; ++p<0.05 compared to the 300 mesh; +++p<0.1 compared to the 300 mesh; Δp<0.1 compared to the 400 mesh; •p<0.1 compared to the 200 mesh; ••p<0.1 compared to the conventional Ti; •••p<0.1 compared to the 400 mesh.
Figure 2.19. Greater osteoblast densities specifically on the nanorough region compared to the conventional region for the varying nanopatterned Ti surfaces after one, three, and five days. Values are mean ±SEM; n=3; *p<0.01 compared to the conventional region on respective days; •p<0.1 compared to the respective regions of 300 mesh; ••p<0.01 compared to the respective regions of 400 mesh; •••p<0.1 compared to the respective regions of 400 mesh; ∆p<0.01 compared to the respective regions of 200 mesh; ∆∆p<0.01 compared to the respective regions on 300 mesh.
2.3.2.4. Total Intracellular Protein Content

In addition to the aforementioned adhesion and proliferation studies, parameters indicating bone cell differentiation, including alkaline phosphatase activity, calcium deposition, and collagen synthesis, was also examined here. But first, to determine these parameters, total protein content must first be found. Figure 2.20 showed the osteoblast total intracellular protein content synthesized on conventional, nanorough, and nanopatterned Ti substrates. As time progressed, total protein synthesis significantly increased on all samples with the greatest amount of osteoblast intracellular protein synthesis observed at day 21. However, except for a few samples on day 14, no one sample type had significantly more osteoblast total intracellular protein than another sample type.
Figure 2.20. Total intracellular protein content of osteoblasts cultured on the varying nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; *p<0.01 compared to the 200 mesh; **p<0.01 compared to the conventional Ti; ***p<0.05 compared to the conventional Ti; #p<0.01 compared to the respective substrates on day 7; ##p<0.05 compared to the respective substrates on day 14; ###p<0.05 compared to the respective substrates on day 7.

Total intracellular protein content for 500,000, 1x10^6, 3x10^6, and 5x10^6 osteoblasts was found. From this data along with total intracellular protein content from the osteoblasts culture on the varying nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti shown above (Figure 2.20), the number of cells on the substrates after 7, 14, and 21 days was found (Figure 2.21). As expected, it was found that the number of osteoblasts on each sample significantly increased over time. Furthermore, the total
number of osteoblasts was found to be equal among all substrates for each time period (with the exception of few samples at day 14). This data was used in the upcoming sections (alkaline phosphatase activity and collagen synthesis) to determine the alkaline phosphatase activity as well as collagen synthesis per cell.

Figure 2.21. Number of osteoblasts at the end of 7, 14, and 21 days on the varying nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces. Values are mean ±SEM; n=4; *p<0.01 compared to the 200 mesh; **p<0.01 compared to the conventional Ti; ***p<0.05 compared to the conventional Ti; #p<0.01 compared to the respective substrates on day 7; ##p<0.05 compared to the respective substrates on day 14; ####p<0.05 compared to the respective substrates on day 7; •p<0.1 compared to the respective substrates on day 7.
2.3.2.5. Alkaline Phosphatase Activity

As mentioned in Figure 1.12, when osteoblasts begin to differentiate, they increase production of alkaline phosphatase. Thus, an early indication of osteoblast differentiation can be determined by examining alkaline phosphatase content. Alkaline phosphatase in this study revealed several facts about the surfaces used. First, compared to day 7, total alkaline phosphatase activity statistically increased on all substrates after 14 and 21 days of culture (Figure 2.22). Similarly, compared to day 14, total alkaline phosphatase activity statistically increased on all substrates after 21 days of culture.

Second, it was noted that total alkaline phosphatase activity after day 7, 14, and 21 was significantly greater when osteoblasts were cultured on non-patterned nanorough Ti substrates created by electron beam evaporation compared to the conventional Ti surfaces as received from the vendor (Figure 2.22).

Third, significantly greater osteoblast total alkaline phosphatase was measured on the 200 mesh substrates compared to the 300 mesh on day 7 and 14 and the 400 mesh on 7, 14, and 21 days (Figure 2.22). Therefore, following the adhesion and proliferation results, varying the width of the nanopatterns affected osteoblast behavior by altering production of alkaline
phosphatase, particularly, with the most synthesized on the 200 mesh containing the largest nanorough region.

Fourth, osteoblasts on the non-patterned nanorough Ti surfaces synthesized more alkaline phosphatase than on the 400 mesh nanopatterned Ti surfaces after day 7, 14, and 21 and the 300 mesh after day 7 and 14 (Figure 2.22). However, no significant difference was observed between the non-patterned nanorough Ti surfaces and the 200 mesh nanopatterned Ti surfaces over the cell culture period. Thus, the dimensions of the nanopatterned grooves does affect osteoblast synthesis of total alkaline phosphatase compared to Ti surfaces with complete nanorough coverage.

Fifth, unlike the nanorough Ti surfaces, compared to the conventional Ti substrates, the 200 mesh nanopatterned Ti substrates (nanopatterns with the most nanorough surface area) caused osteoblasts to produce significantly more total alkaline phosphatase over 7, 14, and 21 days (Figure 2.22). In addition, synthesis of total alkaline phosphatase after day 7 and 14 was also statistically more on the 300 and 400 mesh compared to the conventional Ti surfaces. These results therefore suggested that the presence of nanopatterns improved osteoblast differentiation, with respect to alkaline phosphatase activity, compared to current conventional Ti surfaces.
Figure 2.22. Total alkaline phosphatase synthesis by osteoblasts cultured on the various nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; *p<0.05 compared to the 400 mesh; **p<0.1 compared to the 300 mesh; ***p<0.01 compared to the conventional Ti; #p<0.1 compared to the 400 mesh; ##p<0.05 compared to the conventional Ti; ###p<0.05 compared to the 300 mesh; +p<0.1 compared to the conventional Ti; ++p<0.01 compared to the respective substrates on day 7; +++p<0.1 compared to the respective substrates on day 14; •p<0.05 compared to the respective substrates on day 7; ••p<0.05 compared to the respective substrates on day 14.

When determining the amount of alkaline phosphatase activity by one osteoblast on each surface (found by dividing total alkaline phosphatase activity, Figure 2.22, by total cell number, Figure 2.21), it was found that synthesis of alkaline phosphate per cell remained consistent over the culturing time period, as no statistical difference was revealed (the conventional Ti surfaces were an exception) as demonstrated in Figure 2.23.
It was also found that the trends observed from total alkaline phosphatase synthesis (more activity on the nanopatterned Ti surfaces compared to the conventional Ti surfaces and more activity on the 200 mesh nanopatterned Ti surfaces compared to the 300 and 400 mesh nanopatterned Ti surfaces) were comparable to the alkaline phosphatase synthesis per cell on the surfaces (Figure 2.23). Thus, improved osteoblast alkaline phosphatase activity on the nanopatterned Ti surfaces compared to the conventional Ti surfaces (as well as the 200 nanopatterned Ti surfaces compared to 300 and 400 nanopatterned Ti surfaces) was due to enhanced alkaline phosphatase synthesis rather than increased cell number (which was found to be the same for all surfaces examined, Figure 2.21).
Figure 2.23. Alkaline phosphatase synthesis by each individual osteoblast cultured on the various nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; *p<0.05 compared to the 400 mesh; **p<0.01 compared to the conventional Ti; ***p<0.1 compared to the 400 mesh; #p<0.1 compared to the conventional Ti; ##p<0.01 compared to the 400 mesh; ###p<0.05 compared to the 300 mesh; +p<0.1 compared to the respective substrates on day 7; ++p<0.05 compared to the respective substrates on day 7; +++p<0.01 compared to the respective substrates on day 7.

### 2.3.2.6. Calcium Deposition

Calcium deposition is extremely important in the synthesis of new bone growth. Unlike alkaline phosphatase activity, calcium deposition by osteoblasts is a later indicator for osteoblast differentiation, since it is only after the extracellular matrix development and maturation process that osteoblasts start to differentiate from non-calcium to calcium depositing cells.
(Figure 1.12). However, early alkaline phosphatase activity can be linked to the more rapid calcium deposition by osteoblasts on the substrates examined in this study. Similar trends were revealed from the calcium deposition data in this study (Figure 2.24). First, compared to day 7, the 400 and 200 mesh nanopatterned Ti substrates as well as the non-patterned nanorough Ti substrates had statistically greater calcium deposition after 21 days (Figure 2.24).

Second and more importantly, after any time period tested, calcium content in the extracellular matrix was significantly higher on the non-patterned nanorough Ti surfaces compared to the conventional Ti surfaces (Figure 2.24). This result alone, as seen in previous studies [171, 185, 198, 210, 274], can explain why nanorough surfaces may be the future materials for orthopedic implants. With increasing surface roughness comes increased cell attachment and differentiation, leading to increased deposition of calcium required for the proper integration of new bone into the implanted device.

Third, when examining calcium deposition on the varying nanopatterned substrates, it was found that the 200 mesh contained significantly greater amounts of calcium compared to the 400 mesh nanopatterned Ti substrates after 7, 14, and 21 days and compared to the 300 mesh nanopatterned Ti substrates after 7 and 21 days (Figure 2.24). Osteoblasts on the 300 mesh nanopatterned Ti substrates were found to deposit significantly more calcium
compared to the 400 mesh nanopatterned Ti substrates (only after day 7). In other words, calcium content increased with increasing nanorough region on the nanopatterned substrates with the 200 mesh nanopattern surfaces (2.961 mm² overall nanorough surface area) possessing the most calcium deposition compared to the 300 mesh (2.700 mm² overall nanorough surface area) and the 400 mesh (1.830 mm² overall nanorough surface area).

Fourth, by day 21, it was found that the osteoblasts on the 200 mesh nanopatterned Ti surfaces secreted more calcium than the nanorough Ti surfaces (Figure 2.24). Fifth, similar to alkaline phosphatase production, when comparing calcium synthesis between the 200 mesh and conventional Ti surfaces, significantly greater calcium deposition was found at all time points on the 200 mesh nanopatterned Ti surfaces (Figure 2.24). Thus, not only do surfaces containing increased nanoroughness enhance calcium deposition, the presence of linear nanopatterns with specific dimensions can further improve calcium deposition. Thus, mimicking both nanometer size and orientation of implant surfaces to that of natural bone holds much promise towards increasing new bone juxtaposed to the implant improving the overall efficacy of orthopedic devices used for ITAP.
Figure 2.24. Calcium deposition by osteoblasts cultured on the varying nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; *p< 0.05 compared to the 400 mesh; **p<0.01 compared to the 400 mesh; ***p<0.01 compared to the 300 mesh; #p<0.1 compared to the conventional Ti; ##p<0.1 compared to the 400 mesh; ###p<0.1 compared to the 300 mesh; +p<0.01 compared to the conventional Ti; ++p<0.05 compared to the conventional Ti; +++p<0.05 compared to the 300 mesh; •p<0.05 compared to the nanorough Ti; ••p<0.05 compared to the respective substrates at day 7; •••p<0.01 compared to the respective substrates at day 7; Δp<0.1 compared to the respective substrates at day 7.

Several other calcium tests were completed to qualitatively examine calcium deposition on the numerous substrates used throughout this study. Fluorescently stained calcium deposits on the surfaces after 21 days of culture was examined and revealed that indeed calcium deposition occurred on all surfaces (indicated by the calcein labeled mineralized deposits over the
surface) as shown in Figure 2.25. This experiment further confirmed the capability of the nanopatterned Ti substrates to support osteoblast differentiation. However, no comparison of amount of calcium deposition could be made between the various substrates. Furthermore, the nanopatterned Ti substrates were examined under SEM after 21 days of culture to better examine the calcium deposits at a higher magnification than what was obtained from the fluorescent images (Figure 2.26). Calcium was once again revealed on the nanopatterned Ti surfaces. Interestingly, it seemed that the presence of the nanopatterns directed deposition of the calcium by osteoblasts to the individual regions on the Ti substrates. Such results have also been shown by other researchers who revealed directed mineral deposition by osteoblasts onto patterned carbon nanofibers on polycarbonate urethane surfaces [274]. Thus, it can be concluded from both the quantitative and qualitative data that the use of nanopatterns on the surface of Ti implants can enhance osteoblast differentiation, as revealed through the various calcium deposition studies.
Figure 2.25. Calcein labeling of mineralized deposits on the (a-c) various nanopatterned Ti substrates, (d) nanorough Ti substrates, and (e) conventional Ti substrates at 21 days. Scale bar = 50 µm.
Figure 2. SEM micrographs of calcium deposition by osteoblasts on the various nanopatterned Ti substrates confirming their capability to support osteoblast mineralization. As seen in the images, it appears that the presence of the nanopatterns directs the deposition of calcium phosphate by osteoblast into the separate regions.

2.3.2.7. Collagen Synthesis

As stated, one of the main components of bone is Type I collagen that spontaneously aligns to form fibrils and provide the template for hydroxyapatite crystals precipitation. One of the main motivations behind this research was to mimic the precise organization of such components in bone. Although osteoblasts in their natural state are not aligned, directing
them in a manner where they will secrete collagen into aligned fibrils was examined. Collagen synthesis results from this study revealed several facts about the surfaces used. First, results from the intracellular collagen assay revealed that compared to day 7, total intracellular collagen synthesis statistically increased on all substrates (excluding the 400 mesh nanopatterned Ti substrates) after 21 days of culture (Figure 2.27). Similarly, compared to day 14, total intracellular collagen synthesis statistically increased on all substrates after 21 days of culture.

Second, it was noted that total collagen synthesis after day 7, 14, and 21 was significantly greater when osteoblasts were cultured on non-patterned nanorough Ti substrates created by electron beam evaporation compared to the conventional Ti surfaces as received from the vendor (Figure 2.27).

Third, significantly greater osteoblast collagen synthesis was measured on the 200 mesh nanopatterned Ti substrates compared to the 300 and 400 mesh nanopatterned Ti substrates after day 21 (Figure 2.27). Therefore, following the adhesion and proliferation results along with other osteoblast differentiation markers (alkaline phosphatase activity and calcium deposition), varying the width of the nanopatterns affected osteoblast behavior by altering synthesis of collagen, particularly, with the most synthesized on the 200 mesh nanopatterned Ti surfaces containing the largest nanorough region.
Fourth, unlike results from alkaline phosphatase synthesis and calcium deposition, the non-patterned nanorough Ti substrates did significantly produce more collagen than any of the nanopatterned Ti substrates after 7, 14, and 21 days (Figure 2.27). However, when the nanopatterned Ti substrates were compared to the conventional Ti substrates, it was found that the 200 mesh nanopatterned Ti substrates (nanopatterns with the most nanorough surface area) produced significantly more collagen after day 21. In addition, synthesis of total collagen after day 14 was also noted to be statistically more on the 400 mesh compared to the conventional Ti surfaces (Figure 2.27). These results therefore suggested that the presence of nanopatterns improved osteoblast differentiation, with respect to collagen synthesis, compared to current conventional Ti surfaces.
Figure 2.27. Total collagen synthesis by osteoblasts cultured on the various nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; *p<0.01 compared to the 400 mesh; **p<0.01 compared to the 300 mesh; ***p<0.01 compared to the 200 mesh; #p<0.01 compared to the conventional Ti; ##p<0.05 compared to the 300 mesh; ###p<0.05 compared to the conventional Ti; +p<0.05 compared to the 400 mesh; ++p<0.05 compared to the respective substrates at day 7; +++p<0.1 compared to the respective substrates at day 7; •p<0.1 compared to the respective substrates at day 14; ••p<0.05 compared to the respective substrates at day 14.

When determining the amount of collagen produced per osteoblast on the surfaces of interest to this study (found by dividing total collagen synthesis, Figure 2.27, by total cell number, Figure 2.21), it was found that the synthesis of collagen per cell remained consistent over the culturing time period, a no statistical difference was revealed (the 200 mesh nanopatterned Ti surfaces were an exception) as demonstrated in Figure 2.28. It was also found that the trends observed from total collagen synthesis (more activity on
the 200 mesh nanopatterned Ti surfaces compared to the conventional Ti surfaces and more activity on the 200 mesh nanopatterned Ti surfaces compared to the 300 and 400 mesh nanopatterned Ti surfaces) were comparable to the collagen synthesis per cell on the surfaces (Figure 2.27 and 28). Thus, improved osteoblast collagen synthesis on the nanopatterned Ti surfaces compared to the conventional Ti surfaces (as well as the 200 nanopatterned Ti surfaces compared to the 300 and 400 nanopatterned Ti surfaces) can be linked to enhanced collagen synthesis rather than increased cell number (which was found to be the same for all surfaces examined, Figure 2.21)
Figure 2.28. Collagen synthesis by each individual osteoblast cultured on the various nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces after 7, 14, and 21 days. Values are mean ±SEM; n=4; *p<0.05 compared to the 400 mesh; **p<0.01 compared to the conventional Ti; ***p<0.1 compared to the 400 mesh; #p<0.1 compared to the conventional Ti; ##p<0.01 compared to the 400 mesh; ###p<0.05 compared to the 300 mesh; +p<0.1 compared to the respective substrates on day 7; ++p<0.05 compared to the respective substrates on day 7; +++p<0.01 compared to the respective substrates on day 7.

To qualitatively examine the effect the nanopatterns had on the orientation of the extracellular collagen, collagen was fluorescently stained through an ELISA assay. Interestingly, it was seen that compared to the non-patterned nanorough Ti substrates and the conventional Ti substrates, the extracellular collagen were more aligned (Figure 2.29). Furthermore, upon a closer look, the collagen synthesis was more aligned on the 400 mesh nanopatterned Ti substrates compared to the 200 mesh nanopatterned Ti substrate. From the data examining osteoblast alignment, it was found that the osteoblast aligned
more on the 400 mesh nanopatterned Ti substrates, thus demonstrating that such substrates are better at directing the synthesis of aligned collagen compared to the other nanopatterns where osteoblasts alignment was less. Therefore, by directing osteoblast to align, collagen synthesis can be controlled on nanopatterned Ti surfaces.
Figure 2.29. Fluorescently stained extracellular collagen synthesis on the various nanopatterned Ti surfaces, nanorough Ti surfaces, and conventional Ti surfaces. Qualitatively, directed collagen synthesis was observed on the nanopatterned surfaces, particularly on the 400 mesh substrates (which were observed to have better osteoblast alignment).
2.3.3. Mechanisms for Increased Osteoblast Response

2.3.3.1. Role of Surface Energetics and Wettability

As mentioned in section 1.6.3, surface wettability, surface energy, and surface roughness of biomaterials play a crucial role in enhancing cell adhesion, proliferation, and differentiation. Surface energetics are related to the degree of biological interaction of biomaterials and cells as indicated through a graph relating surface energetics and biocompatibility (Figure 2.30). More specifically, a material that falls in region A would exhibit minimum bioreactivity while the further you go up on the graph, the more biocompatible the material with better adhesive properties [279].

Figure 2.30. Graph revealing the relationship between surface energetics and biocompatibility [279]. A material within region A is considered to possess poor biocompatible properties while a material in region B with higher critical surface tension enhances its properties (such as adhesion, proliferation, and function).
This study has already shown that altering surface roughness alters cell behavior. More specifically, increased surface roughness of a hydrophilic material directly improves osteoblast adhesion, proliferation, and function (such as total protein synthesis, alkaline phosphatase activity, calcium deposition, and collagen synthesis). Increased surface roughness has been found to be linearly associated with surface energy and wettability [234]. In other words, increases in roughness of a hydrophilic material results in a more hydrophilic surface which results in increased surface energy. In this study, surface energy calculations from contact angle data did indeed indicate that increasing surface nanoroughness increased surface energy. The nanorough surfaces, with and without patterns, created through electron beam evaporation had a surface energy significantly higher than that of the conventional regions of the Ti as received from the vendor (Figure 2.31). Such surfaces fall further up on the graph in Figure 2.30 indicating increased bioreactivity and thus increased cell response. It was also found that the nanorough surfaces had lower contact angles for each liquid analyzed (Table 2.4), indicating increased hydrophilicity and, thus, greater wettability compared to the conventional surfaces.
Figure 2.31. Surface energy of the conventional and nanorough Ti surfaces. Surface energy was calculated for each sample by measuring the contact angles of three liquids at the sample surface and entering values into the Owens-Wendt equation. Results indicated higher surface energy for the nanorough Ti surfaces compared to the conventional Ti surfaces. Values are mean ±SEM; n=4; *p<0.01 compared to conventional Ti surfaces.

Table 2.4. Contact angles of three liquids on conventional and nanorough Ti surfaces. Contact angle data was used to determine surface energy via the Owens-Wendt equation.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Contact Angle of DI Water</th>
<th>Contact Angle of Glycerol</th>
<th>Contact Angle of PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional Ti</td>
<td>70.6 ± 1.58</td>
<td>69.3 ± 0.84</td>
<td>41.18 ± 1.20</td>
</tr>
<tr>
<td>Nanorough Ti</td>
<td>59.3 ± 1.13</td>
<td>57.6 ± 0.89</td>
<td>28.3 ± 1.74</td>
</tr>
</tbody>
</table>

2.3.3.2. Role of Protein Adsorption

A key objective of this study was to explain why surfaces possessing more nanorough features (including the nanorough Ti surfaces and the 200 mesh nanopatterned Ti surfaces which contained the most nanorough rough surface area of all the nanopatterned substrates) were more compatible with
osteoblasts compared with surfaces possessing more conventional features. Studies have demonstrated a linear relationship between surface roughness, surface energy, and protein adsorption. More specifically, a surface that is more nanorough has increased surface wettability (more hydrophilic) and corresponding surface energy which influences and increases select protein adsorption [233-235, 280, 281]. This study confirmed the same correlation as it revealed that nanorough Ti substrates (without patterns) created through electron beam evaporation possessed a higher degree of nanometer features, higher surface energy, and increased fibronectin adsorption compared to conventional Ti surfaces as received from the vendor (Figure 2.31). This therefore explains in part why osteoblasts were seen to adhere, proliferate, and differentiate better on the nanorough Ti surfaces compared to the conventional Ti surfaces.

Furthermore, fibronectin adsorption was significantly greater on the 200 and 300 mesh nanopatterned Ti surfaces compared to the 400 mesh nanopatterned Ti surfaces. Since select protein adsorption increases with increasing roughness, this would be expected since the 200 and 300 mesh substrates contained greater nanorough surface areas due to larger hole widths that allows for the deposition of more nanoscale features from electron beam evaporation. This increase in fibronectin adsorption, along with the increase in surface energy and surface roughness of the 200 and 300 mesh
nanopatterned Ti surfaces could explain the observed increased osteoblast adhesion, proliferation, and differentiation on such substrates.

This protein study also revealed that nanorough Ti surfaces contained statistically more fibronectin adsorption compared to all nanopatterned surfaces. This too can be explained by the fact these nanorough Ti surfaces contained the most nanorough surface area, for the entire substrate was modified by electron beam evaporation without the presence of grids, thus, containing nanoscale features everywhere.

Finally, it was seen in the previous sections that osteoblast behavior was better on the 200 and 300 mesh nanopatterned Ti surfaces compared to conventional Ti surfaces which is typical of current orthopedic implant surfaces. These results, like the rest, can be explained by the significantly increased fibronectin adsorption on the 200 and 300 nanopatterned surfaces compared to the conventional Ti surfaces.
Figure 2.32. Fibronectin adsorption on the conventional, nanorough, and nanopatterned Ti surfaces. Values are mean ±SEM; n=4; *p<0.01 compared to the 400 mesh; **p<0.01 compared to the 300 mesh; ***p<0.01 compared to the 200 mesh; #p<0.01 compared to conventional Ti surfaces; ##p<0.05 compared to the 400 mesh; ###p<0.05 compared to conventional Ti.

### 2.4 Conclusions

In summary, the potential of nanopatterned Ti surfaces for improving new bone growth juxtaposed to the implant surface for ITAP was investigated. The motivation behind creating linearly aligned nanopatterned features was inspired by examining the hierarchical structure and organization of natural bone and creating devices that are biomimetic of this nanoscale organization. Collagen Type I, one of the main extracellular matrix components in bone, has nanoscale features and is assembled into aligned fibrils that serve as the
template for hydroxyapatite crystal deposition, another major component in bone. Thus, creating surfaces that possess both nanometer features as well as linear grooves are of interest in promoting osteoblast adhesion, proliferation, and function.

A variety of nanopatterned Ti surfaces with features ranging from 22 to 80 µm were examined. These nanopatterns contained alternating nanorough and conventional regions, thus resulting in surfaces varying in total nanorough surface area. Osteoblast adhesion, proliferation, and differentiation was observed to be the best on the nanopatterned Ti substrates with the largest groove widths (greatest nanorough surface area) compared to the other nanopatterned surfaces as well as the conventional Ti substrates that are similar to current ITAP surfaces. However, when examining the orientation of the cells to the nanopatterns, it was found that the osteoblasts elongated and aligned to the direction of the nanopattern better on the surfaces containing the smallest groove widths (least amount of nanorough surface area).

In addition, the study investigated the underlying mechanism of why surfaces possessing more nanorough features were more compatible with osteoblasts. Results revealed that increasing Ti nanoroughness, surface energy and wettability, fibronectin adsorption can increase. Such proteins
are responsible for mediating cell behavior on an implant surface and can serve as design criteria to improve ITAP efficacy.
Chapter 3

Enhancing Skin Growth on Select Nanotextured Titanium Surfaces

3.1 Introduction

Not only is there a problem with tissue integration at the bone-metal interface, another problematic area of ITAPs is the lack of tissue growth at the skin-metal interface as indicated throughout the introduction. This breaching of the skin at the interface with the implant is due to epithelial (particularly keratinocytes) migration through the dermis rather than adherence throughout the epidermal layers. This results in downgrowth of the skin as a means to regenerate the protective barrier function of the skin (Figure 1.6). The lack of a tight skin seal prevents stabilization of the
abutment of the ITAP, leads to extrusion of the implant, and results in failure of the prosthetic.

Adequate integration between the ITAP surface and the dermal layer require a tight adherence of fibrous tissue in order to avoid the formation of a liquid filled occlusion that contributes to the failure of the implant [282-285]. As mentioned throughout the introduction, fibroblasts are the major cellular component of the fibrous tissue. Therefore, several studies have altered the ITAP surface topography to try and optimize dermal attachment by improving density, morphology, and attachment of fibroblasts [10, 282, 286, 287]. In these in vitro studies, fibroblasts were exposed to a number of titanium (Ti) substrates with varying degrees of micron roughness (specifically, commercially pure Ti, Ti6Al4V, TiTa30, and Ti-6Al-7Nb possessing higher degrees of micron roughness compared to their smooth, electropolished counterparts) [282, 286, 287]. Results indicated that fibroblast behavior decreased on those materials with a higher degree of micron roughness. In particular, fibroblast morphology was less spread, a marker that translates into favorable growth rates. However, in vivo studies using Ti6Al4V screws revealed that the dermal layer attachment was not enhanced by changes in surface roughness [10]. In fact, this in vivo study revealed that only with the addition of a flange to the ITAP design, located immediately below the epithelial layer, was dermal attachment optimized.
However, consistent epithelial (i.e. keratinocyte) attachment, which is considered to be a prerequisite if an ITAP is to be used successfully clinically [11], has not been observed on any surfaces during in vitro or in vivo studies (including the ITAP containing a flange). Early epithelial (i.e. keratinocyte) attachment within the first 24 to 48 hours after implantation is crucial if epithelial downgrowth is to be prevented [11]. Therefore, in order to improve the seal between the skin and the tissue surface, creating implant surfaces to which keratinocytes directly attach, particularly in the initial hours after implantation, is desired. To improve epidermal ingrowth, surfaces with various topographical features have been examined [288, 289]. More specifically, in vivo and in vivo studies examining material surfaces that are porous (carbon with pore diameters 200-250 µm) and grooved (produced by micromachining silicon wafers) indicated that epithelial cells attached more to these surfaces compared to smooth surfaces. Interestingly, one group demonstrated that proliferation, morphology, and attachment of keratinocytes on Ti6Al4V surfaces with various surface topographies (created by machining, polishing, sand-blasting, and acid etching) performed better on the smoother surfaces [11]. More specifically, adhesion was enhanced in addition to a more spread morphology on the smooth surfaces. It was claimed in that study that since spreading of cells is an active process, ridges and troughs present on the Ti6Al4V surfaces provide a physical barrier that slows the progression of migrating cells [11]. In addition, up-regulation of the
intracellular signaling pathways required for focal adhesion and assembly of hemidesmosomes was lacking on these surfaces with higher roughness [11].

Despite these efforts to enhance keratinocyte attachment, further research needs to be conducted. Very little research, if any, has examined keratinocyte response to surfaces containing nanoscale features. As with bone, since the components of skin (such as desmosomes, extracellular cores, collagen, etc.) are in the nanometer regime, it would be expected that surfaces mimicking these features would enhance keratinocyte attachment and, thus, lead to the proper formation of skin at the ITAP implant interface. Since research has failed to examine this important area, it may be ideal to design the surface of the Ti abutment to incorporate nanometer features that will allow for optimal interactions with select proteins and subsequently keratinocytes.

This study therefore examined the effect Ti substrates containing nanometer features have on the adhesion, proliferation, and morphology of keratinocytes as a means to improve skin growth at the implant surface and prevent epidermal downgrowth. In particular, since osteoblast functioned well on the nanorough surfaces created through electron beam evaporation, keratinocytes were also exposed to these identical surfaces made in the Chapter 2. In addition, keratinocytes were also cultured on Ti nanotubular surfaces created through a process known as anodization.
Although not completed in this dissertation, nanotubular Ti surfaces created through anodization has already been shown to enhance osteoblast adhesion and long-term functions (including enhanced alkaline phosphatase activity, synthesis of intracellular collagen, and deposition of calcium-containing minerals) [177, 213, 214, 290, 291]. Specifically, improved osteoblast behavior has been shown on anodized metals (anodized Ti and Ti6Al4V) [213, 214, 290] as well as anodized ceramics (anodized nanoporous alumina) [177]. Additional studies on these anodized Ti surfaces revealed that improved osteoblast response was due to increased nanometer surface roughness as well as increased adsorption of vitronectin and fibronectin compared to unanodized Ti surfaces [213]. Furthermore, anodized Ti rods (inserted in the cancellous bone of the distal femoral epiphysis in rats) were enriched with hydroxyapatite, HA (through a hydrothermal treatment) [291]. These in vitro results revealed that HA grown on the nanotubular Ti surfaces had the highest bone to implant contact when compared to nanotubular Ti enriched with Ca and P [291].

In addition, nanotubular Ti surfaces are also advantageous in that they are good candidates for functionalization with proteins, peptides, or drugs [292-295]. More specifically, nanotubes were loaded with immunoglobulin G for decreasing foreign body responses, bone morphogenetic protein active fragment for promoting new bone growth, and penicillin/streptomycin for antimicrobial properties [294]. That study decreased immunoglobulin G
adsorption and increased adsorption of both bone morphogenetic protein-2 (BMP-2) and penicillin/streptomycin (P/S) on the nanotubular Ti surfaces compared to the unanodized Ti surfaces [294]. The release behavior of each protein was also observed on nanotubular Ti surfaces, and it was found that the drug elution was longer and improved osteoblast density compared to that of the unanodized Ti surfaces [295]. Clearly, the use of anodized nanotubes offers much promise in improving the efficacy of implants, and possibly for enhancing skin growth on the Ti abutment of ITAP.

Thus, the specific objectives of this chapter included the:

- Design and fabrication of Ti substrates with nanorough features created through electron beam evaporation and nanotubular features created through anodization,
- Characterization of the nanotubular Ti substrates using scanning electron microscopy, atomic force microscopy, electron spectroscopy for chemical analysis, and X-ray diffraction (nanorough Ti substrates were characterized in Chapter 2),
- Elucidation of keratinocyte adhesion and proliferation on the nanorough and nanotubular Ti substrates,
- Examination of keratinocyte morphology and spreading on the nanorough and nanotubular Ti substrates,
• Determination of protein adsorption on the nanorough and nanotubular Ti substrates which may explain the results above, and
• Understanding the mechanism for keratinocyte response on the nanorough and nanotubular Ti substrates and the role that nanometer surface roughness, surface wettability, and surface energetics may have in mediating such responses.

3.2 Materials and Methods

3.2.1. Sample Preparation

Similar to section 2.2.1, experiments conducted throughout this chapter involved a series of samples, including (i) conventional Ti substrates as received from the vendor, (ii) nanorough Ti substrates created through electron beam evaporation, and (iii) nanotubular Ti substrates created through anodization. The base material for all sample types were Ti foils (100 x 100 x 1 mm; 99.2% pure; Alfa Aesar, Ward Hill, MA, USA) that were cut into 10 x 10 mm squares using a shear cutter. After cutting into the desired dimensions, all substrates were ultrasonically cleaned with a diluted cleaning solution (Branson; Dabury, CT, USA) for 20 minutes followed by sonication in acetone, 70% ethanol, and deionized water (DI) for 10 minutes. Substrates were then dried in an oven (VWR, Bridgeport, NJ, USA) at 40°C for 15 minutes, or until dry.
3.2.1.1. Titanium Substrates as Received from the Vendor

See Section 2.2.1.1.

3.2.1.2. Electron Beam Evaporated Titanium Substrates

See Section 2.2.1.2.

3.2.1.3. Anodized Titanium Substrates

Anodization involves acid activation and electrolyte anodizing. Acid activation is performed in a mixture of nitric acid (HNO₃) and hydrofluoric acid (HF) to remove surface contaminants and the natural thin Ti oxide layer that forms spontaneously in air. The electrolyte passivation process is carried out in a DC powered electrochemical cell which has a two-electrode configuration – a Ti anode and a platinum cathode. When constant voltage is applied between the anode and cathode, electrode reactions, including oxidation and reduction, in combination with field-driven ion diffusion lead to the growth and formation of an oxide layer on the anode surface (Figure 3.1).
Figure 3.1. Schematic diagram of field-driven ion diffusion during anodization that leads to the formation of an oxide layer on the anode (Ti) surface. (Adapted from [296])

The main chemical reactions that occur during anodization of Ti surfaces include [296]:

at the Ti/Ti oxide interface:

\[ \text{Ti} \leftrightarrow \text{Ti}^{2+} + 2e^- \quad [\text{Eq. 3.1}] \]

at the Ti oxide/electrolyte interface:

\[ 2\text{H}_2\text{O} \leftrightarrow 2\text{O}^2^- + 4\text{H}^+ \quad [\text{Eq.3.2}] \]

\[ 2\text{H}_2\text{O} \leftrightarrow \text{O}_2 + 4\text{H}^+ + 4e^- \quad [\text{Eq. 3.3}] \]

at both interfaces:

\[ \text{Ti}^{2+} + 2\text{O}^2^- \leftrightarrow \text{TiO}_2 + 2e^- \quad [\text{Eq.3.4}] \].

The resulting oxide film properties, such as degree of roughness and surface morphology, is dependent on various parameters, including voltage,
electrolyte composition, and time. Self-ordered nanotubular structures are created using fluorine electrolyte solutions (0.5-1.5 wt%) using a voltage anywhere between 10 and 40 V [297]. The initial pore formation begins when the oxide layer partially dissolves while the unanodized metallic portions remain between these pores (called voids). Both the pores and voids grow as the anodization process continues to form the final nano-tubular structures (Figure 3.2). The substrate metal is not attacked locally during nanotube formation due to the presence of a barrier layer.

Figure 3.2. Schematic illustration of the formation of nanotubes on the Ti surface during anodization in HF: (a) oxide formation, (b) pit formation, (c) pore and void formation, and (d) fully developed into tubes. (Adapted from [296])

In this study, nanotubular Ti substrates were specifically fabricated by first soaking Ti in a dilute acidic mixture of HNO₃ and HF for five minutes (acid activation). The anodization process took place in 1.5 wt% HF for 10 minutes
at a constant voltage of 20 V (Figure 3.3) [213, 214]. These Ti substrates were rinsed with large amounts of DI water immediately after anodization, air dried, and sterilized under ultraviolet light for three hours per substrate side prior to cell culture experiments.

Figure 3.3. Schematic illustration of the anodization process (an electrolytic passivation process used to increase the thickness of the natural oxide layer on metal surfaces) used to create nanotubular surfaces.

3.2.1.4. Reference Material

See section 2.2.1.4.

3.2.2. Sample Characterization

Nanotubular surfaces created by anodization were characterized for roughness, crystallinity, wettability, and surface energetic in the same way the conventional and nanorough Ti surfaces were characterized in Chapter 2. The same techniques used to characterize the conventional Ti surfaces as
received from the vendor and the nanorough Ti surfaces created through electron beam evaporation were also used to characterize the nanotubular Ti surfaces used in this chapter.

3.2.2.1. Scanning Electron Microscopy (SEM)

See section 2.2.2.1.

3.2.2.2. Atomic Force Microscopy (AFM)

See section 2.2.2.2.

3.2.2.3. Electron Spectroscopy for Chemical Analysis (ESCA)

See section 2.2.2.3.

3.2.2.4. X-ray Diffraction (XRD)

See section 2.2.2.4.

3.2.2.5. pH Test

An additional characterization test (not completed in Chapter 2) used was a pH test. Since fluorine from the acidic bath used to anodize the Ti substrates was present in the nanotubes after anodization, the pH of the media could be altered to affect keratinocyte (skin-forming cell) response. Therefore, to determine if fluorine was present in the nanotubes changing the pH of the media that would affect cell responses, a pH test was completed over the
course of five days. For this purpose, ten nanotubular Ti substrates were prepared, of which five of the specimens were dried at room temperature while the other five specimens were dried in a vacuum to determine if either fabrication method influenced the evaporation of fluorine from the nanotubes. Once dry, all specimens were sterilized under UV for a 24 hour time period (12 hours on each side of the Ti specimen). Once sterilization was complete, the specimens were placed into five standard 6-well culture plates with each plate containing a Ti specimen that was air dried and vacuum dried. To each nanotubular Ti specimen, 4 mL of Keratinocyte-Serum Free Medium (KSFM; Invitrogen, Carlsbad, CA, USA) supplemented with 0.05 mg/mL bovine pituitary extract (BPE) and 35 ng/mL epidermal growth factor (EGF) was added and placed under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C). To serve as a control, this media was also added to a single well containing no Ti specimen. The pH of the media was measured using a Delta 320 pH Meter (Mettler-Toledo, Columbus, OH, USA) and recorded at the very beginning of the experiment (Day 0). The pH reading was then taken every 24 hours for the next five days for each nanotubular Ti specimen. The pH of the control was only obtained at the start of the experiment and at the end of the experiment.
3.2.3. Cytocompatibility Assays

3.2.3.1. Cell Culture

Commercially available human keratinocytes (skin-forming cells, CRL-2309, American Type Culture Collection, population number 2-4) were cultured in Keratinocyte-Serum Free Medium (KSFM; Invitrogen) supplemented with 0.05 mg/mL BPE and 35 ng/mL EGF under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C).

3.2.3.2. Cell Adhesion

Keratinocytes were enzymatically lifted from the tissue culture polystyrene flask using 2 mL of a 0.25% (w/v) Trypsin-0.53 mM EDTA solution (Sigma Aldrich, Milwaukee, WI, USA). Keratinocytes were then suspended in KSFM (Invitrogen) in the presence of 0.05 mg/mL BPE and 35 ng/mL EGF and were seeded at a density of 3500 cells/cm² onto the conventional, nanorough, and nanotubular Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C) for four hours. After this prescribed time period, non-adherent keratinocytes were removed by rinsing in phosphate buffered saline (PBS). Keratinocytes adherent on the conventional, nanorough, and nanotubular Ti substrates were fixed with 10% formalin based acetate buffer (Fisher Scientific, Agawam, MA, USA) for ten minutes and then stained with 4',6-diamidino-2-
phenylindole, DAPI dilactate (Sigma Aldrich). The cell nuclei were then visualized and counted in situ using a Leica DM5500 B fluorescence microscope (Bannockburn, IL, USA) with image analysis software captured using a Retiga 4000R camera. Adhesion experiments were run in triplicate and repeated three different times per substrate type. Cell density (cells/cm²) was determined by averaging the number of adherent cells in five random fields per substrate.

3.2.3.3. Cell Proliferation

Keratinocyte proliferation studies were conducted similar to the procedure for keratinocyte adhesion studies. Keratinocytes were enzymatically lifted from the tissue culture polystyrene flask using 2 mL of a 0.25% (w/v) Trypsin-0.53 mM EDTA solution (Sigma Aldrich). Keratinocytes were then suspended in KSFM (Invitrogen) in the presence of 0.05 mg/mL BPE and 35 ng/mL EGF and were seeded at a density of 1500 cells/cm² onto the conventional, nanorough, and nanotubular Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C) for one, three, and five days. The media was changed every other day until the prescribed time elapsed. After this prescribed time period, non-adherent keratinocytes were removed by rinsing in phosphate buffered saline (PBS). Keratinocytes adherent on the conventional, nanorough, and nanotubular Ti substrates were fixed with 10% formalin
based acetate buffer (Fisher Scientific) for ten minutes and then stained with DAPI dilactate (Sigma Aldrich). The cell nuclei were then visualized and counted in situ using a Leica DM5500 B fluorescence microscope with image analysis software captured using a Retiga 4000R camera. Proliferation experiments were run in triplicate and repeated four different times per substrate type. Cell density (cells/cm²) was determined by averaging the number of adherent cells in five random fields per substrate.

3.2.3.4. Cell Morphology

Keratinocyte morphology was first examined by determining the average surface area covered by a single keratinocyte on the varying surfaces to unveil if spreading occurred more on one surface than the other. To calculate the average surface area of each individual keratinocyte, keratinocytes were first enzymatically lifted from the tissue culture polystyrene flask using 2 mL of a 0.25% (w/v) Trypsin-0.53 mM EDTA solution (Sigma Aldrich). Keratinocytes were then suspended in KSFM (Invitrogen) in the presence of 0.05 mg/mL BPE and 35 ng/mL EGF and were seeded per substrate at a density of 3500 cells/cm² for adhesion (four hours) experiments and 1500 cells/cm² for proliferation (three and five days) experiments in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C). The media was changed every other day until the prescribed culturing time period elapsed. After this prescribed
time period, non-adherent keratinocytes were removed by rinsing in phosphate buffered saline (PBS). Keratinocytes adherent on the conventional, nanorough, and nanotubular Ti substrates were fixed with 10% formalin acetate buffer (Fisher Scientific) followed by 0.1% Triton-X-100 (Sigma Aldrich) for ten minutes. The f-actin filaments in the cytoskeleton of the cells were then stained with rhodamine phalloidin (Invitrogen) and visualized in five random fields per substrate using a Leica DM5500 B fluorescence microscope with image analysis software captured using a Retiga 4000R camera. Morphology experiments after four hours was run in triplicate and repeated four different times per substrate type. Morphology proliferation experiments were run in triplicate and repeated three different times per substrate type. Surface area of each cell (µm²) was calculated using Image J software and then averaged over each of the five random fields per substrate.

Keratinocyte morphology on the substrates of interest was also observed using a LEO 1530VP SEM. For this purpose, keratinocytes were first enzymatically lifted from the tissue culture polystyrene flask using 2 mL of a 0.25% (w/v) Trypsin-0.53 mM EDTA solution. Keratinocytes were then suspended in KSFM in the presence of 0.05 mg/mL BPE and 35 ng/mL EGF and were seeded at a density of 7500 cells/cm² onto the conventional, nanorough, and nanotubular Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air
environment at 37°C) for four hours. At the end this time period, the samples were rinsed three times in a 0.1 M sodium cacodylate buffer solution (pH 7.4) to remove any non-adherent keratinocytes. Keratinocytes adherent on the nanotubular, nanorough, and unmodified Ti substrates were fixed with 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer solution (Electron Microscopy Sciences, Hatfield, PA, USA) for 30 minutes at room temperature. After fixation, the samples were rinsed three times in a 0.1 M sodium cacodylate buffer solution. The cells were then dehydrated through a series of ethanol solutions (30, 50, 70, 90, 100, 100, and 100%) for 20 minutes each and subsequently dried via critical point drying (Ladd Research Critical Point Dryer, Williston, VA, USA). The specimens were immersed in liquid CO₂ until there was a complete exchange of liquid CO₂ for the ethanol in the specimens. The specimens were then heated above 34°C under 7.6 MPa, where all liquid CO₂ was converted to gaseous CO₂ until the specimens were dry. Before imaging, all the specimens were sputter-coated with a thin layer of gold-palladium using an ISI PS-2 Coating Unit in a 100 mTorr vacuum argon environment for three minutes and 10 mA of current. SEM images were created using secondary electrons collected with the in-lens detector at a 3 kV acceleration voltage. Magnifications varied according to the distribution of cells.
3.2.4. Mechanisms for Keratinocyte Response

3.2.4.1. Surface Energetics and Wettability

See section 2.2.4.1.

3.2.4.2. Fibronectin Adsorption (ELISA)

See section 2.2.4.2.

3.2.5. Statistics

Data were represented by the mean value with the standard error of the mean (SEM) noted. A one-tailed, paired standard student t-test was used to check statistical significance between means, and p<0.1 was considered statistically significant.

3.3 Results and Discussion

3.3.1. Sample Characterization

3.3.1.1. Scanning Electron Microscopy (SEM)

Low and high scanning electron micrographs of the nanorough and conventional Ti surfaces can be found in section 2.3.1, Figure 2.7. Low and high resolution micrographs of the anodized Ti can be seen in Figure 3.4. The anodized Ti substrates displayed the presence of tube-like structures. As
estimated from the SEM micrographs, the inner diameters of the tubes was approximately 70 to 80 nm in diameter, 200 nm in depth and were uniformly distributed over the entire surface. Thus, throughout the remainder of this dissertation, these Ti substrates will be referred to as nanotubular Ti.

Figure 3.4. SEM micrographs of Ti substrates after anodization. The Ti substrates possessed tube-like features approximately 70 to 80 nm in diameter with a 200 nm depth (and, thus, was termed nanotubular Ti). Scale bar = 3 µm (low resolution), 100 nm (high resolution), and 30 nm (high resolution). SEM micrographs of nanorough Ti and conventional Ti can be found in Figure 2.7.
3.3.1.2. Atomic Force Microscopy (AFM)

Quantitative data, root-mean-square roughness (RMS) and average peak-to-valley roughness (RPV), from AFM further revealed these tube-like surfaces possessed nanometer surface roughness (Figure 3.5). AFM data for the conventional and nanorough Ti surfaces can be found in section 2.3.1, Table 2.2 and Figure 2.9 (RMS and RPV values are relisted here). RMS for the nanotubular Ti surfaces was 12.5 nm and RPV was 30-40 nm (Table 3.1). Thus, when comparing all three surfaces used throughout this section, the nanotubular Ti surfaces had the largest nanometer surface topographies followed by the nanorough Ti surfaces. Table 3.1 summarizes the values found for RMS and RPV of the three sample types used for cell experiments in this chapter. Thus, from the thorough analysis of both SEM and AFM, it can be concluded that during the cell studies (described in the next sections), the keratinocytes will be exposed to surfaces with unique and different surface topographies varying in their degree of nanometer roughness.
Table 3.1. Qualitative data indicated the substrates created by anodization (nanotubular Ti) and electron beam evaporation (nanorough Ti) contained more surface features in the nanometer regime than the Ti surfaces received directly from vendor (conventional Ti). RMS represents root-mean-square roughness while RPV represents peak-to-valley roughness.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RMS (nm)</th>
<th>RPV (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron Beam Evaporated Ti (nanorough topography)</td>
<td>11</td>
<td>30-40</td>
</tr>
<tr>
<td>As Received Ti (conventional topography)</td>
<td>6</td>
<td>10-20</td>
</tr>
<tr>
<td>Anodized Ti (nanotubular topography)</td>
<td>12.5</td>
<td>30-40</td>
</tr>
</tbody>
</table>

Figure 3.5. AFM micrographs and section analyses revealed both qualitative and quantitative topographical nanofeatures on the nanotubular Ti surfaces. Root-mean-square roughness (RMS) and average peak-to-valley roughness (RPV) values can be found in Table 3.1. AFM micrographs for the nanorough Ti and conventional Ti surfaces can be found in Figure 2.9.
3.3.1.3. Electron Spectroscopy for Chemical Analysis (ESCA)

As described in section 2.3.1.3, because it is well-known that alterations in chemistry and/or crystal structures can influence cell function, each of these must be investigated in order to determine which elements may explain the response of keratinocytes observed in the next section.

ESCA, already completed on the nanorough and conventional Ti surfaces (section 2.3.1, Figure 2.10), was performed on the nanotubular Ti surfaces in order to determine if there was a difference in the surface oxide composition. One high resolution ESCA spot was taken on the nanotubular Ti surface, and similar to the conventional and nanorough Ti surface (Figure 2.10), revealed only one identifiable peak, Ti 2p (Figure 3.6). When further examining the outermost layers of the nanotubular Ti surfaces, they mostly contained O and Ti like the nanorough and conventional Ti surface (Table 2.3, Figure 2.10; values are relisted here). However, in addition to O and Ti, a trace amount of F was found in the nanotubular Ti surfaces most likely due to the anodization technique involving the use of HF (Table 3.2).
Table 3.2. Atomic percentage of selective elements in the outermost layers of Ti as received from vendor (conventional Ti), after electron beam evaporation (nanorough Ti), and after anodization (nanotubular Ti) as examined by ESCA.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>O (%)</th>
<th>Ti (%)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanorough Ti</td>
<td>49.34</td>
<td>50.66</td>
<td>0</td>
</tr>
<tr>
<td>Conventional Ti</td>
<td>51.23</td>
<td>48.77</td>
<td>0</td>
</tr>
<tr>
<td>Nanotubular Ti</td>
<td>57.00</td>
<td>33.62</td>
<td>9.38</td>
</tr>
</tbody>
</table>

Figure 3.6. (a) ESCA spectra of elements present in the outermost layer of the nanotubular Ti surfaces (Table 3.2). (b) Binding energy of the high resolution Ti 2p peak for the nanotubular Ti surfaces. Binding energies matched those of the conventional and nanorough Ti surfaces (Figure 2.10).
Although trace amounts of fluorine were found on the surface of the nanotubular Ti specimens, as indicated by ESCA measurements, results from the pH test provided evidence that the presence of residual HF from the acid bath during anodization did not have an effect on the pH of the cell culture media. More specifically, the pH of the media containing the nanotubular specimens remained roughly the same over the course of five days and was almost identical to the pH of the control media at the end of these five days (Table 3.3). In addition, the drying method used for the nanotubular Ti specimens did not affect the evaporation rate of residual HF from the nanotubes, since the pH remained the same for both methods over the course of five days (Table 3.3).

Table 3.3. pH readings of nanotubular Ti specimens soaked in cell culture media (KSFM) over a period of five days in order to determine if the effect of fluorine present on the nanotubular Ti specimens influenced cell culture pH.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>Vacuum Dry</td>
<td></td>
</tr>
<tr>
<td>(nanotubular Ti)</td>
<td>7.43</td>
</tr>
<tr>
<td>Air Dry</td>
<td></td>
</tr>
<tr>
<td>(nanotubular Ti)</td>
<td>7.43</td>
</tr>
<tr>
<td>Control (media only)</td>
<td>7.43</td>
</tr>
</tbody>
</table>
3.3.1.4. X-ray Diffraction (XRD)

Finally, this study determined whether or not there were differences in the phases present on the nanotubular Ti substrates surfaces. XRD spectra for the nanorough and conventional Ti surfaces can be found in section 2.3.1, Figure 2.11. XRD spectra confirmed that all substrates, including the nanorough, conventional, and nanotubular Ti substrates, possessed traces of Ti indicating no phase difference (Figure 3.7).

Figure 3.7. XRD spectra of nanotubular Ti surfaces after anodization. Spectra, when compared to Ti and titania (anatase and rutile) standards, indicated the presence of amorphous TiO$_2$. XRD spectra for the nanorough Ti and conventional Ti surfaces can be found in Figure 2.11 (which contained crystalline TiO$_2$). (Adapted from [298])
3.3.2. Keratinocyte Response

3.3.2.1. Adhesion

Results of this study provided the first evidence that creating Ti surfaces with nanoscale features has a positive influence on keratinocyte adhesion. More specifically, results indicated that keratinocyte adhesion was the highest on the modified Ti substrates containing the nanotubes (anodization) and nanorough coating (electron beam evaporation) compared to the conventional Ti substrates (Figure 3.8). Keratinocyte adhesion was significantly greater on nanotubular Ti and nanorough Ti compared to the conventional Ti (p<0.01) after four hours. No significant difference was observed between the nanotubular and nanorough Ti substrates. Fluorescently stained cell nuclei on the various substrates are shown in Figure 3.9.
Figure 3.8. Keratinocyte adhesion on nanotubular Ti created by anodization, nanorough Ti created by electron beam evaporation, and conventional Ti as received from the vendor after four hours. Values are mean ±SEM; n=3; *p<0.01 compared to the conventional Ti surface; **p<0.01 compared to the etched glass; ***p<0.05 compared to the etched glass.
Figure 3.9. Fluorescent micrographs indicating keratinocyte adhesion properties on the (a) nanotubular Ti surfaces, (b) nanorough Ti surfaces, and (c) conventional Ti surfaces after four hours. Scale bar = 100 µm.
It can be concluded that increased surface roughness promoted keratinocyte adhesion due to the fact these nanometer surfaces possessed biologically-inspired nanometer features of skin, similar to previous studies with osteoblasts demonstrated in earlier chapters. In order to obtain a better understanding of the significance surface topography plays in keratinocyte response, it was necessary to eliminate the influence that surface chemistry can have. More specifically, TiO$_2$ was the only form of Ti oxide present on these surfaces (Figure 2.10, 3.6).

This study provided further evidence that elemental compositions were similar for all substrates (conventional, nanorough, and nanotubular Ti surfaces) although, importantly, the nanotubular Ti had low fluorine content (not present in the other samples) due to the acidic (HNO$_3$ and HF) treatment (Table 3.2). However, upon further investigation, the low amounts of residual HF from the acidic bath did not have an effect on the pH of the cell culture media (Table 3.3). Had the residual HF been a significant factor in the pH of the culture media, conditions not suitable for supporting cell growth would have resulted. It is therefore important to note that although this study demonstrated increased keratinocyte adhesion on Ti metals processed using two different procedures (anodization and electron beam evaporation) with a slight variation in chemistry, the same promising net positive effect on keratinocyte adhesion resulted between the nanotextured Ti surfaces compared to the conventional Ti surfaces.
Therefore, the present results provided strong evidence that surface topography resulting from electron beam evaporation and anodization was a major factor that influenced keratinocyte adhesion. Since epithelial attachment (i.e. keratinocyte attachment) is a mandatory condition for the clinical use of ITAP (especially within the first 24 to 48 hours), this study revealed two promising nanofabrication techniques that may be applied to the Ti abutment of an ITAP as a means of preventing epithelial downgrowth by enhancing keratinocyte attachment. Unlike previous studies described throughout the introduction of this chapter, this study was the first completed that examined keratinocyte behavior on surfaces possessing roughness in the nanometer regime [11, 288, 289]. These studies only examined surfaces with varying micron roughness values and in fact demonstrated improved keratinocyte adhesion on surfaces with smooth features (not those with more rough features) [11]. However, based on the result from this study, when features were created at the nanostructure level, keratinocytes attached better to these surfaces when compared to surfaces that were less rough.

3.3.2.2. Proliferation

Unlike what was observed from keratinocyte adhesion data, over the course of one, three, and five days, results revealed that keratinocyte proliferation was significantly higher on the nanorough Ti substrates (compared to the
nanotubular Ti substrates (p<0.01, Figure 3.10). There still remained a significant difference in keratinocyte proliferation between the nanorough Ti and the conventional Ti (p<0.01 for one and three days and p<0.05 for 5 days, Figure 3.10). Results further demonstrated that conventional Ti promoted keratinocyte proliferation compared to the nanotubular Ti (p<0.1, Figure 3.10). Thus, as time progressed, despite having nanoscale features, keratinocytes were not highly compatible on the nanotubular Ti surfaces. They were however, like osteoblasts, extremely compatible on the nanorough Ti surfaces created through electron beam evaporation still implying the importance surface nanoroughness has on cell behavior due to their biomimetic nature.
3.3.2.3. Morphology

In addition to adhesion and proliferation studies, keratinocyte morphology was also examined on the nanotubular, nanorough, and conventional Ti surfaces. Results of this study also indicated differences in the overall morphology of keratinocytes on the nanotubular and nanorough Ti substrates compared to the conventional Ti substrates after four hours. Keratinocytes were more spread on the nanotubular and nanorough Ti substrates compared to those on the conventional Ti substrates (p<0.01) and, thus, generally, had greater total surface area coverage (Figure 3.11). More specifically, results indicated that on average, the surface area of keratinocytes was 786 µm² and...
792 \( \mu m^2 \) on the nanotubular Ti surfaces and nanorough Ti surfaces, respectively, while the average surface area of keratinocytes on conventional Ti surface was 721 \( \mu m^2 \). SEM images showing keratinocyte morphology (in particular spreading) on the nanotubular, nanorough, and conventional Ti surfaces after four hours is shown in Figure 3.12. Unlike what was observed after four hours, keratinocyte spreading improved on the nanorough Ti substrates compared to the nanotubular Ti substrates after three and five days (\( p<0.01; \) Figure 3.13). There was still a significant difference in keratinocyte spreading between the nanorough Ti and the conventional Ti (\( p<0.01 \)). Results further demonstrated that the conventional Ti surfaces promoted spreading compared to the nanotubular Ti surfaces (\( p<0.05 \) for day five and \( p<0.01 \) for day five). Thus, morphology data also confirmed that keratinocytes do not seem to be as compatible on the nanotubular Ti surfaces compared to the nanorough Ti surfaces despite the fact they both contain features in the nanometer regime. However, as stated from the proliferation data, keratinocytes still preferred the nanofabricated surfaces created through electron beam evaporation, confirming the importance increased surface roughness has on cell behavior. Fluorescently stained f-actin filaments indicative of cell spreading on the nanotubular, nanorough, and conventional Ti surfaces at day five is shown in Figure 3.14.

Although further studies need to be completed to determine why keratinocytes did not perform as well on the nanotubular Ti surface as time
progressed, such results may be explained by decreased surface area on the nanotubular surface. More specifically, the surface area of the nanotubes consists of the outer thin walls created during anodization. As a result, there is little surface for the keratinocyte filopodia to navigate thus resulting in a less spread morphology compared to nanorough Ti (with a larger surface area) where keratinocytes were more spread. It has been shown that cell morphology has a regulatory effect on proliferation, with flattened, more spread cells having improved proliferation properties while those with a more round, less spread morphology have limited proliferation capacity [264]. Thus, a more spread keratinocyte morphology translates to favorable growth rates as indicated by the proliferation results where keratinocyte morphology and proliferation was enhanced on the nanorough Ti surfaces but stunted on the nanotubular Ti surfaces. Compared to this research, other studies, as previously indicated, revealed that smooth-polished surfaces supported monolayers of keratinocytes with a flatter, more round morphology compared with surfaces that possessed an increased surface roughness [11]. However, based on the data revealed by proliferation and morphology in this study, surfaces with increased nanoroughness enhanced keratinocyte behavior thereby suggesting that materials biomimetic of natural skin can enhance their performance and improve tissue integration with the ITAP.
Figure 3.11. Average surface area of keratinocytes (indicative of cell spreading) on the nanotubular Ti, nanorough Ti, and conventional Ti surfaces after four hours. Values are mean ±SEM; n=3; *p<0.05 compared to conventional Ti surfaces.
Figure 3.12. SEM images of keratinocytes adhered on the (a-d) nanotubular Ti, (e-h) nanorough Ti, and (i-k) unmodified Ti after four hours of culture. Keratinocytes on the nanofabricated Ti substrates (nanotubular and nanorough Ti surface) were flatter and more spread with more defined filopodia. Keratinocytes on the conventional Ti substrates were more round (decreased spreading) with less directed filopodia. Scale bars = (a,i) 10 µm, (e) 20 µm, (b,f,j,k) 2 µm, (c,g) 1 µm, and (d,h) 200 nm.
Figure 3.13. Average surface area of keratinocytes (indicative of cell spreading) on the nanotubular Ti, nanorough Ti, and conventional Ti surface after three and five days. Values are mean ±SEM; n=3; *p<0.01 compared to nanotubular Ti surfaces; **p<0.01 compared to conventional Ti surfaces; ***p<0.05 compared to nanotubular Ti surface.
Figure 3.14. Fluorescent micrographs of the f-actin filaments in the cytoskeleton of the keratinocytes demonstrating cell spreading on the (a) nanotubular Ti, (b) nanorough Ti, and (c) conventional Ti surfaces. Scale bar = 100 µm.
3.3.3. Mechanisms for Increased Keratinocyte Response

3.3.3.1. Role of Surface Energetics and Wettability

As mentioned numerous times throughout this dissertation, surface wettability, surface energy, and surface roughness of biomaterials, such as the ITAP, play a crucial role in enhancing cell adhesion, proliferation, and differentiation. Like Chapter 2, surface energy calculations from contact angle measurements indicated that greater surface roughness at the nanoscale correlated to increased surface energy. Specifically, nanotubular and nanorough Ti surfaces had a surface energy significantly higher than that of conventional Ti surfaces (Figure 3.15; Table 3.4).

![Figure 3.15](image)

Figure 3.15. Surface energy of the conventional, nanorough Ti, and nanotubular Ti surfaces. Surface energy was calculated for each sample by measuring the contact angles of three liquids at the sample surface and entering values into the Owens-Wendt equation. Results indicated greater surface roughness at the nanoscale correlated to increased surface energy. Values are mean ±SEM; n=4; *p<0.01 compared to conventional Ti surfaces; **p<0.01 compared to nanorough Ti.
Table 3.4. Contact angles of three liquids on conventional Ti, nanorough Ti, and nanotubular Ti surfaces. Contact angle data was used to determine surface energy via Owens-Wendt equation.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Contact Angle of DI Water</th>
<th>Contact Angle of Glycerol</th>
<th>Contact Angle of PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional Ti</td>
<td>70.6 ± 1.58</td>
<td>69.3 ± 0.84</td>
<td>41.18 ± 1.20</td>
</tr>
<tr>
<td>Nanorough Ti</td>
<td>59.3 ± 1.13</td>
<td>57.6 ± 0.89</td>
<td>28.3 ± 1.74</td>
</tr>
<tr>
<td>Nanotubular Ti</td>
<td>26.5 ± 2.40</td>
<td>21.9 ± 1.32</td>
<td>11.1 ± 0.80</td>
</tr>
</tbody>
</table>

3.3.3.2. Role of Protein Adsorption

As already thoroughly described and seen in Chapter 2, greater nanoscale roughness leads to more surface area and more reactive sites to influence initial protein interactions to control subsequent cellular adhesion. More specifically, the initial adsorbed fibronectin concentration was shown to increase on the nanotextured Ti surfaces due to the greater presence of surface defects, and consequently higher surface energy as shown above (Figure 3.16). More specifically, fibronectin adsorption was statistically higher on the nanostructured surfaces (nanotubular and nanorough Ti surfaces) compared to the conventional Ti surfaces. It was also found that the nanotubular Ti surfaces had significantly more fibronectin adsorption than the nanorough Ti surface. It has been demonstrated that in the presence of fibronectin, keratinocyte attachment and spreading was enhanced [299]. Such results indicate the possible importance of fibronectin in mediating keratinocyte behavior, especially during wound repair after the
implantation of the Ti abutment of an ITAP. Furthermore, when fibronectin
was covalently coupled to Ti surfaces through silanization, keratinocyte
adhesion improved [300]. As a result, because fibronectin adsorption was
enhanced on the nanorough Ti surfaces, keratinocyte behavior was enhanced
suggesting that altering the surfaces of Ti abutments through electron beam
evaporation can improve the integration between the skin and the implant
surface, thus, restoring the epithelial seal and improving ITAP success.

Figure 3.16. Fibronectin adsorption on the conventional, nanorough, and
nanotubular Ti surfaces. Values are mean ±SEM; n=4; *p<0.01 compared to
conventional Ti surfaces; **p<0.05 compared to nanorough Ti surfaces.
3.4 Conclusions

In summary, there is a high need for investigating ways to improve keratinocyte attachment to the abutment of ITAPs. Currently, rather than attaching, keratinocytes migrate downward into the dermis that results in breaching of the skin at the implant interface. This lack of a tight skin seal leads to instability of the abutment preventing the amputee from properly being able to use the prosthetic. Minimal research has been completed to improve this area, particularly examining the effect surface topographies with nanoscale dimensions can have on keratinocytes since the components of skin are nanometer in size. Developing surfaces with biologically inspired nanometer features has already shown to be a success so creating surfaces that mimic the natural environment of skin could enhance keratinocyte function.

Increased keratinocyte adhesion was seen on two different types of nanostructured surfaces (nanotubular surfaces created from anodization and nanorough surfaces created from electron beam evaporation) compared to conventional Ti surfaces. In addition, surface area of an average keratinocyte was larger on these nanostructured surfaces indicating there was more spreading, another parameter that measures their behavior. The proliferation rate of keratinocytes was also enhanced on the nanorough Ti surfaces created from electron beam evaporation (a similar trend seen with
osteoblasts in Chapter 2). However, the nanotubular Ti surfaces were not as compatible.

In addition, this study investigated the underlying mechanism of why Ti surfaces possessing more nanorough features were more compatible with keratinocytes than those with conventional, micron rough features. Results revealed that with increasing roughness of a hydrophilic material, surface energy and wettability of the surfaces were enhanced which promoted the adsorption of fibronectin. This in turn influenced keratinocyte behavior which could result in a better skin seal at the implant interface improving the overall efficiency of the ITAP.
Chapter 4

Functionalization of Titanium Surfaces with Fibroblast Growth Factor-2 for Improving Keratinocyte Function

4.1 Introduction

Chemical modification of titanium (Ti) surfaces is becoming common for overcoming numerous problems associated with currently used Ti specifically by attaching peptides, proteins, and antibiotics. For example, coupling gentamicin and penicillin to Ti showed a significant reduction in the Staphylococcus aureus while coupling with collagen promoted osteoblast adhesion and proliferation [301]. In this study, a trichlorosilane coupling agent was immobilized on the oxidized Ti surface followed by coupling of
gentamicin, penicillin, or collagen via carbodiimide chemistry. In addition, hydroxyapatite nanoparticles have been functionalized with the cell adhesive arginine-glycine-aspartic (Arg-Gly-Asp) acid (RGD) peptide sequence, known to mediate cell attachment [302], which resulted in increased osteoblast adhesion by approximately 75% [303]. This study used a three step reaction procedure involving silanization, cross-linking using maleimide chemistry, and peptide immobilization. Also, when fibronectin was chemically coupled to Ti silanized surfaces, fibroblasts had significantly larger cell areas and more focal contact markers (markers indicative for improved cell attachment) thereby potentially preventing epithelial downgrowth on ITAP [304]. These biological molecules cannot be merely adsorbed onto the Ti surface because once the device is implanted into the body, such molecules will be displaced leading to diffusion from the implant site and may be distributed systematically resulting in detrimental consequences [305]. In addition, the conformation of proteins simply adsorbed to the surfaces may be altered or denatured [306-310], thus changing their bioactivity [311, 312]. Therefore, to solve these limitations, chemical modification processes, as previously described, have been used and commonly involve a three step reaction: silanization, usually with 3-aminopropyltriethoxysilane (APTES), addition of a cross-linker through maleimide chemistry, and finally immobilization of the peptide and/or protein. Due to the two functional groups at each end (silane and amino groups), APTES is the most commonly used chemical. With the
presence of these two functional groups, the silane group is covalently attached to the Ti oxide surface while the amino groups are used to couple biological molecules at the opposite end.

Chemical functionalization may be ideal for the Ti surfaces possessing nanotubes since these features did not enhance keratinocyte proliferation and migration or decreased bacteria adhesion as indicated in Chapter 3 and 4. Determining the biological molecules to attach to Ti to specifically increase keratinocyte function was found by examining how the body naturally promotes keratinocyte proliferation and migration. Human fibroblasts growth factors (FGF) are heparin-binding proteins that participate in an array of processes, including skin development, by mediating cell proliferation, survival, migration, or differentiation. There are 22 members of the FGF family in humans, of which FGF-1 through FGF-10 bind to fibroblasts growth factor receptors (FGFRs) on cell membranes. In addition, keratinocyte growth factor receptor (KGFR) is a tyrosine kinase protein only expressed in epithelial cells that belong to the family of FGFRs and in particular is a splice variant of FGFR-2 [313]. KGFR specifically binds to keratinocyte growth factor (KGF/FGF7), which has been shown to stimulate the migration of keratinocytes as well as proliferation [314-317]. One study indicated that when FGFR was blocked, organization of epidermal keratinocytes was disrupted in addition to epidermal hyperthickening [318]. More specifically, studies have tested the effect of basic fibroblast growth
factor (also known as FGF-2) on keratinocyte behavior [319, 320]. It was found that keratinocyte cultures were enhanced and stimulated in the presence of FGF-2 compared to cultures that lacked FGF-2 [319, 320]. Because these findings indicate that FGF-2 is a crucial growth factor for keratinocyte, this study attached FGF-2 to the surface of the Ti abutment in an ITAP device as an alternative means to enhance skin growth at the tissue-implant interface.

Thus, the specific objectives of this chapter included the:

- Functionalization of electron beam evaporated and anodized Ti surfaces with FGF-2,
- Characterization of the FGF-2 functionalized Ti surfaces created using electron beam evaporation and anodization, and
- Completion of keratinocyte studies after 24 hours.

4.2 Materials and Methods

4.2.1. Sample Preparation

Experiments conducted throughout this chapter involved a series of samples, including conventional Ti substrates, nanorough Ti substrates, and nanotubular Ti substrates. As in Chapter 2, all sample types were created from Ti foils (100 x 100 x 1 mm; 99.2% pure; Alfa Aesar, Ward Hill, MA, USA) that were cut into 10 x 10 mm squares using a shear cutter. After
cutting into the desired dimensions, all substrates were ultrasonically cleaned with a diluted cleaning solution (Branson, Dabury, CT, USA) for 20 minutes followed by sonication in acetone, 70% ethanol, and deionized water (DI) for 10 minutes. Substrates were then dried in an oven (VWR, Bridgeport, NJ, USA) at 40°C for 15 minutes, or until dry.

4.2.1.1. Titanium Substrates as Received from the Vendor

See Section 2.2.1.1.

4.2.1.2. Electron Beam Evaporated Titanium Substrates

See Section 2.2.1.2.

4.2.1.3. Anodized Titanium Substrates

See section 3.2.1.3.

4.2.1.4. Titanium Surface Functionalization

The functionalization of FGF-2 involved a three step reaction: silanization with 3-aminopropyltriethoxysilane (APTES; Sigma Aldrich, Milwaukee, WI, USA), cross-linking with N-succinimidyl-3-maleimido propionate (SMP; Sigma Aldrich), and finally FGF-2 immobilization (Millipore, Billerica, MA, USA). The schematic of this three step reaction can be found in Figure 4.1.
4.2.1.4.1. Silanization

Grafting of the aminofunctional organosilane (APTES; Sigma Aldrich) onto the surface of Ti substrates was the first step. Silanization was performed by heating Ti substrates at 120ºC for three hours in refluxing toluene (30 mL) containing 0.5 mL (2.15 mmol) APTES. After the reaction was complete, the substrates were rinsed thoroughly in DI water (ten washes at five minutes each under constant agitation).

4.2.1.4.2. Cross-linker

N-succinimidyl-3-maleimido propionate (SMP; Sigma Aldrich) was used as a hetero-bifunctional cross-linking agent between the amino group of the APTES and the actual biological molecule, in this case FGF-2. SMP, once attached to the amino group, exposed maleimide groups that are perfect targets for the attachment of biological molecules. Coupling of the SMP was completed by soaking the samples in a 28mM solution of SMP in N, N-dimethylformamide (DMF) for two hours under dry conditions. After this reaction was complete, substrates were rinsed five times in DMF and then times times in sterile DI water. To prevent hydrolysis, the next reaction (addition of FGF-2) was immediately completed.
4.2.1.4.3. Biological Molecule

Couple of FGF-2 to the maleimide groups was completed by soaking the Ti substrates in a 0.001 mM solution of FGF-2 (Millipore) diluted in sterile PBS (pH 7.4) for two hours. Once the reaction was complete, samples were rinsed three times in sterile DI water.

![Reaction schematic](image)

Figure 4.1. Reaction schematic for functionalizing FGF-2 on to nanotubular Ti substrates (created through anodization), electron beam evaporated Ti substrates (created through electron beam evaporation), and conventional Ti substrates (as received from the vendor).

4.2.2. Sample Characterization

Functionalized conventional, nanorough, and nanotubular Ti surfaces were characterized by fluorescence microscopy and scanning electron microscopy after each reaction (silanization, addition of cross-linker, and addition of FGF-2).
4.2.2.1. Fluorescence Microscopy

A novel 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) fluorescence technique was used to characterize the chemically functionalized Ti surfaces (Molecular Probes, Eugene, OR, USA) following manufacturer's instructions. A 40 mM CBQCA reagent solution was prepared by dissolving the reagent in DMSO. Potassium cyanide was dissolved in distilled water to give a 20 mM solution. Each of these solutions was diluted to 10mM using PBS (pH 9.3). The Ti surfaces were then exposed to a solution of PBS (pH 9.3), 10 mM CBQCA, and 10mM potassium cyanide for one hour at room temperature with constant shaking. CBQCA in its natural state is a non-fluorescence molecule, but upon reaction with amine groups in the presence of cyanide molecules, CBQCA fluoresces (Figure 4.2). Functionalized Ti surfaces with and without the addition of CBQCA was examined using a Leica DM5500 B fluorescence microscope (Bannockburn, IL, USA) with image analysis software captured using a Retiga 4000R camera.
Figure 4.2. Reaction demonstrating how CBQCA transforms from a non-fluorescence molecule into a fluorescence molecule when reacting with amine groups in the presence of cyanide molecules.

4.2.2.2. Scanning Electron Microscopy (SEM)

See section 2.2.2.1.

4.2.3. Cytocompatibility Assays

4.2.3.1. Cell Culture

See section 3.2.3.1.

4.2.3.2. Cell Proliferation

Keratinocytes were enzymatically lifted from the tissue culture polystyrene flask using 2 mL of a 0.25% (w/v) Trypsin-0.53 mM EDTA solution (Sigma Aldrich). Keratinocytes were then suspended in Keratinocyte-Serum Free Medium (KSFM; Invitrogen, Carlsbad, CA, USA) in the presence of 0.05 mg/mL bovine pituitary extract (BPE) and 35 ng/mL epidermal growth factor (EGF) and were seeded at a density of 1500 cells/cm² onto the conventional,
nanorough, and nanotubular Ti substrates as well as the functionalized conventional, nanorough, and nanotubular Ti substrates in a standard 24-well culture plate under standard cell culture conditions (5% CO₂/95% humidified air environment at 37°C) for one day. After this prescribed time period, non-adherent keratinocytes were removed by rinsing in phosphate buffered saline (PBS). Keratinocytes adherent on the conventional, nanorough, and nanotubular Ti substrates were fixed with 10% formalin based acetate buffer (Fisher Scientific) for ten minutes and then stained with DAPI dilactate (Sigma Aldrich). The cell nuclei were then visualized and counted in situ using a Leica DM5500 B fluorescence microscope with image analysis software captured using a Retiga 4000R camera. Proliferation experiments were run in duplicate and repeated three different times per substrate type. Cell density (cells/cm²) was determined by averaging the number of adherent cells in five random fields per substrate.

4.2.4. Statistics

Data were represented by the mean value with the standard error of the mean (SEM) noted. A one-tailed, paired standard student t-test was used to check statistical significance between means, and p<0.1 was considered statistically significant.
4.3 Results and Discussion

4.3.1. Sample Characterization

4.3.1.1. Fluorescence Microscopy

Characterization by fluorescence microscopy (CBQCA) demonstrated the ability to functionalize the conventional, nanorough, and nanotubular Ti substrates with FG2. Substrates were first examined after silanization to determine if the amino groups from the APTES were functionalized on the surfaces. Specifically, in the absence of CBQCA, APTES treated substrates did not fluoresce (Figure 4.3). In contrast, in the presence of CBQCA, the same materials fluoresced indicating that the CBQCA reacted with the amino groups present on the surface (Figure 4.3). Qualitatively, the nanofabricated surfaces fluoresced more than conventional Ti surfaces functionalized with APTES. Substrates were then examined after adding the maleimide cross-linker, SMP. Unlike the silanized surfaces, SMP treated surfaces did not fluoresce in the presence (or absence) of CBCQA indicating that the exposed amine groups present after silanization were completely covered by the SMP moiety (Figure 4.4). Finally, the FGF-2 functionalized Ti surfaces produced excellent fluorescence (Figure 4.5) indicating the presence of the FGF-2 moiety on each Ti surface via the CBQCA reaction with a terminal amine group (at the N-terminus). When in the absence of CBQCA, there was no
fluorescence observed on the FGF-2 functionalized Ti surface (Figure 4.5). Similar to the silanized surfaces, qualitatively, the intensity of fluorescence was more on the nanorough and nanotubular FGF-2 functionalized Ti surfaces compared to the FGF-2 functionalized conventional Ti surfaces.
Figure 4.3. Fluorescent micrographs of the chemically functioned nanotubular, nanorough, and conventional Ti substrate after step 1 of the process (addition of APTES) with and without CBQCA. Scale bar = 50 µm.
Figure 4.4. Fluorescent micrographs of the chemically functioned nanotubular, nanorough, and conventional Ti substrate after step 2 of the process (addition of maleimide cross-linker) with and without CBQCA. Amine groups present after silanization were completely covered by the SMP moiety thus nothing was found to fluorescence. Scale bar = 50 µm.
Figure 4.5. Fluorescent micrographs of the chemically functioned nanotubular, nanorough, and conventional Ti substrate after step 3 of the process (addition of FGF-2) with and without CBQCA. Scale bar = 50 µm.
4.3.1.2. Scanning Electron Microscopy (SEM)

SEM images of the nanotubular, nanorough, and conventional Ti substrates were also taken after each time point (silanization, addition of cross-linker, and addition of FGF-2) during the chemical functionalization process. These images also qualitatively revealed that the presence of silane, maleimide, and FGF-2 did not cause an overt change in surface structure, but the presence of certain features indicated they were present on the surfaces after each step (Figure 4.6). Specifically, the walls of the nanotubes were seen to thicken compared to their original, non-functionalized state (seen in Figure 3.4) while the surface of the nanorough and conventional Ti surfaces possessed particulates as well as regions of a black hue compared to their original non-functionalized state (seen in Figure 2.7).
APTES Functionalized Ti

Nanotubular Ti

Low Magnification, 10 K

High Magnification, 50 K

(a)
APTES Functionalized Ti + SMP

Nanotubular Ti

Low Magnification, 10 K

High Magnification, 50 K

(b)
Figure 4.6. SEM micrographs of the nanotubular, nanorough, and conventional Ti surfaces after (a) silanization, (b) addition of maleimide cross-linker, and (c) addition of FGF-2. Scale bar = 1 µm (low magnification) and 200 nm (high magnification).
4.3.2. Keratinocyte Proliferation

Results of this study provided evidence of increased keratinocyte proliferation after 24 hours on nanotubular, nanorough, and conventional Ti surfaces coupled with FGF-2 compared to their non-functionalized counterparts (Figure 4.7). Results also confirmed those shown in Chapter 3 whereby non-functionalized nanorough Ti substrates (created by electron beam evaporation) promoted keratinocyte proliferation better than the nanotubular Ti substrates as well as conventional Ti substrates. In addition, this study indicated that the non-functionalized conventional Ti increased keratinocyte proliferation compared to nanotubular Ti surface, a trend also shown in Chapter 3. It was this result that motivated the functionalization of these surfaces. Interestingly, when the nanotubular Ti substrates were immobilized with FGF-2, they contained significantly higher (p<0.05) keratinocytes compared to the non-functionalized conventional Ti surfaces (Figure 4.7). Also revealed from this data was that similar to the non-functionalized Ti surfaces, nanorough Ti surfaces functionalized with FGF-2 contained significantly higher (p<0.01) keratinocyte densities compared to the functionalized conventional Ti surfaces, while no significant difference was found between the functionalized nanorough and nanotubular Ti surfaces. Fluorescently stained cell nuclei on the various substrates are shown in Figure 4.8. Although this is the first study to examine the effect
that FGF-2 functionalized Ti surfaces has on keratinocyte proliferation, such results confirm those studies who have examined functionalized Ti surfaces on osteoblast adhesion using similar chemical functionalization processes [292, 301, 303]. More specifically, nanotubular Ti surfaces functionalized with RGD or bone morphogenetic protein-2 (BMP-2) enhanced osteoblast adhesion. Thus, chemical modification of Ti surfaces holds much promise of improving the Ti properties for enhancing cell behavior.

Figure 4.7. Keratinocyte densities on the nanotubular Ti, nanorough Ti, and conventional Ti surfaces as well the functionalized nanotubular Ti, nanorough Ti, and conventional Ti surfaces with FGF-2 after 24 hours. Values are mean ±SEM; n=3; *p<0.01 compared to the nanotubular Ti; **p<0.05 compared to the conventional Ti; ***p<0.1 compared to the nanorough Ti; #p <0.01 compared to the conventional Ti; ##p <0.01 compared to the functionalized conventional Ti; ###p<0.1 compared to the conventional Ti.
Figure 4.8. Fluorescent micrographs indicating keratinocyte proliferation (after 24 hours) properties on the nanotubular Ti surfaces, nanorough Ti surface, and conventional Ti surface as well as the functionalized nanotubular Ti surfaces, nanorough Ti surface, and conventional Ti surface with FGF-2. Scale bar = 50 µm.
4.4 Conclusions

In summary, the motivation behind this chapter stemmed from the results found in Chapter 3 – the proliferation rate of keratinocytes was not compatible with nanotubular Ti surfaces (created through anodization). Because chemical modification of Ti surfaces is becoming common for overcoming numerous problems associated with currently used Ti, this chapter examined the effect of such medication on keratinocyte proliferation. This particular study used a three step chemical reaction to attach FGF-2 onto the surfaces of nanotubular, nanorough, and conventional Ti: silanization with 3-aminopropyltriethoxysilane (APTES), cross-linking with N-succinimidyl-3-maleimido propionate (SMP), and finally FGF-2 immobilization.

Specifically, this study showed the successful attachment of FGF-2 to the nanotubular, nanorough, and conventional Ti surfaces. In addition, keratinocyte studies completed on these surfaces revealed that the presence of FGF-2 enhanced keratinocyte proliferation for surfaces compared to their non-functionalized counterparts. In addition, it was found that by immobilizing the nanotubular Ti surfaces, keratinocyte proliferation was enhanced compared to the conventional Ti surfaces. Finally, it was found that keratinocyte densities on functionalized nanorough Ti surfaces increased compared to functionalized nanorough Ti surfaces. This study thereby
provides an alternate solution for influence keratinocyte behavior which could further enhance skin growth at the implant surface thereby increasing the success of ITAP.
Chapter 5

Decreased Bacterial Attachment on Select Nanofabricated Titanium Surfaces

5.1 Introduction

Infection is a common problem for both the bone-anchored metal implant and abutment of the ITAP. Infection of the bone-anchored implant of an ITAP is similar to that of all orthopedic joint prosthetics. Prosthetic joint replacements, like ITAPs, are being used with increasing frequency to alleviate pain, to promote mobility, and improve the quality of life. Yet, prosthetic joint implantation suffers from the added risk of infection occurring in about 1.5-2.5% of all hip and knee arthroplasties resulting in failure of the device and, thus, a need for revision surgery [321]. Joint
prosthetic infection costs about $50,000 United States dollar per episode while the associated mortality may be as high as 2.5% [321]. In addition, if the infection persists into the deep tissue, amputation may also be required resulting in the need for an ITAP.

To put these percentages into perspective, it is important to note that in 2004, 265,441 total hip arthroplasties (THA) and 496,018 total knee arthroplasties (TKA) were performed in the United States alone [322]. Of these, an estimated 3352 THA (1.23%) and 5838 TKA (1.21%) were treated for infection [322]. It was further revealed that of these total arthroplasties performed in 2004, 38,629 THA and 36,425 TKA were due to revision surgeries [322]. Revision surgeries are a result of implant failure that can be caused from stress-strain imbalances, implant migration, wear debris, lack of integration, and infection listed throughout section 1.2. Of these implant failure modes, about 8% of THA and 15% of TKA revision surgeries were a direct result of infection [322].

In addition to infection occurring at the bone-metal interface, infection at the skin-metal is another major problem of ITAP [27, 28, 63-65]. This is due to epithelial downgrowth (Figure 1.6) mentioned in Chapters 1 and 3 that prevents a tight skin seal from being formed. As a result, this provides a clear route for invasion of bacteria [61, 62]. As bacteria colonize either the implant surface or adjacent damaged tissue sites, biomaterial exit sites
become the gateway to infection [66-76], possibly leading to bacteria spreading internally and causing osteomyelitis [76, 77]. According to previous studies, the occurrence of osteomyelitis is anywhere between 0 to 4% [66-77]. In addition to osteomyelitis, infection leads to bone implant loosening [72-75] and fracture malunion or nonunion leading to early failure of the device.

Current methods aimed at preventing bacterial infection include altering surgical techniques, modifying implant design, and coating the device with a new material. Coating approaches to improve anti-bacterial properties of ITAP include using fibroblast growth factor-2 embedded apatite composites [323] as well as silver [324-327] and hydroxyapatite coated implants [72, 328-334]. For example, titanium (Ti) screws coated with fibroblast growth factor-2 embedded apatite composites showed a 50% decrease in infection compared to those without the composite layer [323]. Similarly, one studied revealed that 62% of silver coated pins became infected compared to the 84% of uncoated pins that became infected [325]. Clearly, these coatings possess properties that minimize the presence of bacteria compared to their uncoated counterparts, yet the presence of infection is still high (due to delamination concerns) indicating there is a need for an alternate means to decrease infection rates.
Reduction of microbial adhesion to an implant without the use of drugs could be an attractive method for reducing infection. Planktonic (suspended) bacteria present in the body can be cleared by host defenses mechanisms and are more susceptible to antibiotics [335]. However, once bacteria bind to the biomaterial surface, changes in their functions occur. More specifically, bacteria gene expression changes and growth rates are altered leading to host defense mechanisms that are no longer able to remove them from the body and the formation of an antibiotic-resistant biofilm makes pharmaceutical treatments difficult [335-339]. Development of this biofilm is responsible for many chronic infections [335-339] and prevents proper integration of the implant to the surrounding tissue. In addition, antibiotic resistant strains cannot be treated by antibiotic therapy after adhesion to the prosthetic surface. Multiple antibiotic resistant strains including *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) have already been documented in the clinical orthopedic setting [298, 299]. Thus, it can be argued that the prevention of bacteria adhesion without drugs may be one of the best ways to reduce implant infection [340].

As shown throughout the previous chapters of this dissertation, altering the surface roughness of an implant material from one that possesses conventional, micron size features to one that possesses nanometer size features has been shown to enhance the adhesion of certain cells and subsequent cellular functions (such as osteoblasts and keratinocytes) while
simultaneously decreasing competitive cell function, such as fibroblasts (cells that create the fibrous tissue around an implanted material preventing proper integration) and bacteria [172, 187, 200, 201, 254, 341]. More importantly, previous studies have also shown that by varying the surface roughness of the biomaterial, bacterial adhesion is decreased [187]. However, more research is required to understand the underlying factors for such phenomenon and translating such results to metals commonly used in orthopedics.

Based on the evidence from the previous studies in this dissertation, this chapter explored the adhesion of multiple bacteria species well-known to lead to orthopedic implant infection as well as initial protein adsorption events on conventional Ti substrates as received from the vendor, nanorough Ti substrates created by electron beam evaporation, and nanotubular and nanotextured Ti substrates created by anodization. Specifically, gram positive *S. aureus* and *S. epidermidis* along with gram negative *Pseudomonas aeruginosa* (*P. aeruginosa*) were examined since these strains have been shown to be clinically prevalent in orthopedic prosthetic infection [340]. With selectively improved cellular responses, as already demonstrated, and decreased bacterial adhesion, integration between the living tissue and implant surface would be promoted, thus, improving the success rate of orthopedic prosthetics.
Thus, the specific objectives of this chapter included the:

- Design and fabrication of Ti surfaces with nanorough features created through electron beam evaporation and nanotubular and nanotextured features created through anodization,
- Characterization of the nanotextured Ti substrates using scanning electron microscopy, atomic force microscopy, electron spectroscopy for chemical analysis, and X-ray diffraction (all other surfaces were previously characterized in Chapter 2 and 3),
- Elucidation of bacteria adhesion on the nanorough, nanotubular, and nanotextured Ti substrates,
- Determination of protein adsorption on the nanorough, nanotubular, and nanotextured Ti substrates which may explain the results above, and
- Understanding the mechanisms for bacteria response on the nanorough, nanotubular, and nanotextured Ti substrates and the role that surface roughness, surface wettability, and surface energetics may have in mediating such responses.
5.2 Materials and Methods

5.2.1. Sample Preparation

As described in section 2.2.1 and 3.2.1, experiments conducted throughout this chapter involved a series of samples, including (i) conventional Ti substrates as received from the vendor, (ii) nanorough Ti substrates created through electron beam evaporation, and (iii) nanotubular and nanotextured Ti substrates created through anodization. The base material for all sample types were Ti foils (100 x 100 x 1 mm; 99.2% pure; Alfa Aesar, Ward Hill, MA, USA) that were cut into 10 x 10 mm squares using a shear cutter. After cutting into the desired dimensions, all substrates were ultrasonically cleaned with a diluted cleaning solution (Branson, Dabury, CT, USA) for 20 minutes followed by sonication in acetone, 70% ethanol, and deionized water (DI) for 10 minutes. Substrates were then dried in an oven (VWR, Bridgeport, NJ, USA) at 40°C for 15 minutes, or until dry.

5.2.1.1. Titanium Substrates as Received from the Vendor

See Section 2.2.1.1.

5.2.1.2. Electron Beam Evaporated Titanium Substrates

See Section 2.2.1.2.
5.2.1.3. Anodized Titanium Substrates

In this study, nanotubular Ti substrates were fabricated the same way as described in section 3.2.1.3. Nanotextured Ti substrates were fabricated using anodization but rather than being completed in 1.5 wt% HF for 10 minutes at a constant voltage of 20 V (the process used to fabricate nanotubular Ti substrates), these samples were anodized in 0.5 wt% HF for 1 minute at a constant voltage of 20 V [213, 214]. These Ti substrates were rinsed with large amounts of DI water immediately after anodization, air dried, and sterilized under ultraviolet light for three hours per substrate side prior to cell culture experiments.

5.2.2. Sample Characterization

Nanotextured surfaces created by anodization were characterized for roughness, crystallinity, wettability, and surface energetics in the same way the conventional Ti, nanorough Ti, and nanotubular Ti surfaces were characterized in Chapters 2 and 3. Techniques used to characterize the conventional Ti surfaces as received from the vendor, nanorough Ti surfaces created through electron beam evaporation, and nanotubular Ti surfaces created through anodization were also used to characterize the nanotextured Ti surfaces used in this chapter.
5.2.2.1. Scanning Electron Microscopy (SEM)

See section 2.2.2.1.

5.2.2.2. Atomic Force Microscopy (AFM)

See section 2.2.2.2.

5.2.2.3. Electron Spectroscopy for Chemical Analysis (ESCA)

See section 2.2.2.3.

5.2.2.4. X-ray Diffraction (XRD)

See section 2.2.2.4.

5.2.3. Cytocompatibility Assays

5.2.3.1. Cell Culture

Bacteria cell lines used in this study were *Staphylococcus epidermidis* (*S. epidermidis*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Staphylococcus aureus* (*S. aureus*) obtained in freeze-dried form the American Type Culture Collection (35984, 25668, and 25923 respectively). The dry pellet was rehydrated in 6 mL of Luria broth (LB) consisting of 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter double distilled water with the pH adjusted to 7.4 (all chemicals were obtained from Sigma Aldrich, Milwaukee, WI, USA).
The bacteria solution was agitated under standard cell culture conditions (5% CO₂/95% humidified air at 37°C) for 24 hours until the stationary phase was reached. The second passage of bacteria was diluted at a ratio of 1:200 into fresh LB and incubated until it reached stationary phase. The second passage was then frozen in one part LB and one part glycerol (Sigma Aldrich) and stored at -18°C. All experiments were conducted from this frozen stock. One day before bacterial seeding for experiments, a sterile 10 µl loop was used to withdraw bacteria from the frozen stock and to inoculate a centrifuge tube with 3 mL of fresh LB.

5.2.3.2. Cell Adhesion

Prior to seeding, sterilized substrates (including conventional Ti, nanotubular Ti, nanotubular Ti, and nanotextured Ti substrates) were placed into a standard 24-well culture plate and were washed twice with phosphate buffer saline (PBS). Bacteria were seeded on the conventional Ti, nanotubular Ti, nanotubular Ti, and nanotextured Ti substrates at a density of 1x10⁷ bacteria/mL (as estimated by the McFarland scale) by diluting the LB bacteria cultures to an optical density of 0.52 at 562 nm and then further diluted at a ratio of 1:90 in Dulbecco’s Modified Eagle’s Medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone), 1% penicillin-streptomycin (P/S; Hyclone), 50 µg/mL L-ascorbate acid (Sigma Aldrich), and 10 mM β-glycerophosphate (Sigma Aldrich). The
bacteria were allowed to adhere for one hour under standard cell culture conditions (5% CO$_2$/95% humidified air at 37°C) with constant shaking at 200 rpm to prevent settling of the cell solution. At the end of the prescribed time period, the substrates were rinsed twice with Tris-buffered saline (TBS) comprised of 42 mM Tris·HCl, 8 mM Tris Base, and 0.15 M NaCl (Sigma Aldrich) and then incubated for 15 minutes with the BacLight Live/Dead solution (Life Technologies Corporation, Carlsbad, CA, USA) dissolved in TBS at the concentration recommended by the manufacturer. This solution consisted of propidium iodide (PI) and SYTO 9, which will diffuse into the cell and fluoresce upon binding to nucleic acids [342, 343]. More specifically, SYTO 9 is membrane permeable and therefore stains both viable and nonviable bacteria (green fluorescence) while PI has a higher affinity for nucleic acids (red fluorescence) but is excluded from viable cells by membrane pumps [342, 343]. Substrates were then rinsed twice with TBS and placed into a 50% glycerol solution in TBS prior to imaging. Bacteria were then visualized and counted in situ using a Leica DM5500 B fluorescence microscope (Bannockburn, IL, USA) with image analysis software captured using a Retiga 4000R camera. Adhesion experiments were run in duplicate and repeated three different times per substrate type. Total bacteria colonies were determined by summing the number of live and dead bacteria colonies found using Image J.
5.2.4. Mechanisms for Bacteria Response

5.2.4.1. Surface Energetics and Wettability

See section 2.2.4.1.

5.2.4.2. Fibronectin Adsorption (ELISA)

See section 2.2.4.2.

5.2.5. Statistics

Data were represented by the mean value with the standard error of the mean (SEM) noted. A one-tailed, paired standard student t-test was used to check statistical significance between means, and p<0.1 was considered statistically significant.

5.3 Results and Discussion

5.3.1. Sample Characterization

5.3.1.1. Scanning Electron Microscopy (SEM)

Low and high scanning electron micrographs of the nanorough and conventional Ti surfaces can be found in section 2.3.1 (Figure 2.7) while section 3.3.1 (Figure 3.4) presented the micrographs for nanotubular Ti surfaces. Low and high resolution micrographs of the Ti surfaces anodized
for 1 min in 0.5 % HF at 20 V can be seen in Figure 5.1. These surfaces contained nanotextured surface features ranging from about 30 to 40 nm in diameter. Thus, throughout the remainder of this dissertation, these Ti substrates will be referred to as nanotextured Ti.

Figure 5.1. SEM micrographs of Ti substrates after anodization for 1 minute in 0.5% HF at 20 V. The Ti substrates possessed nanotextured features (and, thus, were termed nanotextured Ti). Scale bar = 2 µm (low resolution) and 200 nm (high resolution). SEM micrographs of nanorough Ti and conventional Ti can be found in Figure 2.7 and nanotubular Ti can be seen in Figure 3.4.

5.3.1.2. Atomic Force Microscopy (AFM)

Quantitative data, root-mean-square roughness (RMS) and depth profiles (RPV), from AFM adapted from [214] revealed these nanotextured surfaces possessed nanometer surfaces roughness (Figure 5.2). Although relisted here, AFM data for the conventional and nanorough Ti surfaces can be found in section 2.3.1 (Table 2.2 and Figure 2.9), and AFM data for the nanotubular
surfaces can be found in section 3.3.1 (Table 3.1 and Figure 3.5). RMS for the nanotextured Ti surfaces was found to be 9.75 nm while depth profiles were approximately 30-40 nm \[214\] (Table 5.1). Thus, when comparing all four surface types used throughout this section, the nanotubular Ti surfaces had the largest nanometer surface topographies followed by the nanorough Ti surfaces. Table 5.1 summarizes the AFM values found for conventional Ti, nanorough Ti, nanotubular Ti, and nanotextured Ti surfaces used for cell experiments in this chapter. Thus, from the thorough analysis of both SEM and AFM, it can be concluded that during the cell studies (described in the next sections), bacteria were exposed to surfaces with unique and different surface topographies varying in their degree of nanometer roughness.

Table 5.1. Qualitative data indicated the substrates created by anodization (nanotubular and nanotextured) and electron beam evaporation (nanorough Ti) contained more surface features in the nanometer regime than the Ti surfaces received directly from the vendor (conventional Ti). RMS represents root mean square roughness while RPV represents peak-to-valley roughness.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RMS (nm)</th>
<th>RPV (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron Beam Evaporated Ti (nanorough topography)</td>
<td>11</td>
<td>30-40</td>
</tr>
<tr>
<td>As Received Ti (conventional topography)</td>
<td>6</td>
<td>10-20</td>
</tr>
<tr>
<td>Anodized Ti (nanotubular topography)</td>
<td>12.5</td>
<td>30-40</td>
</tr>
<tr>
<td>Anodized Ti (nanotextured topography)</td>
<td>9.75</td>
<td>30-40</td>
</tr>
</tbody>
</table>
AFM micrographs and section analyses revealed topographical nanofeatures on the nanotextured Ti surfaces [214]. Root-mean-square roughness (RMS) and average peak-to-valley roughness (RPV) values can be found in Table 5.1. AFM micrographs for the nanorough Ti and conventional Ti surfaces can be found in Figure 2.9 and in Figure 3.5 for the nanotubular Ti surfaces.

5.3.1.3. Electron Spectroscopy for Chemical Analysis (ESCA)

As described in section 2.3.1.3, because it is well-known that alterations in chemistry and/or crystal structures can influence cell function, each of these must be investigated in order to determine which elements may explain the response of keratinocytes observed in the next section.

ESCA, already completed on the nanorough and conventional Ti surfaces (section 2.3.1, Figure 2.10) as well as nanotubular Ti surfaces (section 3.3.1, Figure 3.6), was performed on the nanotextured Ti surfaces in order to determine if there was a difference in the surface oxide composition. One high resolution ESCA spot was taken on the nanotextured Ti surfaces, and
similar to the conventional Ti, nanorough Ti, and nanotubular Ti surfaces (Figure 2.10 and 3.6), revealed only one identifiable peak, Ti 2p (Figure 5.3), indicating all surfaces possessed similar oxide compositions. When further examining the outermost layers of the nanotextured Ti surfaces, they mostly contained O and Ti like the nanorough Ti, conventional Ti, and nanotubular Ti surfaces (Table 3.2, Figure 2.10 and 3.6; values relisted here). However, in addition to O and Ti, a trace amount of F was found in the nanotextured Ti surfaces, same as the nanotubular surfaces, most likely due to the anodization technique involving the use of HF (Table 5.2). The nanotubular Ti surfaces had a slightly higher percentage than the nanotextured Ti possibly due to the fact that anodization was completed in a higher HF solution (1.5% compared to 0.5%).

Table 5.2. Atomic percentage of selective elements in the outermost layers of Ti as received from the vendor (conventional Ti), after electron beam evaporation (nanorough Ti), and after anodization (nanotubular and nanotextured Ti) as examined by ESCA.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>O (%)</th>
<th>Ti (%)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanorough Ti</td>
<td>49.34</td>
<td>50.66</td>
<td>0</td>
</tr>
<tr>
<td>Conventional Ti</td>
<td>51.23</td>
<td>48.77</td>
<td>0</td>
</tr>
<tr>
<td>Nanotubular Ti</td>
<td>57.00</td>
<td>33.62</td>
<td>9.38</td>
</tr>
<tr>
<td>Nanotextured Ti</td>
<td>56.27</td>
<td>34.95</td>
<td>6.03</td>
</tr>
</tbody>
</table>
Figure 5.3. (a) ESCA spectra of elements present in the outermost layer of the nanotextured Ti surfaces (Table 5.2). (b) Binding energy of the high resolution Ti 2p peak for the nanotextured Ti surfaces. Binding energies matched those of the conventional Ti, nanorough Ti, and nanotubular Ti surfaces (Figure 2.10 and 3.6).

5.3.1.4. X-ray Diffraction (XRD)

Finally, this study determined whether or not amorphous or crystalline phases were present on the nanotextured Ti substrates surfaces. XRD spectra for the nanorough, conventional, and nanotubular Ti surfaces can be found in section 2.3.1 and 3.3.1 (Figures 2.11 and 3.7). XRD spectra revealed
that all surfaces contained the same phase structure (Ti) with no traces of anatase or rutile found (Figure 5.4).

Figure 5.4. XRD spectra of nanotextured Ti surfaces after anodization. Spectra, when compared to Ti and titania (anatase and rutile) standards, indicated the presence of amorphous TiO$_2$ (also observed in nanotubular Ti surfaces, Figure 3.7). XRD spectra for the nanorough Ti and conventional Ti surfaces can be found in Figure 2.11 (contained crystalline TiO$_2$). (Adapted from [298])

5.3.2. Bacteria Adhesion

Figure 5.5 represents total bacteria colonies, including both live and dead bacteria, attached after one hour. The results of this study revealed that bacteria adhered the least to the nanorough Ti substrates. More specifically, when normalized to the projected surface area, there was a significantly lower attachment of colonies for all bacteria lines (*S. aureus, S. epidermidis,*
and *P. aeruginosa* on the nanorough Ti substrates compared to the conventional, nanotubular, and nanotextured Ti substrates (Figure 5.5). In addition, when further examining bacteria behavior on the anodized Ti surfaces, results indicated that bacteria significantly adhered more to the nanotubular Ti compared to the nanotextured Ti (Figure 5.5). Figure 5.6 qualitatively highlights the decreased attachment of *S. aureus*, *S. epidermidis*, and *P. aeruginosa* on the nanorough Ti substrates. Interestingly, data also indicated that the nanotubular and nanotextured Ti substrates had the highest number of bacterial colonies for all cell lines compared to conventional and nanorough Ti substrates (Figure 5.5). Figure 5.6 also visually highlights the significantly greater number of *S. aureus*, *S. epidermidis*, and *P. aeruginosa* present on the nanotextured and nanotubular Ti substrates compared to conventional and nanorough Ti substrates.
Figure 5.5. Total *S. aureus*, *S. epidermidis*, and *P. aeruginosa* colonies on nanorough Ti, conventional Ti, nanotubular Ti, and nanotextured Ti after one hour. Values are mean ±SEM; n=3; *p<0.01 compared to nanorough Ti; **p<0.01 compared to conventional Ti; ***p<0.01 compared to nanotextured Ti; #p<0.1 compared to nanotextured Ti; ##p<0.05 compared to nanotextured Ti for respective bacteria lines.
S. Aureus

(a) Conventional Ti  
(b) Nanorough Ti

(c) Nanotextured Ti  
(d) Nanotubular Ti
(e) Conventional Ti
(f) Nanorough Ti

(g) Nanotextured Ti
(h) Nanotubular Ti
Figure 5.6. Fluorescent micrographs of (a–d) *S. aureus*, (e–h) *S. epidermidis*, and (i–l) *P. aeruginosa* colonies on conventional, nanorough, nanotextured, and nanotubular Ti surfaces. It was found that bacteria colonies decreased on the (b,f,i) nanorough Ti surfaces compared to all other substrates and increased on the (c,g,k) nanotextured and (d,h,l) nanotubular Ti surfaces compared to the (a,e,i) conventional Ti after one hour.
Figure 5.7 (a) reports the number of live bacteria colonies present on the Ti surfaces after one hour while Figure 5.7 (b) reports the number of dead bacteria colonies present on the Ti surfaces after one hour. Results indicated that nanorough Ti substrates had the least amount of living bacteria after one hour. In other words, when normalized to the projected surface area, there was a significantly lower attachment of live bacteria colonies for all strains (*S. aureus, S. epidermidis*, and *P. aeruginosa*) on the nanorough Ti substrates compared to conventional, nanotubular, and nanotextured Ti substrates (Figure 5.7 (a)). In addition, results showed that the nanotubular and nanotextured Ti substrates had more live colonies for each bacteria line compared to conventional Ti substrates (Figure 5.7 (a)). Furthermore, upon examining the amount of dead bacteria present on the surfaces, nanotubular and nanotextured Ti substrates contained the greatest number of dead bacteria colonies for all bacteria lines, while the nanorough Ti substrates contained the least amount of dead bacteria colonies (Figure 5.7 (b)).
Figure 5.7. (a) Live and (b) dead colonies of *S. aureus, S. epidermidis,* and *P. aeruginosa* on nanorough Ti, conventional Ti, nanotubular Ti, and nanotextured Ti surfaces after one hour. Values are mean ±SEM; n=3; *p<0.05 compared to the nanorough Ti; **p<0.01 compared to the nanorough Ti; ***p<0.01 compared to the conventional Ti; #p<0.05 compared to the conventional Ti; ##p<0.01 compared to the nanotextured Ti; ###p<0.05 compared to the nanotextured Ti for respective bacteria lines.
Figure 5.8 shows the percentage of live bacteria, as calculated from the data provided in Figure 5.7. The results indicated that the nanorough Ti substrates contained a significantly higher percentage of live bacteria for all strains attached to the surface after one hour (Figure 5.8). When examining this data, as well as the total number of bacterial colonies (Figure 5.5), it can be concluded that the nanorough Ti substrates (created by electron beam evaporation) were the best surfaces for inhibiting bacteria compared to the conventional, nanotubular, and nanotextured Ti substrates.
Figure 5.8. Percentage of live bacteria colonies for *S. aureus*, *S. epidermidis*, and *P. aeruginosa* attached on nanorough Ti, conventional Ti, nanotubular Ti, and nanotextured Ti surfaces after one hour. Values are mean ±SEM; n=3; *p<0.1 compared to the nanotextured Ti; **p<0.01 compared to the nanotextured Ti; ***p<0.05 compared to the nanotubular Ti; #p<0.05 compared to the conventional Ti; ##p<0.01 compared to the nanotubular Ti; ###p<0.1 compared to the conventional Ti; ++p<0.1 compared to the nanotubular Ti for respective bacteria lines.

5.3.3. Mechanisms for Decreased Bacteria Response

Results from this study indicated that the presently prepared nanorough Ti surfaces were the best surfaces for inhibiting bacterial adhesion. Compared to conventional surfaces, nanostructured materials have excellent biocompatibility properties due to enhanced select protein interactions (including adsorption and conformation) resulting in improved cellular adhesion and tissue growth [233, 235]. It has been demonstrated here that
there was a linear relationship between roughness, surface energy, and fibronectin adsorption. More specifically, a surface with extensive nanorough features increases surface energy which leads to greater fibronectin adsorption [183, 196, 214, 233-235, 280, 344]. This study also confirmed the same correlation as it revealed that nanorough, nanotubular, and nanotextured Ti possessed higher degrees of nanometer features, higher surface energy, and increased fibronectin adsorption compared to conventional Ti. Specifically, nanotubular, nanotextured, and nanorough Ti had a surface energy significantly higher than that of conventional Ti (Figure 5.9; Table 5.3). Regarding protein adsorption, fibronectin was statistically higher on the nanofabricated surfaces (nanotubular, nanotextured, and nanorough Ti surfaces) compared to the conventional Ti surfaces. It was also found that the nanotubular Ti surfaces had significantly more fibronectin adsorption than the nanorough Ti surface (Figure 5.10).
Figure 5.9. Surface energy of the conventional Ti, nanorough Ti, nanotubular Ti, and nanotextured Ti surfaces. Surface energy was calculated for each sample by measuring the contact angles of three liquids at the sample surface and entering values into the Owens-Wendt equation. Results indicated greater surface roughness at the nanoscale correlating to increased surface energy. Values are mean ±SEM; n=4; *p<0.01 compared to conventional Ti; **p<0.01 compared to nanorough Ti; ***p<0.05 compared to nanotextured Ti.

Table 5.3. Contact angles of three liquids on conventional Ti, nanorough Ti, nanotubular Ti, and nanotextured Ti surfaces. Contact angle data was used to determine surface energy via Owens-Wendt equation.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Contact Angle of DI Water</th>
<th>Contact Angle of Glycerol</th>
<th>Contact Angle of PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional Ti</td>
<td>70.6 ± 1.58</td>
<td>69.3 ± 0.84</td>
<td>41.18 ± 1.20</td>
</tr>
<tr>
<td>Nanorough Ti</td>
<td>59.3 ± 1.13</td>
<td>57.6 ± 0.89</td>
<td>28.3 ± 1.74</td>
</tr>
<tr>
<td>Nanotubular Ti</td>
<td>26.5 ± 2.40</td>
<td>21.9 ± 1.32</td>
<td>11.1 ± 0.80</td>
</tr>
<tr>
<td>Nanotextured Ti</td>
<td>29.5 ± 1.13</td>
<td>25.4 ± 1.35</td>
<td>17.2 ± 1.43</td>
</tr>
</tbody>
</table>
Figure 5.10. Fibronectin adsorption on the conventional Ti, nanorough Ti, nanotubular Ti, and nanotextured Ti surfaces. Values are mean ±SEM; n=3; *p<0.01 compared to the conventional Ti; **p<0.1 compared to the conventional Ti; ***p<0.05 compared to the nanorough Ti; #p<0.01 compared to the nanorough Ti; ##p<0.1 compared to the nanotextured Ti.

Research has also shown that increased protein adsorption, such as fibronectin, results in decreased bacteria attachment [345, 346]. More specifically, fibronectin coated PMMA-based teropolymers inhibited S. aureus up to 98% compared to In the present study, this trend was observed between the nanorough Ti, which promoted the least amount of bacterial attachment, and conventional Ti. Compared to conventional Ti, nanorough Ti possessed no chemical difference, and, thus, the presence of nanometer features alone (higher surface energy) resulted in increased fibronectin adsorption which decreased bacterial attachment.
Based on this thinking, decreased bacterial attachment would also be expected for the nanotubular and nanotextured Ti substrates since these surfaces had greater surface roughness, surface energy, and fibronectin adsorption. However, increased bacteria attachment was observed on both the nanotubular and nanotextured Ti compared to the nanorough and conventional Ti. It is possible that fluorine present on the nanotubular and nanotextured on these surfaces (Table 5.2) increased bacterial adhesion compared to conventional and nanorough Ti surfaces. Further examining bacteria attachment on the anodized nanotubular surfaces revealed the highest bacteria attachment compared to the anodized nanotextured surfaces (Figure 5.5), correlating well to the possible role of greater bacteria attachment with fluorine concentration (Table 5.2). Other studies have confirmed this trend that fluorine present at a material surface increases bacterial adhesion [347-350]. Specifically, Katsikogianni and colleagues examined bacteria (S. epidermidis) function on polymers with and without the presence of fluorine [350]. They showed that the polymers containing fluorine (created by plasma spraying in the presence of CF$_4$/H$_2$) increased bacteria attachment [350]. Li and colleagues showed that surface ions present on metal oxides encouraged the binding for both gram positive (Bacillus subtilis) and gram negative (two P. aeruginosa strains, three Escherichia coli, two Burkholderia cepacia) bacteria to glass or metal oxide surfaces [349]. Such metal oxides examined in that study included TiO$_2$,
SnO$_2$, SnO$_2$:F, Al$_2$O$_3$, and Fe$_2$O$_3$, of which those possessing fluorine ions resulted in the highest bacterial adhesion. This observation was not affected by the surface charge or hydrophobicity/hydrophilicity of the bacteria surface [349]. These findings may explain the results from this current study which demonstrated that nanotubular and nanotextured Ti surfaces containing fluorine ions increased bacteria attachment despite observed increases in fibronectin adsorption. It is also interesting to note that a previous study by Popat and colleagues were able to decrease the adhesion of bacteria (*S. epidermidis*) by 70% after one hour on nanotubular Ti (prepared by an anodization similar to the process used in this study) compared to conventional counterparts, only after loading antibiotics (specifically, gentamicin) into the nanotubes [351]. The nanotubes were loaded with 600 µg of gentamicin, which was completely released within 90 minutes.

Although total bacteria adhered the most to the anodized nanotubular surfaces, this study also revealed that the anodized surfaces (nanotubular and nanotextured Ti) decreased the percentage of living cells compared to the non-fluorinated surfaces (nanorough and conventional Ti). This could be a result of the antibacterial effects caused by the presence of fluorine, as shown by other studies [352-354]. For example, Raulio and colleagues found that by coating stainless steel with fluoropolymers, it was possible to reduce biofilm formation of several bacteria strains (up to 90%), including *S. epidermidis* [353]. In addition, Yoshinari and colleagues found fluorine ion-implanted Ti
surfaces contained fewer viable bacteria colony forming units further suggesting the antibacterial properties of fluorine. This study demonstrated that fluorine ions were not released suggesting that the formation of metal fluoride bonds were sufficient for producing antibacterial effects.

Another reason for the observed increase in total bacteria colonies on nanotubular and nanotextured Ti surfaces compared to nanorough and conventional Ti surfaces could be explained by the large number of adherent dead bacteria (Figure 7(b)). Dead bacteria bound to a biomaterial surface can aid in the adhesion of subsequent live bacteria [355, 356]. Specifically, dead or dying *P. aeruginosa* can release intracellular lectins to promote the adhesion of living bacteria [355]. In a similar manner, *S. Aureus* can release an intercellular protein upon death to enhance the adhesion of other microorganisms [356]. Since nanotubular and nanotextured Ti surfaces contained fluorine that enhanced the percentage of dead bacteria, this may have resulted in the release of some chemical or compound that further promoted subsequent live bacterial adhesion in this study (Figure 7(a)), which explains why these materials had the highest number of live bacteria. Clearly, this study revealed that fluorine was an important factor that had a prominent effect on bacteria response. Further experiments involving certain proteins and amino acid sequences (such as LPXTG) known to contribute to the colonization of bacteria should be completed to better understand the role of fluorine in this study [356]
Thus, a simple means for the reduction of bacterial adhesion and subsequent infection of Ti, nanometer sized surface roughness was considered here for orthopedic applications. In summary, results of this *in vitro* study demonstrated the decreased adhesion of *S. aureus*, *S. epidermidis*, and *P. aeruginosa* (bacteria that limit orthopedic implant function and efficacy) on nanorough Ti surfaces created through electron beam evaporation while nanotubular and nanorough Ti created through anodization resulted in an increase of bacteria attachment. This research demonstrated that through the careful selection of nanometer surface properties to increase fibronectin adsorption, while maintaining favorable chemistry (i.e. decreased fluorine ions present on the surface) and crystallinity (specifically, anatase TiO$_2$), it is possible to decrease bacteria colonization without drugs. This study, thus, provided further knowledge to the orthopedic field on ways to reduce bacteria adhesion, a prerequisite for infection, on metals (without the use of drugs) which should be further be investigated as a means to improve the longevity of ITAP.

### 5.4 Conclusions

Infection of ITAP devices is undesirable and carries a significant burden for the success of prosthetics. Reducing the adhesion of a broad range of bacteria could be an attractive means to decrease infection and allow tissue integration with the biomaterial surface. In this study, nanometer sized
topographical Ti surfaces, which have been previously investigated for enhancing protein adsorption and osteoblast and keratinocyte function (as indicated through previous chapters of this dissertation) were investigated as a means to simultaneously reduce bacterial adhesion.

This study examined bacteria adhesion (S. aureus, S. epidermidis, and P. aeruginosa) on conventional Ti, nanorough Ti produced by electron beam evaporation, and nanotubular and nanotextured Ti produce by anodization in a hydrofluoric acid bath. This study found that the nanorough Ti surfaces produced by electron beam evaporation decreased the adherence of bacteria the most while those produce by anodization resulted in the highest attachment of bacteria. Titanium chemistry and crystalline phases (TiO₂) were found to be similar when compared to the conventional Ti.

More importantly, it was also found that the nanotubular and nanotextured Ti had fluorine ions present on their surface in addition to the presence of amorphous titania, both of which have been linked to the promoted adhesion of bacteria on their surfaces. Therefore, the results of this study demonstrated that certain nanometer sized Ti topographies may be useful for the reduction of bacteria adhesion while maintaining tissue growth and, thus, should be further investigated for improving the efficacy of ITAP devices.
Chapter 6

In Vivo Study Demonstrating Enhanced Bone and Skin Growth on Nanorough and Nanotubular Ti6Al4V Pins

6.1 Introduction

As mentioned, ITAP provide a more permanent prosthetic option for individuals undergoing the loss of a major limb. The in vitro data described in the previous chapters, revealed that electron beam evaporated (nanorough) titanium (Ti) surfaces enhanced osteoblast and keratinocyte behavior compared to conventional Ti surfaces and inhibited bacteria adhesion. In addition, anodized (nanotubular) Ti surfaces have been shown by other researchers to enhance osteoblast functions (such as alkaline phosphatase
activity, calcium deposition, and collagen synthesis). In this study, despite the decreased keratinocyte behavior on these surfaces over time compared to the nanorough Ti, this study revealed that nanotubular Ti surfaces enhanced keratinocyte adhesion, a time window that is crucial if epithelial downgrowth is to be prevented. Thus, from the results of the in vitro data, electron beam evaporated (nanorough) Ti surfaces as well as anodized (nanotubular) Ti surfaces were used in a small animal (rat) amputation model. Canines, rabbits, and rats are the more commonly used animal models for orthopedic applications. Particularly, these models have been used to examine a variety of Ti implants (fluoride ion modified TiO$_2$, RGD coated Ti implants, machined smooth-surfaced Ti, plasma sprayed rough surfaced Ti, nanohydroxyapatite Ti coatings, anodically oxidized Ti, etc.) inserted into either the femur or tibia of the animal in order to examine the effect that surface topography and coatings can have on bone growth [357-366]. This particular study chose the rat model due to the fact that rats are more easily housed, easily accessible, easily reproducible, and less expensive. The goal of this in vivo study was to determine if these nanofabricated Ti surfaces could achieve better device integration at the bone- and skin-implant interface compared to conventional Ti surfaces currently used today. This experiment was designed so that both bone and skin growth on these nanofabricated surfaces could be examined simultaneously. Based on the in vitro data, it was hypothesized that better bone and skin growth along with reduced infection would be found on the
nanorough and nanotubular Ti surfaces compared to the conventional Ti surfaces.

Thus, the specific objectives of this chapter included the:

- Design and fabrication of orthopedic Ti alloy (Ti6Al4V) pins with nanorough features created through electron beam evaporation and nanotubular features created through anodization,
- Characterization of the nanofabricated orthopedic Ti alloy (Ti6Al4V) pins using electron beam evaporation and anodization,
- Surgical implantation of the orthopedic Ti alloy (Ti6Al4V) pins into a rat amputee model, and
- Examination of bone and skin growth on the various orthopedic Ti alloy (Ti6Al4V) pins through a series of histological stains.

6.2 Materials and Methods

6.2.1. Sample Preparation

6.2.1.1. Control Ti6Al4V Pins

Partially threaded implant pins were made from a medical grade Ti alloy rod stock (ASTM F136 Ti6Al4V Eli Grade 5) with a 2 mm diameter and a length of 30 mm. The rod consisted of three regions: the bone anchored region consisted of a conical tip and a 15 mm long threaded section (2 M x 0.4,
oversized), the percutaneous region consisted of a 10 mm smooth section, and the distal region consisted of a 5 mm threaded section (2 M x 0.4) (Figure 6.1). Machined samples were rinsed and sonicated successively in 70% ethanol, alconox solution, and deionized water followed by chemical passivation in 23% nitric acid for 20 min at 80°C. Lastly, machined samples were rinsed and sonicated for five minutes in deionized water and air dried at 80°C to produce control surfaces.

![Figure 6.1](image)

Figure 6.1. Photograph of the Ti6Al4V pins indicating the dimensions and the different regions that will be exposed to bone and skin in the animal model.

6.2.1.2. Electron Beam Evaporated Ti6Al4V Pins

Electron beam evaporation (section 2.2.1.2) was performed on the control implant surface described above. The process was performed twice on each pin in order to coat the entire Ti surface.

6.2.1.3. Anodized Ti6Al4V Pins

Anodization was performed on the control implant surface described above. In this study, nanotubular Ti6Al4V pins were fabricated the same way as
described in section 3.2.1.3 to prepare nanotubular Ti substrates. However, rather than using 1 platinum mesh, the pin was placed parallel between 2 platinum meshes (Figure 6.2). These Ti6Al4V pins were rinsed with large amounts of DI water immediately after anodization and air dried.

Figure 6.2. Schematic illustration of the anodization process used to create nanotubular features on the Ti6Al4V pins.

6.2.2. Sample Characterization

After electron beam evaporation and anodization, the Ti6Al4V pins were examined under scanning electron microscopy (SEM, section 2.2.2.1) to confirm that the desired surface features were present.
6.2.3. Animal Study

Eighteen animals (Sprague Dawley, Charles River, 425-450 gm) were used in a unilateral through-the-knee, amputation rat model for evaluating tissue integration to the ITAP devices. This study was conducted with approval from the Rhode Island Hospital Institutional Animal Care and Use Committee (IACUC). Animals were divided into three groups of six animals and received a Ti alloy (Ti6Al4V) implant (Figure 6.1) with either a standard surface (control) or one of two surface modifications (electron beam evaporation, referred to as nanorough Ti, and anodization, referred to as nanotubular Ti). Animals were anesthetized by Isoflurane inhalation and received preoperative Buprenorphine hydrochloride (SQ, 0.1mg/kg), Ancef (Cefazolin) (IM, 20mg/Kg) for antibiotic prophylaxis and Rymadyl (Carprofen) (SQ, 5 mg/kg) to control inflammation. The entire right hindlimb, caudal portion of the ventral side of the abdomen, and caudal portion of the back was shaved, prepped with povidone iodine scrub (Betadine), then 70% alcohol, and povidone iodine was applied, and then steriley draped. A through-knee amputation was then performed. Briefly, two semi-circumferential skin incisions were made at the level of the mid-tibia to form an anterior to posterior skin flap. The femoral artery was ligated and a through-knee amputation performed by circumferential sectioning the underlying muscles, clamped vessels, nerves and knee ligaments. The muscles were released and
scraped from the distal end of the femur, before removal of the condyles at the epiphysis and trimming using a rongeur. Drill bits (1.5 - 2 mm diameter) were used to open and size the intramedullary canal, as needed before threading the implant halfway into the canal (~15 mm) by hand and tightening with Kelly forceps. Muscles and tendons were drawn up around the end of the femur and sutured (Vicryl). The distal end of the ITAP was passed through a biopsy punched hole (2 mm, Sklar Instruments, West Chester, PA, USA) in the skin created in a neutral position near the distal femur. The skin was closed with wound clips and/or nylon non-absorbable sutures. A 2 mm id Teflon o-ring was slipped onto the end of the implant followed by a 2 mm nylon nut and a 2 mm stainless steel nut to prevent the distal end of the implant from becoming submerged under the skin immediately after surgery.

Buprenorphine was administered twice per day for the first couple of days (Day 1; 0.1mg/kg) and (Day 2-3; 0.05mg/kg) in conjunction with 3 days of Rimadyl (5 mg/kg). Doxycycline (SQ, 25 mg/kg) was given as a fluorescent bone label to half of the animals, 4 days prior to sacrifice (day 24).

After surgery, the animals were housed in single cages and were allowed unrestricted activity and unrestricted access to food and water. Figure 6.3 shows photographs of the rats standing and walking on the ITAP devices after implantation. Healing was followed with weekly high-resolution lateral
radiographs (Faxitron MX 20 X-ray, Faxitron X-ray Corporation, Wheeling, IL) and photographic documentation of the exit site condition. All of the animals were euthanized four weeks post-operatively. The femurs with implants were harvested by simple dissection then fixed in 10% neutral buffered formalin or 70% ethanol for poly (methyl methacrylate) embedding, hard section histology. Toluidine blue and hematoxylin and eosin (H&E) stained sections were used to measure the percent tissue contact with implant, soft tissue thickness, and epithelial down growth. Image J was used to calculate the percentage of new bone growth revealed from the toluidine blue histology slides.
Figure 6.3. Photographs showing the rats actively using the implanted pin as a leg prosthetic. This particular rat contained a pin that had been modified by anodization (nanotubes).
6.3 Results and Discussion

6.3.1. Sample Characterization

Low and high scanning electron micrographs of the control, electron beam evaporated nanorough, and anodized nanotubular Ti6Al4V pins were taken (Figure 6.4). As expected, the high resolution images revealed that the control pins were very smooth when compared to the electron beam pins which clearly possessed a high degree of nanometer roughness. These surface features were observed on both the grooved (valley) and raised (peak) regions of the threads. The anodized pins revealed the presence of nanotubes as expected on both the grooved (valley) and raised (peak) regions of the threads. Thus, results demonstrated for the first time that these nanofabrication methods (i.e. electron beam evaporation and anodization) not only can be applied to 2-D surfaces for in vitro applications, they can also be applied on larger scale 3-D materials used for in vivo applications.
Figure 6.4. SEM micrographs of Ti6Al4V pins (a) before modification (control), (b) after electron beam evaporation (referred to as nanorough Ti), and (c) after anodization (referred to as nanotubular Ti). After electron beam evaporation, the Ti pins possessed a higher degree of nanometer roughness compared to the smooth control surfaces. The presence of nanotubes was also observed on the Ti surfaces after anodization.
6.3.2. X-rays

X-rays of all Ti6Al4V pins (conventional, nanorough, and nanotubular) implanted in rat femurs were taken each week to show the placement of the pins and to better understand the healing process (Figure 6.5).

![X-ray images of rat femurs showing pins at different stages of healing.](image)

**Figure 6.5.** X-ray micrographs of the (a) conventional (control), (b) nanorough (electron beam evaporation), and (c) nanotubular (anodization) Ti6Al4V pins implanted in rat femurs at 14, 21, and 28 days.
6.3.3. Bone Growth

Bone growth was examined using two different methods. First, a toluidine blue stain was used to measure the percent bone contact with the implant (in particular the threads of the pin). Toluidine blue specifically stains proteoglycans, the major component of the extracellular matrix that forms large complexes with collagen in bone. Toluidine blue has been used in numerous other in vivo studies to histologically evaluate new bone formation (direct bonding of the implant to bone) on the Ti implant [357-366]. Compared to conventional Ti6Al4V pins (10.22%), images revealed a higher percentage of toluidine blue at the threads on the nanofabricated Ti6Al4V pins (nanorough, 34.63%, and nanotubular, 20.96%) as indicated in Figure 6.6. Thus, in vivo data demonstrated that the nanorough and nanotubular (20.96 Ti6Al4V pins enhanced bone-to-implant contact indicating better bone growth on these nanofabricated materials compared to the conventional Ti6Al4V pins.
Figure 6.6. Toluidine blue histology stains of the (a) conventional (control), (b) nanorough (created through electron beam evaporation), and (c) nanotubular (created through anodization) Ti6Al4V pins after 28 days. Clearly, these slides revealed that the nanofabricated surfaces promoted bone growth around the threads of the pins (indicated by the blue stain) compared to the control pins that showed minimal new bone growth (lack of blue stain around the threads).
The second method involved injecting doxycycline (a fluorescent bone label also known as tetracycline) into the rats on the 24th day (4 days prior to sacrifice). The doxycycline is absorbed into bone and forms a stable complex with calcium phosphate to provide dynamic information about bone formation and remodeling. Like toluidine blue, tetracycline is another common histological stain used to examine bone remodeling activity and obtain better timeline for the development of new bone growth [359, 367]. These images revealed bone formation was active for the nanorough and nanotubular Ti6Al4V pins up until the time of animal sacrifice (Figure 6.7). In fact, bone bridging between the gaps of the threads was seen in the images. For the control pins, bone formation was decidedly less at the time of animal sacrifice as indicated by the lack of fluorescence from the dye (Figure 6.7).
Figure 6.7. Doxycycline (tetracycline) histology stains of the (a) conventional (control), (b) nanorough (created through electron beam evaporation), and (c) nanotubular (created through anodization) Ti6Al4V pins after 28 days. These slides revealed bone formation was active at the time of animal sacrifice for the nanofabricated surfaces. For the control pins, the bone formation in the last week of the experiment was decidedly less as indicated by the lack of fluorescence from the dye. Magnification = 20x.
6.3.4. Skin Growth

To examine skin growth, hematoxylin and eosin (H&E) stain was used (a common dye used to examine the results of in vivo studies [362]) as indicated in Figure 6.8. Due problems that occurred during processing (as described in the next sections), the results were inconclusive. However, photographs of the conventional, nanorough, and nanotubular Ti6Al4V pins were taken of each rat at weeks 2, 3, and 4 to examine the ITAP exit site through the skin. Despite proper histology, better skin growth was qualitatively observed throughout the duration of the experiment on the nanorough and nanotubular Ti6Al4V pins compared to the conventional Ti6Al4V pins as revealed in the photographs (Figure 6.9). Drainage and inflammation was seen in all rats containing the control Ti6Al4V preventing proper skin growth around the tissue-implant interface. A lack of drainage and little inflammation resulted in what appeared to be better skin integration for most rats containing the nanorough and nanotubular Ti6Al4V pins compared to conventional Ti6Al4V.
Figure 6.8. Hematoxylin and eosin (H&E) stains of the (a) conventional (control), (b) nanorough (created through electron beam evaporation), and (c) nanotubular (created through anodization) Ti6Al4V pins after 28 days. Due to problems encountered during processing, these slides were inconclusive. However, photographs seen in Figure 6.9, revealed improved skin function on the nanorough and nanotubular pins compared to the conventional pins.
Figure 6.9. Photographs taken of the rats over the duration of the experiment (14, 21, and 28 days) to monitor the area at the exit site of the (a) conventional, (b) nanorough, and (c) nanotubular Ti6Al4V pins. These photographs qualitatively revealed better skin growth for the nanofabricated pin surfaces by decreased puss and inflammation and improved skin closure.
6.3.5. Problems Encountered During In Vivo Study

Although this in vivo study provided qualitative evidence that Ti6Al4V pins, designed to mimic an ITAP, fabricated through electron beam evaporation and anodization improved bone and skin growth compared to conventional Ti6Al4V pins, quantitative data was inconclusive. This was due to some problems encountered during this in vivo experiment. First, the surgical technique was inconsistent and needs to be further optimized. In this study, the pin did not consistently fill the intramedullary canal nor was consistently in contact with cortical bone. This was a result of a long, curved rat femur that prevented the pin from being properly inserted consistently from implantation to implantation. Future studies using a shorter, wider pin would be more ideal for this particular animal model. In addition, the method by which the transcutaneous piece was inserted through the skin varied during surgery. For example, some of the skin was left loose while at other times, it was folded back. Piercing the distal end with a hole that would enable the skin to be held to the pin through sutures would resolve this problem. Furthermore, future studies should remove all hair from around the surgical area on the animal to prevent disruption of the natural wound healing process.

There were also some histological processing difficulties of the pins in this study. More specifically, the pins were intended to be cut transversally
rather than sagittally into three sections for each of the three histological stains examined (Toluidine blue, tetracycline, and hematoxylin and eosin). Further studies should involve better processing techniques to improve histological examination and provide more conclusive data.

Due to these problems, toluidine and tetracycline stains could not be quantified and thus observations were strictly qualitative. In addition, ways to measure dermal attachment to the ITAP devices quantitatively needs to be determined. More generally speaking, using a different, larger animal model, such as the miniature guinea pig, may be more ideal for such an in vivo experiment [368-370]. In fact, it has been demonstrated that the guinea pig is a suitable model for histological evaluation skin and bone to the implant of an ITAP [368]. However, it is important to note that despite these problems encountered, this preliminary study provided promising results that surfaces created by electron beam evaporation as well as anodization could enhance bone and skin growth for ITAP devices and, thus, should be further investigated.

6.4 Conclusions

Throughout the previous chapters of this dissertation, in vitro data revealed the positive effect of nanorough and nanotubular Ti, created through electron beam evaporation and anodization respectively has, on bone and skin growth as well as infection. To obtain a better understanding of the influence of such
surface features, a preliminary in vivo study using a rat model was completed over the course of 28 days. Although problems were noted during the experiment, qualitative data revealed that nanofabricated Ti6Al4V surfaces promoted bone ingrowth compared to conventional surfaces. Histology studies revealed prominent bone growth around the threads of the screws and bone formation was continuous up until the time of animal sacrifice only for the nanofabricated pins. For skin growth, it was found that conventional pins resulted in drainage and extensive inflammation that prevented what appeared to be the formation of a skin seal as occurred on the nanofabricated pins. Therefore, the results from this in vivo study provided evidence that certain nanofabrication techniques (such as electron beam evaporation and anodization) should be further researched as a means to improve ITAP Ti implant longevity and efficacy.
Chapter 7

Conclusions and Future Direction

This study developed a new process, electron beam evaporation, which was shown to improve the function of titanium (Ti) for bone growth. More specifically, results demonstrated greater osteoblasts adhesion, proliferation, and differentiation on nanopatterned Ti substrates (with alternating regions of nanorough and conventional surfaces) with the most nanorough surface area (largest nanopattern width) compared to conventional Ti surfaces (control). Furthermore, greater osteoblasts alignment to the Ti nanopatterns was observed on those with the smallest groove dimensions. It was also found that these nanopatterns directed collagen synthesis thereby mimicking the natural orientation of bone. It was found that the mechanism for such osteoblast behavior was due to greater surface energy on the nanorough
regions which lead to improved fibronectin adsorption, a protein known to enhance osteoblast interaction with the implant surface. Therefore, from this part of the research, design recommendations for improving the Ti fixture of the ITAP implanted into the femur included creating nanopatterned surfaces with small grooves possessing only nanofeatures.

This study also examined two nanofabrication techniques, electron beam evaporation and anodization, for improving function of Ti for skin growth. Results demonstrated greater adhesion (and morphology) of keratinocytes on surfaces with nanofeatures (created through both electron beam evaporation and anodization) compared to conventional surfaces. It was further revealed that greater keratinocyte proliferation (and morphology) occurred on the nanorough Ti surface created by electron beam evaporation compared to conventional Ti surfaces. Similar to the osteoblast studies, it was found that greater surface energy lead to improved fibronectin adsorption which enhanced keratinocyte behavior. Therefore, it is recommended to design a Ti abutment with nanorough features created through electron beam evaporation.

To further improve keratinocyte function, Ti surfaces were functionalized with growth factors. More specifically, this research demonstrated that fibroblast-growth-factor-2 (FGF-2) was successfully bound to the surfaces of the nanotubular and nanorough Ti samples. Furthermore, this chemical
functionalization of FGF-2 was shown to enhance keratinocyte proliferation compared to their non-functionalized counterparts. Thus, another design recommendation would be if using nanotubular Ti surface (created through anodization) for enhancing skin growth, they should be functionalized with FGF-2.

In order to examine the effect that these nanofabricated Ti surfaces may have on infection, bacteria adhesion studies were completed. Results demonstrated decreased bacteria attachment on nanorough Ti surfaces created by electron beam evaporation compared to conventional Ti surfaces. It also demonstrated that bacteria increased on surface created through anodization. Such results identified that bacteria is linked to both chemistry and crystallinity of a surface. Thus, these results further confirm the idea of modifying the Ti abutment with nanorough features created through electron beam evaporation in order to improve keratinocyte function as well as to decrease bacteria attachment.

Finally, an in vivo study was completed on model ITAP using Ti implants modified by these novel nanofabrication methods. Bone histology demonstrated better bone growth on these nanostructure surfaces while histology also showed better skin growth on these nanostructure surfaces, particularly demonstrated through the lack of inflammation and formation of puss compared to the conventional Ti implants used as the control.
Thus, from the data completed in this research, it is concluded that the current ITAP surfaces should be modified so that the Ti fixture implanted into femur possess nanopatterned Ti surfaces of alternating nanorough regions and nanotubular regions with groove widths being smaller than the average size of an osteoblast (10-40 µm). SEM micrographs of such surfaces can be found in Figure 7.1. The Ti abutment should be modified to contain nanorough features created through electron beam evaporation as a means of enhancing skin growth. The design can be seen in Figure 7.2.
Figure 7.1. SEM micrographs of nanopatterned Ti substrates with alternating regions of nanotubular and nanorough features. The dimensions are 22 µm for the nanorough regions and 40 µm for the nanotubular regions.
Figure 7.2. Newly modified ITAP through the use of electron beam evaporation and anodization as a means of improving bone and skin growth. The Ti fixture contains nanopatterns of alternating nanorough and nanotubular regions while the Ti abutment consists of only nanorough Ti features.
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Appendix A – Versatility of Electron Beam Evaporation

To determine the versatility of electron beam evaporation, altering the surface of polymers found in biomaterials (including polyethylene (PE), silica, and Teflon) to possess nanorough features created by this process was examined. Results successfully showed that, in addition to metals, polymers can also be coated through electron beam evaporation. SEM revealed that, similar to Ti, polymers with an electron beam coated surface contained nanorough topographies when compared to their conventional counterparts (Figure A1).

Figure A1. SEM micrographs of polyethylene (PE) (a) before and (b) after electron beam evaporation. These images reveal a distinct difference in surface topography with the original PE containing conventional features while the electron beam evaporated PE surfaces contained nanorough features. Scale bar = 1 µm.

Contact angles were completed on the PE surfaces and revealed that the nanorough PE surfaces created through electron beam evaporation were more hydrophilic than the conventional PE (Table A1). These contact angles
were used to determine surface energy of these polymers, and it was found that nanorough PE possessed higher surface energy than the conventional PE surfaces (Figure A2).

Table A1. Contact angles of three liquids on conventional Ti, nanorough Ti, nanotubular Ti, and nanotextured Ti surfaces. Contact angle data was used to determine surface energy via Owens-Wendt equation.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Contact Angle of DI Water</th>
<th>Contact Angle of Glycerol</th>
<th>Contact Angle of PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional PE</td>
<td>95.60 ± 0.32</td>
<td>69.79 ± 1.18</td>
<td>41.40 ± 0.78</td>
</tr>
<tr>
<td>Nanorough PE</td>
<td>55.08 ± 1.64</td>
<td>69.90 ± 1.52</td>
<td>18.80 ± 1.71</td>
</tr>
</tbody>
</table>

Figure A2. Surface energy of the conventional PE and nanorough PE surfaces. Surface energy was calculated for each sample by measuring the contact angles of three liquids at the sample surface and entering values into the Owens-Wendt equation. Results indicated greater surface roughness at the nanoscale correlated to increased surface energy. Data are mean ±SEM; n=4. *p<0.01 compared to conventional PE.

To further test these substrates, cellular assays were completed. More specifically, osteoblast adhesion (four hours) was performed (Figure A3) and revealed significantly greater (p<0.01) osteoblast adhesion on nanorough Ti surfaces compared to the conventional Ti surfaces (as seen with nanorough Ti compared to conventional Ti demonstrated in Chapter 2). Thus, electron
beam evaporation is a versatile nanofabrication method that can be use on a variety of materials (metals, polymers, etc) used to make prosthetic devices and should be further investigated.

Figure A3. Greater osteoblast adhesion on nanorough PE (created through electron beam evaporation) compared to conventional PE surfaces after four hours. Data are mean ±SEM; n=3; *p<0.01 compared to conventional PE.
Appendix B · Problems Experienced with Titanium Foils from Alfa Aesar

Early on in this research, Alfa Aesar discontinued carrying the exact Ti substrates used throughout the preliminary studies and began sending substrates that had a very distinct surface difference. The catalog number was the same, and it was claimed by several Alfa Aesar representatives that they had not changed their items. They researched the products and said that the chemical treatment used to create the Ti substrates has not changed. However, the Ti substrates used throughout preliminary studies had a pickled, rough surface while the new Ti had a smooth, polished surface. SEM images comparing the surface topography of old and new Ti samples were also taken to verify this difference at higher magnifications (Figure B1). The micrographs revealed a clear difference in the roughness as revealed to the naked eye.
Figure B1. SEM images comparing the original Ti samples (those used throughout the preliminary study) and the new Ti samples (item number was the same and no alteration in chemical treatment was found by Alfa Aesar). There was a clear, distinct difference in the topography despite the fact Alfa Aesar claimed they were the same samples.

Alterations in the surface topography led to problems in the research. More specifically, after electron beam evaporation, there was a large amount of flaking on the new Ti specimens. In other words, once samples were removed from the electron beam evaporator, the deposited material could be easily blown off. Furthermore, upon cleaning, Ti particles were seen floating in the solution and the original surface was exposed. This too was verified by SEM images which revealed the original micron rough areas in addition to the nanorough areas that were still maintained (Figure B2). Because the Ti coating was not adhering to the Ti substrate surface, cellular experiments could not be completed because they could easily engulf the Ti particles which would lead to cell death.
Therefore, the goal was to now determine a way to create a more energetically favorable surface for the Ti to deposit onto. One method was to treat/rinse the Ti samples in nitric acid (HNO₃) as a way to remove the thin Ti oxide layer that spontaneously forms in the air. More specifically, Ti specimens were soaked directly into a solution of 100% HNO₃ for two minutes and then rinsed thoroughly with deionized water. Furthermore, due to the rising popularity and interest of anodized Ti, such specimens were also used to examine the strength of attachment of Ti nanoparticles. To examine the adhesion strength of each of these specimens (the old Ti, the new Ti, the HNO₃ treated Ti, and anodized Ti), standard scotch tape was placed firmly on the substrates after electron beam evaporation and then pulled off (ASTM standard B 571 – 97, Standard Practice for Qualitative Adhesion Testing of
Metallic Coatings. Upon analyzing the tape, the strength of adhesion of the Ti on the surface was examined. The anodized Ti specimens had a surface that created the strongest adherence (indicated by minimal coating on the tape), followed by the untreated specimens and then the HNO₃ treated samples (Figure B3). The HNO₃ treated specimens were thought to create an energetically favorable surface and the new Ti specimens were thought to have the least energetically stable surface for adherence of the Ti coating. However, based on visual appearance after electron beam deposition; the exact opposite occurred. Based on the tape test, 100% of the deposited Ti nanoparticles were removed on the HNO₃ specimens, an environment that does not support cell survival.

Interestingly, the substrates sent from Alfa Aesar (same item number) were once again different in their surface appeared. This change was to the advantage of this research as strong coating adhesion was found for the new Ti samples. Thus, the Ti foils were ordered in large bulk amounts to prevent this problem from happening again and were used for all experiments described throughout this dissertation.
samples directly after electron beam deposition

 grids were gently removed, and scotch tape was placed onto the specimens and removed

Old Ti Substrate

New Ti Substrate

Anodized Ti Substrate

HNO₃ Ti Substrate

Figure B3. Tape test demonstrating the strength of Ti coating after electron beam evaporation. Dark regions = Ti coating not adherent to surface (indicating lack of adhesion).