

NANO-FABRICATION AND CHARACTERIZATION OF NOVEL TITANIUM
SURFACES FOR VASCULAR STENT APPLICATION

BY

JING LU

B.S., SHANGHAI JIAOTONG UNIVERSITY, 2003

M.S., ECOLE CENTRALE DE LYON, 2005

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN ENGINEERING AT BROWN UNIVERSITY

PROVIDENCE, RHODE ISLAND

MAY 2010

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This dissertation by Jing Lu is accepted in its present form
by the Division of Engineering as satisfying the
dissertation requirement for the degree of Doctor of Philosophy.

Date_____

Thomas J. Webster, Advisor

Recommended to the Graduate Council

Date_____

Robert Hurt, Reader

Date_____

Derek Stein, Reader

Date_____

Christian Franck, Reader

Date_____

Eric Darling, Reader

Date_____

Jeffrey Karp, Reader

Approved by the Graduate Council

Date_____

Sheila Bonde, Dean of the Graduate School

CURRICULUM VITAE

Jing Lu was born on September 9, 1981, in Shanghai, P.R. China. She attended Shanghai Jiaotong University in 1999 and got her B.S. degree in Materials Science and Engineering in June, 2003. She entered Ecole Centrale de Lyon in September 2003 and received her M.S. degree in Materials Engineering and her second M.S. degree in Friction Engineering in September, 2005. She was admitted to the Biomedical Engineering program at Brown University in January, 2006.

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1. **Lu J**, Rao MP, MacDonald NC, Khang DW and Webster TJ. Improved endothelial cell responses on nanometer patterned titanium surface features. *Acta Biomaterials*, 2008 Jan;4(1):192-201.
2. **Lu J**, Khang D, Yao C, Haberstroh KM and Webster TJ. The role of nanometer and sub-micron surface features on vascular and bone cell adhesion on titanium, *Biomaterials*, 2008 Mar;29(8):970-83
3. Samaroo H, **Lu J** and Webster TJ. Enhanced Endothelial Cell Adhesion and Proliferation on Nitinol Surfaces with Sub-Micron to Nanometer Roughness.

International Journal of Nanomedicine, 2008 Mar;3(1):75-82

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6. Lu J and Webster TJ. Endothelial cell adhesion on highly controllable compared to random nanostructured titanium surface features. 2006 Materials Research Society Conference Proceedings, 0951-E12-29

7. Lu J and Webster TJ. Highly controlled versus random nanometer roughness on Ti for vascular stent applications. 2006 e-MRS Conference Proceeding.

8. Lu J, Khang DW and Webster TJ. Enhanced Vascular Endothelial Cell Function on Nanostructured Titanium Surface Features: the Roles of Nano to Submicron Roughness, 2008 Materials Research Society Conference Proceedings, 1136-DD04-04

ACKNOWLEDGMENTS

First of all, I would like to thank my advisor Prof. Thomas J. Webster for his guidance and support in my graduate research. In addition, I would also like to thank Prof. Robert Hurt, Prof. Derek Stein, Prof. Christian Franck and Prof. Jeffrey Karp from Harvard-MIT Division of Health Sciences and Technology (HST) for serving as my committee members and for their suggestions during my thesis preparation. Similarly, I would like to thank Prof. Jeffrey Morgan and Prof. Edith Mathiowitz for being my preliminary exam committee members.

I would like to thank Harry Samaroo and Margaret Merritt, the two undergraduates at Brown University, for their help in the projects of NiTi surface characterization and flow system set-up, respectively. I would thank Anthony McCormick who taught me to use the scanning electron microscope, as well as Dr. Robert Creton and Geoffrey Williams who assisted me in using the fluorescence and confocal microscopes.

I would also like to extend my acknowledgement to Sandra Spinacci, Diane Felber, Patricia Capece, Cheryl Carvalho and the other secretaries in Division of Engineering for their assistance. Moreover, I would thank all the members of

Prof. Webster's Nanomedicine Laboratory for their help, suggestions and friendship during past four years.

Finally, this thesis is dedicated to my parents, Defu Lu and Fanling Kong, and my fiancé Chang.

TABLE OF CONTENTS

	Page
COPYRIGHT PAGE.....	ii
SIGNATURE PAGE.....	iii
CURRICULUM VITAE	iv
ACKNOWLEDGMENTS	vi
TABLE OF CONTENTS.....	viii
LIST OF TABLES	xviii
LIST OF FIGURES	xix
CHAPTER 1. INTRODUCTION.....	38
1.1. Artery Physiology.....	38
1.1.1. Anatomy of the Artery.....	38
1.1.2. The Arterial Wall	40
1.1.2.1. The Tunica Intima.....	40
1.1.2.2. The Tunica Media.....	41
1.1.2.3. The Tunica Adventitia.....	43
1.1.3. Arterial Cells	44
1.1.3.1. Endothelial Cells.....	44
1.1.3.2. Smooth Muscle Cells.....	46
1.1.4. Function of the Normal Artery.....	50

1.1.4.1. Vascular Remodelling	50
1.1.4.2. Hemodynamics of Blood Flow	52
1.2. Atherosclerosis	55
1.2.1. An Overview of Atherosclerosis	55
1.2.2. Atherosclerosis Initiation	57
1.2.3. Previous Atherosclerosis Theories	59
1.2.4. Roles of Cells Involved in Atherosclerotic Lesions	60
1.2.4.1. The Functions of the Endothelium in Vascular Disease	60
1.2.4.2. Factors that Induce Endothelium Dysfunction	61
1.2.4.3. Smooth Muscle Cells	63
1.2.4.4. Macrophages	65
1.2.4.5. Overview of Cytokines	66
1.2.4.6. TNF- α	70
1.2.4.7. IL-1	70
1.2.4.8. IL-6	71
1.3. Treatments to Atherosclerosis	72
1.3.1. The Need for More Efficient Atherosclerosis Treatments	72
1.3.2. Coronary Artery Bypass Grafting	74
1.3.3. Balloon Angioplasty and Restenosis	74
1.3.4. Vascular Stenting and In-stent Restenosis	76
1.3.5. Improvements on Current Vascular Stents	79
1.3.5.1. Drug-eluting Stents	79
1.3.5.2. Other Therapies	82

1.4. Titanium Used for Cardiovascular Applications	83
1.4.1. Titanium Chemistry and Crystal Structure	84
1.4.2. Physical and Mechanical Properties	85
1.4.3. Chemical Properties	87
1.5. Nano/microtextured Template Techniques in Tissue Engineering and Cardiovascular Device Design	88
1.5.1. Review of Nano/microtextured Template	88
1.5.2. Micro/nano Fabrication in Tissue Engineering	91
1.5.2.1. Photolithography	92
1.5.2.2. Electron Beam Evaporation	94
1.6. Tissue-Implant Interactions	96
1.6.1. Protein-Implant Interactions	97
1.6.2. Cell-Implant Interactions	98
1.6.3. Cell-Implant Interactions Under Shear Stress	100
1.7. Objectives	103
CHAPTER 2. Materials and Methods	106
2.1. Substrate Preparation	106
2.1.1. Etched Glass	106
2.1.2. Random Nanostructured Titanium	106
2.1.3. Random Nanostructured Nitinol (NiTi)	107
2.1.4. Highly Controllable Nanostructured (Nano/micropatterned) Titanium Surface Features	108

2.1.5. Nano and Sub-micron Titanium Surface Roughness on Borosilicate Glass Coverslips.....	110
2.1.6. Highly Aligned Patterns of Titanium Flat, Nano and Sub-micron Surface Roughness	111
2.2. Surface Chemistry and Topography Characterization (FESEM, EDS and AFM).....	113
2.2.1. Scanning Electron Microscopy	113
2.2.2. Atomic Force Microscopy	113
2.2.3. Surface Energy and Contact Angles	114
2.3. Vascular Cell Responses	115
2.3.1. Vascular Cell Culture.....	115
2.3.1.1. Rat Aortic Endothelial Cell (RAEC) Culture.....	115
2.3.1.2. Rat Aortic Smooth Muscle Cell (RASMC) Culture.....	115
2.3.2. RAEC Responses on Highly Controllable Nanostructured Surface Features: The Role of Pattern Dimension on RAEC Functions.....	116
2.3.2.1. RAEC Adhesion on Highly Controllable Nanostructured Surface Features	116
2.3.2.2. RAEC Proliferation on Highly Controllable Nanostructured Surface Features	116
2.3.2.3. RAEC Viability on Highly Controllable Nanostructured Surface Features	117
2.3.3. RAEC Responses on Random Nanostructured NiTi (Nitinol) Surface Features	117

2.3.3.1. RAEC Adhesion on Random Nanostructured NiTi (Nitinol) Surface Features	117
2.3.4. RAEC and RASMC Responses on Non-patterned and Patterned Titanium Substrates with Different Roughness by E-beam Evaporation: The Role of Nano and Sub-micron Surface Features.....	118
2.3.4.1. RAEC Adhesion Bulk and Patterned Titanium Substrates with Different Roughness.....	118
2.3.4.2. Co-culture Cell Adhesion on Bulk Titanium Substrates with Different Roughness.....	119
2.3.4.3. RAEC and RASMC Proliferation on Bulk Titanium Substrates with Different Roughness.....	120
2.3.4.4. Co-culture Cell Proliferation.....	121
2.3.4.5. RAEC and RASMC Morphology on Bulk Titanium Substrates with Different Roughness.....	121
2.3.4.6. Immunofluorescence of Vinculin during RAEC Attachment on Bulk Titanium Substrates with Different Roughness	122
2.3.4.7. Protein Adsorption (Fibronectin, Vitronectin and Fibrinogen) on Bulk Titanium Substrates with Different Roughness	123
2.3.4.8. RAEC NOx and Endothelin-1 Functional Assays on Bulk Titanium Substrates with Different Roughness	125
2.3.4.9. Long-term RAEC and RASMC Functional Assays on Bulk Titanium Substrates with Different Roughness	127

2.3.4.10. RAEC Mobility Assay on Bulk Titanium Substrates with Different Roughness	130
2.3.5. Osteoblast Responses	131
2.3.5.1. Human Osteoblast (HOB) Culture	131
2.3.5.2. Osteoblast (HOB) Responses on Patterned Titanium Substrates Created with Different Roughness by E-beam Evaporation	132
2.3.6. Immune Responses	133
2.3.6.1. Mouse Macrophage Culture	133
2.3.6.2. Human Monocytes	133
2.3.6.3. Macrophage Density Assays	133
2.3.6.4. Monocyte Adhesion on Titanium Substrates with Different Roughness	134
2.3.6.5. Monocyte Adhesion on RAEC Monolayers on Titanium Substrates with Different Roughness	135
2.3.6.6. Monocyte Differentiation on Titanium Substrates with Different Roughness	136
2.3.6.7. Cytokine Release from Macrophages Attached on Titanium Substrates with Different Roughness	137
2.4. Laminar Flow in the Vessel Model	138
2.4.1. The Vessel Flow Model in a Home-made Flow System	138
2.4.2. Coating Stability Test	142
2.4.3. Dynamic RAEC Adhesion Test on Titanium Substrates with Different Roughness	142

2.4.4. Dynamic Monocyte Adhesion Test on Titanium Substrates with Different Roughness	143
2.5. Statistical Analysis	144
CHAPTER 3. RESULTS	145
3.1. Characterization of Nanostructured Titanium Surfaces	145
3.1.1. Random Nanostructured Titanium.....	145
3.1.2. Random Nanostructured Nitinol (NiTi).....	146
3.1.3. Highly Controllable Nanostructured (Nano/Micropatterned) Titanium Surface Features Created by TIDE	147
3.1.4. Nano and Sub-micron Titanium Surface Roughness on Borosilicate Glass Coverslips Created by E-beam Deposition	150
3.1.5. Highly Aligned Patterns of Flat, Nano and Sub-micron Surface Roughness Created by E-beam Deposition	155
3.1.6. Surface Chemistry and Surface Energy	158
3.2. Vascular Cell Responses on Various Titanium Surfaces Created in the Present Study	160
3.2.1. RAEC Responses on Highly Controllable Nanostructured Surface Features: The Role of Pattern Dimension on RAEC Functions.....	160
3.2.2. RAEC Responses on Random Nanostructured NiTi (Nitinol) Surface Features	167
3.2.3. RAEC and RASMC Responses on Non-patterned and Patterned Titanium Substrates with Different Roughness by E-beam Evaporation: The Role of Nano and Sub-micron Surface Features.....	171

3.2.3.1. RAEC Adhesion on Titanium Substrates with Different Roughness Created by E-beam Deposition	171
3.2.3.2. RAEC Adhesion on Patterned Titanium Substrates with Different Roughness	173
3.2.3.3. RAEC and RASMC Proliferation on Titanium Substrates with Different Roughness Created by E-beam Deposition.....	176
3.2.3.4. RAEC and RASMC Morphology on Titanium Substrates with Different Roughness Created by E-beam Deposition.....	179
3.2.3.5. Co-culture of HAEC and RSMC Adhesion on Titanium Substrates with Different Roughness Created by E-beam Deposition	182
3.2.3.6. Co-culture Cell Proliferation on Titanium Substrates with Different Roughness	183
3.2.3.7. Protein Adsorption (Vitronectin, Fibronectin and Fibrinogen) on Titanium Substrates with Different Roughness Created by E-beam Deposition.....	185
3.2.3.8. RAEC NO _x and Endothelin-1 Functional Assays on Titanium Substrates with Different Roughness Created by E-beam Deposition	187
3.2.3.9. Long-term Cell Function Assays of RAEC and RASMC on Titanium Substrates with Different Roughness Created by E-beam Deposition	189
3.2.3.10. RAEC Mobility Assay on Titanium Substrates with Different Roughness	193
3.2.4. Immune Cell Responses	196

3.2.4.1. Macrophage Density Assays Titanium Substrates with Different Roughness	196
3.2.4.2. Monocyte Adhesion	201
3.2.4.3. Monocyte Adhesion on Titanium Substrates with Different Roughness Created by E-beam Deposition	202
3.2.4.4. Monocyte Adhesion on RAEC Monolayers on Titanium Substrates with Different Roughness Created by E-beam Deposition	203
3.2.4.5. Monocyte Differentiation on Titanium Substrates with Different Roughness Created by E-beam Deposition	204
3.2.4.6. Cytokine Release from Macrophages on Titanium Substrates with Different Roughness Created by E-beam Deposition.....	205
3.3. The Vessel Flow Model in a Home-made Flow System	209
3.3.1. Coating Stability Test.....	209
3.3.2. Dynamic RAEC Adhesion Test Under Laminar Flow	210
3.3.3. Dynamic Monocyte Adhesion Test Under Laminar Flow.....	213
3.4. Osteoblast Responses	214
3.4.1. Osteoblast Adhesion Titanium Substrates with Different Roughness	214
CHAPTER 4. DISCUSSION.....	216
4.1. Comparison of Micro/Nanofabrication Techniques Used in the Current Study	216
4.2. Using E-beam Deposition to Create Different Roughness and Patterns: Mechanisms and Surface Properties.....	220

4.3. Nanostructured NiTi Created by Cold Compaction Methods for Regulating Vascular Responses	227
4.4. Surface Topography (Nano/Sub-micron Titanium Patterning) Created by TIDE Lithography and E-beam Deposition for Regulating Vascular Cell Functions	230
4.4.1. Vascular Cell Function on TIDE Created Patterned Surfaces	230
4.4.2. Vascular Cell Function on E-beam Deposition Created Patterned Surfaces	232
4.5. Surface Roughness and Surface Energy for Regulating Vascular Endothelial Cell Functions on E-beam Deposition Created Surfaces	234
4.6. Comparison of Vascular Endothelial Cell and Smooth Muscle Cell Functions on E-beam Deposition Created Surfaces with Various Roughness and Surface Energy	241
4.7. Protein Adsorption	244
4.8. Role of Sub-micron and Nano Surface Structures on Endothelial Cell Migration	249
4.9. Immune Responses	251
4.10. Endothelial Cell and Monocyte Adhesion Under Flow	254
4.11. Future Directions	257
CHAPTER 5. CONCLUSIONS	264
LIST OF REFERENCES	268

LIST OF TABLES

Table	Page
Table 1-1 Compounds secreted by the endothelium and their respective activities.....	46
Table 1-2 List of important pro- and anti-inflammatory cytokines (Adapted from [66]).	67
Table 1-3 Physical properties of titanium.	86
Table 1-4 Mechanical properties of selected titanium, titanium alloys (Adapted from [122]).	87
Table 3-1 EDS analysis for flat, nano and sub-micron titanium surfaces. There were no significant changes in surface chemistry for flat (F-Ti), nano (N-Ti) and sub-micron (S-Ti) structured titanium surfaces (errors were $\pm 0.031\%$). Some Si and K signals were noticed due to the presence of the glass substrate beneath the titanium deposition.....	158
Table 4-1 Comparison of three fabrication techniques used in the present study.	220

LIST OF FIGURES

Figure	Page
Figure 1.1 Schematic showing the structure of the aorta (Retrieved and redrawn from [1]).	39
Figure 1.2 Three-layered anatomy of the arterial wall (Adapted and redrawn from [6])......	41
Figure 1.3 The longitudinal view of the arterial wall. (A) Micrograph of the tunica intima, media and adventitia (Adapted and redrawn from [10]), bar=100 μm ; (B) Micrograph of the endothelial cell lining (Retrieved and redrawn from [11]), bar=10 μm	42
Figure 1.4 Collage type I and III in the tunica media (Adapted and redrawn from [12]) bar=10 μm	43
Figure 1.5 The tunica adventitia of the artery (Adapted and redrawn from [13]), bar=10 μm	44
Figure 1.6 Surface of the endothelial cell layer covered with atherosclerotic plaques (indicated with arrows) (Adapted and redrawn from [11]), bar=100 μm	45
Figure 1.7 Characteristics of the vascular smooth muscle cell phenotype (Adapted and redrawn from [22])......	49

Figure 1.8 The classification of vascular remodeling (circles represent top view of blood vessels). A) Decreased vessel luminal dimensions in response to a decreased blood flow velocity; B) Increased vessel luminal dimensions in response to an increased flow velocity; C) Increased medial thickness in response to vascular hypertension and vascular hypertrophy; and D) Unchanged medial thickness in response to vascular hypertension (Adapted and redrawn from [35]).	52
Figure 1.9 Schematic of parabolic flow through a blood vessel.	53
Figure 1.10 Atherosclerosis development process: (A). Structure of the normal arterial wall; (B). The initial event in atherosclerosis development; (C). Formation of fatty streak lesions; (D). Formation of proliferative or fibrous lesions; (E). Formation of a complicated lesion (Adapted from [45]).	57
Figure 1.11 Cytokines involved in atherosclerosis and their cellular source and targets (adapted and redrawn from [68]).	69
Figure 1.12 Leading causes of death in the United States in 2004 [77] (CLR: Chronic Lower Respiration; CVD: Cardiovascular Disease).	73
Figure 1.13 Schematic of balloon angioplasty (Adapted from [82]).	75
Figure 1.14 Schematic of a vascular stenting procedure. A) Implantation of a cardiac catheter in an inflatable balloon and wire-mesh stent; B) Inflation of the balloon to expand the stent and dilate the artery; and C) Deflation and withdrawn of the balloon with the catheter to leave the stent expanded against the arterial wall. (Adapted from [92]).	77

Figure 1.15 Risks associated with drug-eluting stents and bare-metal stents: Drug-eluting stents can inhibit in-stent restenosis in bare-metal stents, however, uncovered struts may be prone to late thrombosis after the discontinuation of drug release. (Adapted from [111]).....	81
Figure 1.16 Crystal structures of (a) alpha and (b) beta phase titanium. Lattice parameters for hcp alpha titanium are: $a = 2.95 \text{ \AA}$, $c = 4.68 \text{ \AA}$ [120] (Adapted from [121]).	85
Figure 1.17 Crystallographic unit cell of rutile and anatase titanium dioxide: (a) rutile; (b) anatase. White small balls represent titanium cations while dark large balls represent oxygen anions (Adapted from [124]).....	88
Figure 1.18 Scanning electron micrograph of the HeartMate [®] integrally textured fiber surface. Bar = 200 μm (Adapted from [133])	91
Figure 1.19 Atomic force microscope (AFM) images of a PLGA cast nanosphere (200 nm) surface. Bar = 150 nm (Adapted and redrawn from [134]).....	91
Figure 1.20 Schematic of micro/nanopatterning by (a) photolithography; and (b) microcontact printing (Adapted and redrawn from [137]).	94
Figure 1.21 Schematic of E-beam evaporation process (Adapted and redrawn from [143]).	96
Figure 1.22 Schematic illustration of the electrochemical double layer formed at the titanium/electrolyte interface. IHP and OHP, inner and outer Helmholtz planes, respectively. (Adapted and redrawn from [146]).	98
Figure 1.23 Schematic of the interaction of cells to an implanted surface as mediated by proteins (Adapted and redrawn from [151]).	100

Figure 2.1 Pattern dimension distribution in the two kinds of multiplexed chip design. (A) Pattern dimensions from 750 nm to 1 μ m; and (B) Pattern dimensions from 1 μ m to 100 μ m.	109
Figure 2.2 Design schematic of the Panasonic E640-ICP dry etching system [167].....	110
Figure 2.3 Fabrication of flat, nano and sub-micron surface roughness on bulk and aligned titanium surfaces. (a) A conventional e-beam deposition rate (2 $\text{\AA}$ /sec) generated flat titanium (F-Ti) surfaces on glass coverslips (50nm of thickness). (b) A high e-beam deposition rate (20 $\text{\AA}$ /sec) generated nano surface roughness (N-Ti) after a thickness of 50nm on F-Ti. High deposition rates with a thickness of 1 μ m generated sub-micron surface structures (S-Ti). The inset image shows different transparency for these substrates because of the different deposition thickness on glass coverslips. Sub-micron titanium surfaces were opaque. (c) Schematic of aligned micron patterns of different surface roughness (submicron, nano surface or flat). A titanium thin layer (50nm) on glass coverslips was used as a base substrate. A metal grid (Ni/Au/Cu or shadow mask) with aligned grooves (width of 30 μ m) was placed on F-Ti (see (a)). Nano or sub-micron rough titanium was coated through the open grooves (respectively) as described in (a) and (b). Then, the shadow mask was removed from F-Ti to generate aligned patterns of two different surface roughness values on titanium.	112

Figure 2.4 Parallel flow chamber showing (A) images of the installed flow chamber with the fittings. And (B) schematic diagram of the flow chamber structure.....	140
Figure 2.5. Image of the medium reservoir with the 3-hole cap.	141
Figure 3.1 SEM image of random nanostructured titanium surface features at (a) low magnification, bar = 1 μm and at (b) high magnification bar = 200 nm.	146
Figure 3.2 SEM images of micro and sub-microstructured NiTi surfaces. (a) Microstructured NiTi, bar = 20 μm . And (b) sub-microstructured, bar = 20 μm	147
Figure 3.3 SEM images of the titanium TIDE patterned surface with groove widths of (a) 750 nm, bar = 1 μm ; (b) 2 μm , bar = 1 μm ; (c) 5 μm , bar = 1 μm ; (d) 25 μm , bar = 20 μm ; (e) 75 μm , bar = 20 μm ; and (f) 100 μm , bar = 20 μm . The thin, non-uniform bright lines and spots observed in the etched grooves (darker stripes) were artifacts of the dry etching process.	148
Figure 3.4 AFM images of the titanium TIDE patterned surface with groove widths of: (a) 0.75 μm , scan scale = 10 μm ; (b) 1 μm , scan scale = 10 μm ; (c) 2 μm , scan scale = 10 μm and (d) 5 μm , scan scale = 30 μm	149
Figure 3.5 SEM images of titanium surface roughness. (a) Flat surface (F-Ti), bar = 2 μm ; (b) Flat surface (F-Ti), bar = 200 nm; (c) Nanorough surfaces (N-Ti), bar = 2 μm and (d) Nanorough surfaces (N-Ti), bar = 200 nm; (e) Sub-micron rough surfaces, bar = 1 μm and (f) Sub-micron rough surfaces (S-Ti), bar = 200 nm.	152

Figure 3.6 AFM characterization of titanium surface roughness. (a)-(b) AFM

analysis of F-Ti showed no surface features at the sub-micron and nano scale. A sectional analysis showed that F-Ti had flat (or smooth) structures in both the nano and sub micron scales. Depth analysis (inset image) showed negligible hits of depth features (less than 1 nm). (c)-(d) AFM analysis showed clear differences in surface features at the submicron and nano scales. AFM sectional analysis showed that N-Ti was very rough at the nano scale (structure widths were 30-40 nm) whereas surface roughness was less on S-Ti. Depth analysis (inset image) showed that a majority of hits were distributed within 2-6 nm. (e) AFM analysis showed that S-Ti had very rough surfaces at the sub-micron scale but it had rather smooth surface structures at the nano-scale: less lateral scale variation compared to N-Ti in the nano scale in (f). The widths of lateral structures were above 100 nm. Depth analysis (inset image) showed that the depth of titanium surface sizes were around 20-40 nm. All depth profiles were measured from $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ AFM scans. 154

Figure 3.7 Transparency of e-beam deposited titanium on glass. (a) Shows

different transparency for these substrates because of the different deposition thickness on glass coverslips. Sub-micron titanium surfaces were opaque. (b) Shows that the aligned titanium patterned surfaces were still transparent (SEM image shows aligned patterns with $30\text{ }\mu\text{m}$ spaces: aligned dark regions are nano or sub-micron layers and bright regions correspond to flat surfaces (F-Ti). 155

Figure 3.8 SEM characterization of titanium surface roughness. (a) Highly aligned micron patterns of nano structures (N-Ti) on flat titanium (F-Ti). Inset image (upper) showed surface spots due to nano scale surface structures (N-Ti). The flat region (below inset) did not show any surface structures at the nano scale. (b) Highly aligned micron patterns of S-Ti (sub-micron-titanium) on flat titanium (F-Ti). Inset magnified images showed a very rough morphology of S-Ti in the submicron scale. (c)-(d) Depth analysis showed the height difference between S-Ti and F-Ti and the difference was about $1\mu\text{m}$ (confirmed deposition thickness). (d)-(e) AFM analysis at the aligned intersection between S-Ti and F-Ti. (e) AFM sectional analysis of (d) showed two clear differences in surface roughness (flat and sub-micron). 157

Figure 3.9 Surface roughness, area and energy characterization showing contact angle measurements on (a) flat (F-Ti), (b) nano (N-Ti) and (c) sub-micron (S-Ti) titanium surface features. (d) Surface roughness (RMS) versus surface area (%) variance on the substrates. (e) Surface energy versus contact angles. All error bars are mean $\pm$ SEM; $n=3$; $*p<0.01$ (compared to flat) and $**p<0.01$ (compared to nano). 159

Figure 3.10 Increased rat aortic endothelial cell (RAEC) adhesion after 4 hours culture on nano to sub-micrometer patterns fabricated by the TIDE process compared to micrometer-scale patterns and random nanostructured titanium features. Data = mean $\pm$ SEM; $N = 6$; $*p < 0.01$ and $**p < 0.01$ (compared to titanium patterns of $100\mu\text{m}$ and random nanostructured titanium, respectively). 161

Figure 3.11 Increased rat aortic endothelial cell (RAEC) cell density after the first day of culture on the TIDE-made patterned titanium surface features. Data = mean $\pm$ SEM; N = 3; * $p < 0.05$ (compared to the patterns of 100 and 75 μm) and ** $p < 0.05$ (compared to random nanostructured surface features).

..... 163

Figure 3.12 Rat aortic endothelial cell (RAEC) cell density after (i) Four hours; (ii) One (iii) Three and (iv) Five days of culture on titanium patterns of (a) 750 nm; (b) 2 μm ; (c) 5 μm ; (d) 75 μm ; (e) 100 μm and (f) random nanostructured titanium surfaces. (a) to (f) bars = 50 μm . Arrows indicate groove alignment direction on patterned samples. 165

Figure 3.13 SEM images of RAEC morphology on the 750 nm nanopatterns after 4 hours of culture: The filopodia extended along the nanolines. Bars = 10 μm 166

Figure 3.14 SEM images of RAEC morphology after the fifth day of culture on titanium patterns of (A) 1 μm and (B) 100 μm . Bar = 20 μm 167

Figure 3.15 RAEC density increased on fine grained NiTi substrates with greater submicron to nanometer surface features after 5 hours, * $p < 0.01$ compared to all other substrates. The difference in endothelial cell adhesion between coarse grain NiTi, c.p. Ti, and the control was not significant. Data = mean $\pm$ SEM; N = 3. Control = tissue culture polystyrene..... 168

Figure 3.16 RAEC density increased on fine grained NiTi substrates with greater sub-micron to nanometer surface features after 1 day. The difference in the number of endothelial cells between fine and coarse grain NiTi substrates

was significant, $*p < 0.01$. However, there was no significant difference in endothelial cell density between fine grain NiTi and c.p. Ti. Data = mean $\pm$ SEM; N = 3. Control = tissue culture polystyrene.....169

Figure 3.17 Fluorescence microscope images of endothelial cells after (a, c, e) 1 day (Bars = 100 μ m) and (b, d, f) 5 days (Bars = 50 μ m) on fine and coarse grain NiTi and c.p. Ti substrates. Endothelial cells were more confluent on fine grain NiTi after 5 days compared to coarse grain NiTi.170

Figure 3.18 RAEC adhesion on titanium substrates with different roughness. Adhesion density showed that sub micron structures led to the best RAEC adhesion density (seeding density was 3500cells/cm²) and highest aspect ratio. All error bars are mean $\pm$ SEM; N=3; $*p<0.01$ and $**p<0.05$172

Figure 3.19 RAEC morphology on titanium substrates with different roughness. (a) Fluorescence RAEC density on submicron structured surfaces (S-Ti); (b) Magnified fluorescence RAEC image of submicron surfaces shows high cell aspect ratios. Inset image shows SEM surface structures (bar is 100nm); (c) Fluorescence RAEC density on flat surfaces (F-Ti) and (d) Magnified fluorescence cell images of RAEC on flat surfaces show small cell aspect ratios (more round cellular shapes than on sub micron surfaces). Bar = 100 nm. All yellow bars in (a) and (b) are 100 μ m and yellow bars in (b) and (d) are 20 μ m.173

Figure 3.20 Endothelial cell (RAEC) adhesion on aligned patterns. (“top” indicates upper coated layers and “bottom” indicates lower layers). All error bars are mean $\pm$ SEM; N=3; $*p<0.01$ (compared to the respective bottom

layer) and $**p<0.05$ (compared to the respective bottom layer). No significant p values were observed for the flat top and flat bottom (both 50nm and 1µm height difference). S-Ti is submicron featured surface, N-Ti is nanofeatured surface, and F-Ti is flat titanium surface..... 175

Figure 3.21 RAEC adhesion on aligned patterns. (a) Fluorescence cell image showed RAEC adherent more on aligned submicron spaces (S-Ti) compared to aligned flat titanium (F-Ti). Bar is 50µm. (b) Selected cell images (magnified) from (a) showed F-actin filaments oriented more on aligned submicron structured patterns than aligned flat surfaces (bar is 50µm). (c) Selective adhesion of endothelial cells showed more cells adherent on nanorough surfaces (N-Ti) than F-Ti (height was 50nm). (d) Non-selective cell adhesion on the top layer (F-Ti) and bottom layer (F-Ti) showed no preferential attachment of RAEC cells. All scale bars in (c) and (d) are 60nm. 176

Figure 3.22 RAEC proliferation on submicron (S-Ti), nanometer (N-Ti), flat (F-Ti) titanium surface features and borosilicate glass (Glass) after 1, 3 and 5 days of culture. RAEC seeding density was 10,000 cells/cm². All error bars are mean ± SEM; N=9; $*p<0.1$, $**p<0.05$ and $***p<0.01$ 178

Figure 3.23 RASMC proliferation on submicron (S-Ti), nanometer (N-Ti), flat (F-Ti) titanium surface features and borosilicate glass (Glass) after 1, 3 and 5 days of culture. RASMC seeding density was 10,000 cells/cm². All error bars are mean ± sem; N=9; $*p<0.1$ and $**p<0.05$ 178

Figure 3.24 Fluorescence cell images showing RAEC morphology on submicron (S-Ti), nanometer (N-Ti) and flat (F-Ti) titanium surface features after 1, 3 and 5 days of culture. All scale bars are 100 μm	180
Figure 3.25 Fluorescence cell images showing RASMC morphology on submicron (S-Ti), nanometer (N-Ti) and flat (F-Ti) titanium surface features after 1, 3 and 5 days of culture. All scale bars are 100 μm	181
Figure 3.26 RAEC coverage on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) surfaces after one day, showing that the area RAEC covered on S-Ti was more than three times as large as on F-Ti. The spreading area was normalized by the area of the substrates. All error bars are mean $\pm$ SEM; N=3; $**p<0.05$	182
Figure 3.27 Competitive RAEC and RASMC co-culture adhesion on submicron (S-Ti), nanometer (N-Ti), flat (F-Ti) titanium surface features and borosilicate glass (Glass) after 4 hours. The cell seeding densities were 4,500 cells/cm ² . All error bars are mean $\pm$ SEM; N=9; $**p<0.05$	183
Figure 3.28 Competitive RAEC and RASMC co-culture proliferation on submicron (S-Ti), nanometer (N-Ti), flat (F-Ti) titanium surface features and borosilicate glass (Glass) after 1, 3 and 5 days of culture. The cell seeding densities were 2,000 cells/cm ² . All error bars are mean $\pm$ SEM; n=9; $*p<0.1$, $**p<0.05$, $***p<0.01$, $****p<0.005$ and $*****p<0.001$	184
Figure 3.29 Vitronectin adsorption on S-Ti, N-Ti, and F-Ti substrates after 4 hours of exposure. Data=mean $\pm$ SEM, N=3. $**p<0.01$. Y axis is optical	

density readings from a spectrophotometer which are related to protein adsorption amount.....	186
Figure 3.30 Fibronectin adsorption on S-Ti, N-Ti, and F-Ti substrates after 4 hours of exposure. Data=mean+/-SEM, N=3. $*p<0.05$ and $**p<0.01$	186
Figure 3.31 Fibrinogen adsorption on S-Ti, N-Ti, and F-Ti substrates after 4 hours of exposure. Data=mean+/-SEM, N=3. $*p<0.05$ and $**p<0.01$	187
Figure 3.32 NOx concentration synthesized by RAEC on sub-micron rough (S-Ti), nanorough (N-Ti), flat (F-Ti) titanium substrates Data=mean+/-SEM, N=3. $*p<0.05$	188
Figure 3.33 Endothelin-1 concentrations synthesized by RAEC on sub-micron rough (S-Ti), nanorough (N-Ti) and flat (F-Ti) titanium substrates Data=mean+/-SEM, N=3.	189
Figure 3.34 Collagen content secreted by endothelial cells after 1 and 2 weeks of culture on titanium substrates with different roughness. Results were normalized with cell numbers. Data=mean+/-SEM, N=3. For each substrate, collagen content at second week was significantly higher than the first week. $**p<0.01$ and $****p<0.001$	190
Figure 3.35 Elastin content secreted by endothelial cells after 1 and 2 weeks of culture on titanium substrates with different roughness. Results were normalized with cell numbers. Data=mean+/-SEM, N=3. For each substrate, elastin content at second week was significantly higher than the first week. $**p<0.01$	191

Figure 3.36 Collagen content secreted by smooth muscle cells after 1 and 2 weeks of culture on titanium substrates with different roughness. Results were normalized with cell numbers. Data=mean+/-SEM, N=3. ** $p<0.01$	192
Figure 3.37 Elastin content secreted by smooth muscle cells after 1 and 2 weeks of culture on titanium substrates with different roughness. Results were normalized with cell numbers. Data=mean+/-SEM, N=3. For each substrate, elastin content at second week was significantly higher than the first week. * $p<0.05$ and ** $p<0.01$	193
Figure 3.38 RAEC migration on S-Ti, N-Ti, and F-Ti substrates over 12, 24 and 96 hours. Cells were stained with Calcein AM. Scale bars = 200 μ m. Dash lines indicate starting points at time zero.....	194
Figure 3.39 RAEC migration on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) titanium surfaces after 12, 24 and 96 hours, showing that RAEC motility greatly increased on S-Ti compared with that on N-Ti and F-T. All error bars are mean $\pm$ SEM; N=3; * $p<0.05$ (compared to F-Ti after 12 hours), ** $p<0.05$ (compared to N-Ti and F-Ti after 24 and 96 hours), # $p<0.05$ (compared to F-Ti after 12, 24 and 96 hours).	195
Figure 3.40 Immunofluorescent images of vinculin, which indicated focal adhesion of RAEC, on titanium surfaces of different roughness. (a) Sub-micron rough titanium (S-Ti); (b) Nanorough titanium (N-Ti) and (c) Flat titanium (F-Ti). All yellow scale bars = 100 μ m.....	196

Figure 3.41 IC-21 macrophage attachment on various e-beam titanium coatings after 12 and 24 hours without LPS. Data=mean+/-SEM, N=3. * $p<0.05$ and ** $p<0.01$	197
Figure 3.42 IC-21 macrophage attachment on various e-beam titanium coatings after 12 and 24 hours. Data=MEAN+/-SEM, N=3. * $p<0.05$ and ** $p<0.01$. 200ng/ml LPS was added in the culture medium to activate macrophage function.	198
Figure 3.43 Fluorescence images showing macrophages adherent onto (a, c) Sub-micron rough (S-Ti), (b, e) Nanorough (N-Ti), and (c, f) Flat (F-Ti) titanium surfaces after 12 hours. First line is high magnification (x20) and second line is respective low magnification image (x10). Cells were stained with DAPI (blue) and rhodamine-phalloidin (red). Scale bars = 100 μ m.	199
Figure 3.44 Fluorescence images showing macrophages adherent onto (a, d) Sub-micron rough (S-Ti), (b, e) Nanorough (N-Ti), and (c, f) Flat (F-Ti) after 12 hours with LPS activation. First line is high magnification (x20) and second line is respective low magnification image (x10). Cells were stained with DAPI (blue) and rhodamine-phalloidin (red). Scale bars = 100 μ m.	200
Figure 3.45 The single cell area of IC-21 macrophages on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) surfaces under flow after 12 hours, showing that the cell area on S-Ti was the smallest of all the substrates. All error bars are mean $\pm$ SEM; N=3; ** $p<0.05$	201
Figure 3.46 THP-1 monocyte adhesion on S-Ti, N-Ti, and F-Ti after 4 hours with TPA activation. Seeding density was 100,000 cells/cm ² . TPA was added at a	

concentration of 1µg/ml. Data=mean+/-SEM, N=3. ** $p<0.01$ and **** $p<0.001$.	202
Figure 3.47 THP-1 monocyte adhesion on endothelial monolayers on S-Ti, N-Ti, and F-Ti after 4 hours with TPA activation. Seeding density was 3,500 cells/cm ² . TPA was added at a concentration of 1µg/ml. Data=mean+/-SEM, N=3. ** $p<0.01$, **** $p<0.001$ and ***** $p<0.0005$.	204
Figure 3.48 Fluorescent images of monocytes on S-Ti, N-Ti, and F-Ti after (a-c) 4 hours and (d-f) 5 days. Cells were stained with DAPI (blue) and rhodamine-phalloidin (green). Scale bars = 100 µm.	205
Figure 3.49 Concentration of IL-1β secreted by macrophages after 4, 12, 24, and 36 hours of culture on titanium substrates with different roughness. Seeding density was 1,000,000 cells/cm ² . Data=mean+/-SEM, N=3. ** $p<0.01$, *** $p<0.005$ and # $p<0.05$.	206
Figure 3.50 Concentration of IL-6 secreted by macrophages after 4, 12, 24, and 36 hours of culture on titanium substrates with different roughness. Seeding density was 1,000,000 cells/cm ² . Data=mean+/-SEM, N=3. ** $p<0.01$ and # $p<0.05$.	207
Figure 3.51 Concentration of TNF-α secreted by macrophages after 4, 12, 24, and 36 hours of culture on titanium substrates with different roughness. Seeding density was 1,000,000 cells/cm ² . Data=mean+/-SEM, N=3. ** $p<0.01$ and # $p<0.05$.	208
Figure 3.52 SEM images of (a, d) Submicron-rough (S-Ti), (b, e) Nano-rough (N-Ti), and (c, f) Flat titanium (F-Ti) coatings before and after exposure to flow	

conditions for 24 hours. The shear stress was set at 20 dynes/cm ² . Scale bars in (a), (c), (d) and (f) are 1µm; in (b) and (e) are 100 nm.	210
Figure 3.53 Double stained endothelial cells remaining on (a) sub-micron rough (S-Ti), (b) nanorough (N-Ti), (c) flat (F-Ti) titanium surfaces, and (d) glass after 24 hour of laminar flow. Shear stress was kept at 10 dynes/cm ² . All substrates were pre-cultured with endothelial cells for 36 hours to form a complete layer of endothelial cells.....	212
Figure 3.54 RAEC Coverage on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) surfaces under flow after 4 hours, showing that the area RAEC covered on both S-Ti and N-Ti was twice as large as on F-Ti. The spreading area was normalized by the area of the substrates. All error bars are mean ± SEM; N=3; ** <i>p</i> <0.05.	213
Figure 3.55 THP-1 monocyte adhesion on S-Ti, N-Ti, F-Ti, and glass under flow conditions. All substrates were pre-coated with a layer of endothelial cells and the shear stress was kept at 5 dynes/cm ² for 4 hours. Cell density in the culture medium is 50,000 cells/ml. N=3, Data = Mean +/- SEM. ** <i>p</i> <0.05, **** <i>p</i> <0.005 and ***** <i>p</i> <0.001.	214
Figure 3.56 Bone cell (HOB) adhesion on aligned patterns. Fluorescence live cell images, (a) and (b), showed that osteoblasts adhered more on sub-micron rough surfaces (S-Ti) than flat titanium (F-Ti) and, thus, provided evidence of selective responses of bone cells on sub-micron regions. The SEM images (c), (d) and (e) indicated different cell adhesion. (f) HOB adhesion density analysis showed that both nano and sub-micron density led to selective	

adhesion compared to flat surfaces. Greater adhesion probability on sub micron roughness than nano surfaces was observed. All error bars are mean $\pm$ SEM; N=3; * $p<0.01$, and ** $p<0.05$215

Figure 4.1 Schematic using e-beam deposition to create to N-Ti, S-Ti, and F-Ti surfaces by altering deposition rate. High deposition rate in (a) is 20 Å/sec while low deposition rate in (b) is 0.5-1 Å/sec. Small particles in the schematic indicate titanium atoms. F-Ti is flat titanium, N-Ti is nano-rough titanium and S-Ti is submicron rough titanium.224

Figure 4.2 Relationship between e-beam deposited surface roughness and e-beam deposition parameters (specifically, deposition rate and deposition thickness).225

Figure 4.3 Correlations between lateral width and depth versus RAEC adhesion: Importance of nano surface structures with respect to surface energy and cell adhesion. (a) RAEC adhesion on nano surface structures (less than 50nm for both vertical and lateral scales) increased about 50% compared to flat (F-Ti) surfaces. Also, RAEC adhesion densities on submicron structures (S-Ti) increased about 200% compared to flat titanium. The cell adhesion difference between nano (N-Ti) and S-Ti was 40% (more cells on sub-micron compared to nano structures). Although vertical depths in sub-micron structures (S-Ti) were below 100nm, a majority of S-Ti features in the lateral dimension showed structures larger than 100nm. Criteria of sub-micron and nano features were determined by the current definition of nanometer less than 100nm. (b) Vertical depth showed negligible differences between flat and

nano titanium (4nm) while lateral widths increased about 40nm which is almost ten times greater	237
Figure 4.4 Relationship between the biological interactions and the critical surface tension of a biomaterial. Area “A” represents a non-adhesive zone; area “B” represents biomaterials with good bioadhesion to their biological environment (Adapted and redrawn from [184]).	239
Figure 4.5 Ratio of NOx to endothelin-1 released by RAEC after 24 hours on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) surfaces. All error bars are mean $\pm$ SEM; N=3; $**p<0.05$	241
Figure 4.6. Protein adsorption (vitronectin and fibrinogen) proportional to surface energy on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) surfaces. Flat (54mJ/m^2), nano (62mJ/m^2) and submicron (69mJ/m^2) titanium had increased surface energy. Data=mean $\pm$ SEM, N=3. $**p<0.01$, $^{\#}p<0.01$ (compared to F-Ti), $^{\&}p<0.01$ (compared to F-Ti) and $^{\&\&}p<0.05$ (compared to N-Ti).	248
Figure 4.7 Future directions of the present study focusing on developing the next generation vascular stents (without drugs) using e-beam deposition method.	258
Figure 4.8 Schematics of a cell culture system to study vascular stents combining 3D culture, co-culture and dynamic flow conditions. VEGF is vascular endothelial growth factor.	262
Figure 5.1 Correlation of surface energy, roughness, protein adsorption and vascular endothelial cell functions.	266

CHAPTER 1. INTRODUCTION

1.1. Artery Physiology

1.1.1. Anatomy of the Artery

The arteries in the human body consist of systemic arteries and pulmonary arteries (Figure 1.1). Pulmonary arteries are responsible for transporting the deoxygenated blood from the heart to the lungs via the right ventricle [1-3]. The size of the average pulmonary artery is on the about 3 cm in diameter and 5 cm in length [1-3]. Systemic arteries deliver the oxygenated blood from the heart to the rest of the body. The main trunk of the systemic artery is the aorta, whose diameter ranges from 3 cm to about 1.75 cm [2, 3]. The aorta, coming from the left ventricle, is divided into the ascending aorta, the aorta arch and the descending aorta. At the site near the connection of the ascending aorta and the left ventricle, the aorta branches off into the coronary arteries, while the descending aorta is divided into two common iliac arteries [1-3].

In both humans and animals, atherosclerosis preferentially occurs in arteries (including arterial branch points, aortic arch and aortic branch points (Figure 1.1)). The coronary arteries play a crucial role in supporting

the heart with nutrients and oxygen. The right coronary artery supports the right atrium and the right ventricle with nutrients and oxygen, and the left coronary artery supplies the left atrium and the left ventricle with nutrients and oxygen [1].

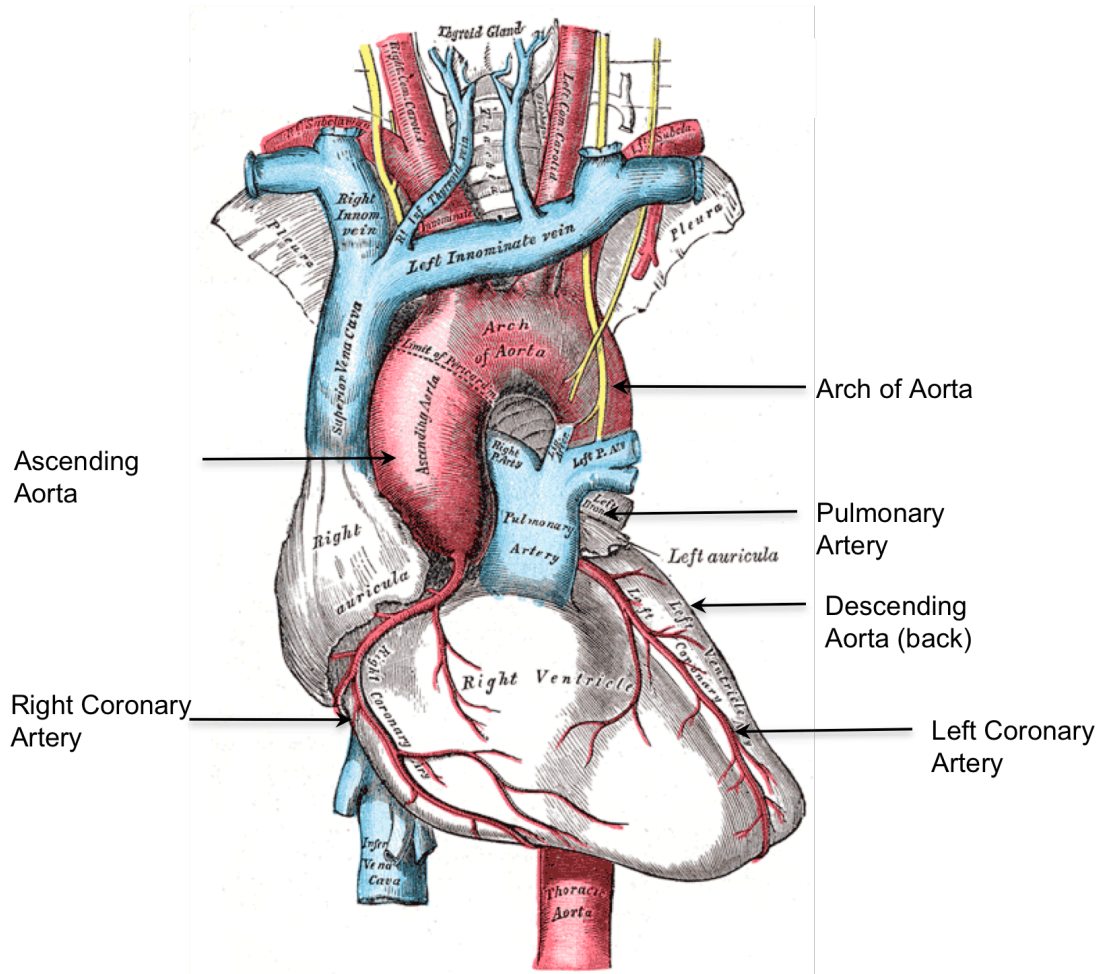


Figure 1.1 Schematic showing the structure of the aorta (Retrieved and redrawn from [1]).

1.1.2. The Arterial Wall

The arterial wall consists of three different circumferential layers: the tunica intima, the tunica media and the tunica adventitia. This anisotropic, multi-layered and non-elastic structure leads to the high non-linearity of the mechanical properties of the arterial wall [2, 4, 5]. The schematic diagram of the arterial wall structure is shown in Figure 1.2 and further described below.

1.1.2.1. The Tunica Intima

The tunica intima is the innermost layer of the arterial wall. It also consists of three layers. The innermost section is the endothelium, which is a layer of endothelial cells. The middle section is a thin sub-endothelial layer of areolar tissue (Figure 1.2 and 1.3). In arteries larger than 2 mm, the connective tissue is largely developed and the outermost section is a thin membrane of connective tissue with elastic fibers [1, 2]. The tunica intima does not significantly contribute to the mechanical properties of the arterial wall [4].

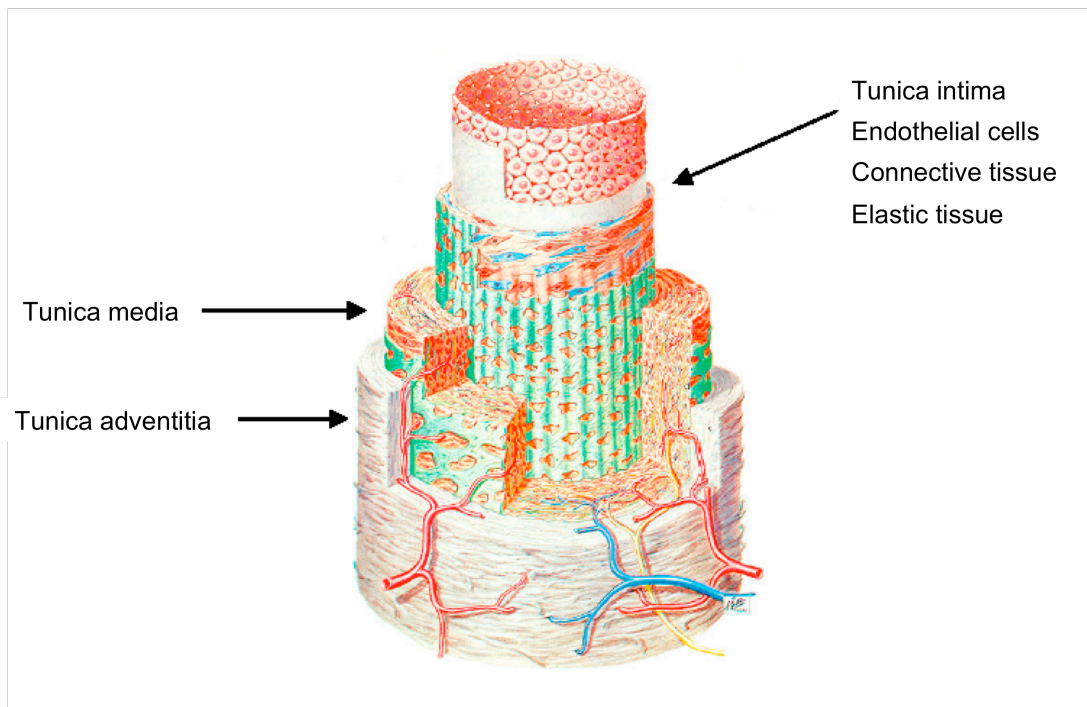


Figure 1.2 Three-layered anatomy of the arterial wall
(Adapted and redrawn from [6]).

1.1.2.2. The Tunica Media

The tunica media is the thickest layer between the tunica intima and tunica adventitia (about 0.3 mm for the human left coronary artery and 0.1 mm for the human right coronary artery [7]). It is composed of connective tissue, elastic tissue and primarily, circumferentially oriented smooth muscle cells, which are embedded in an extracellular matrix of elastin and collagen (type I and III; Figure 1.4). Importantly, the collagen, elastin and proteoglycans of the tunica media determine the mechanical properties of the arteries, especially the elasticity and strength of the arterial wall [4, 8, 9]. The layer of smooth muscle cells changes blood pressure and blood flow by contracting and relaxing, which are regulated by intercellular

calcium concentration. In large arteries, the concentration of elastic fibers is much more than that of small arteries [2, 3].

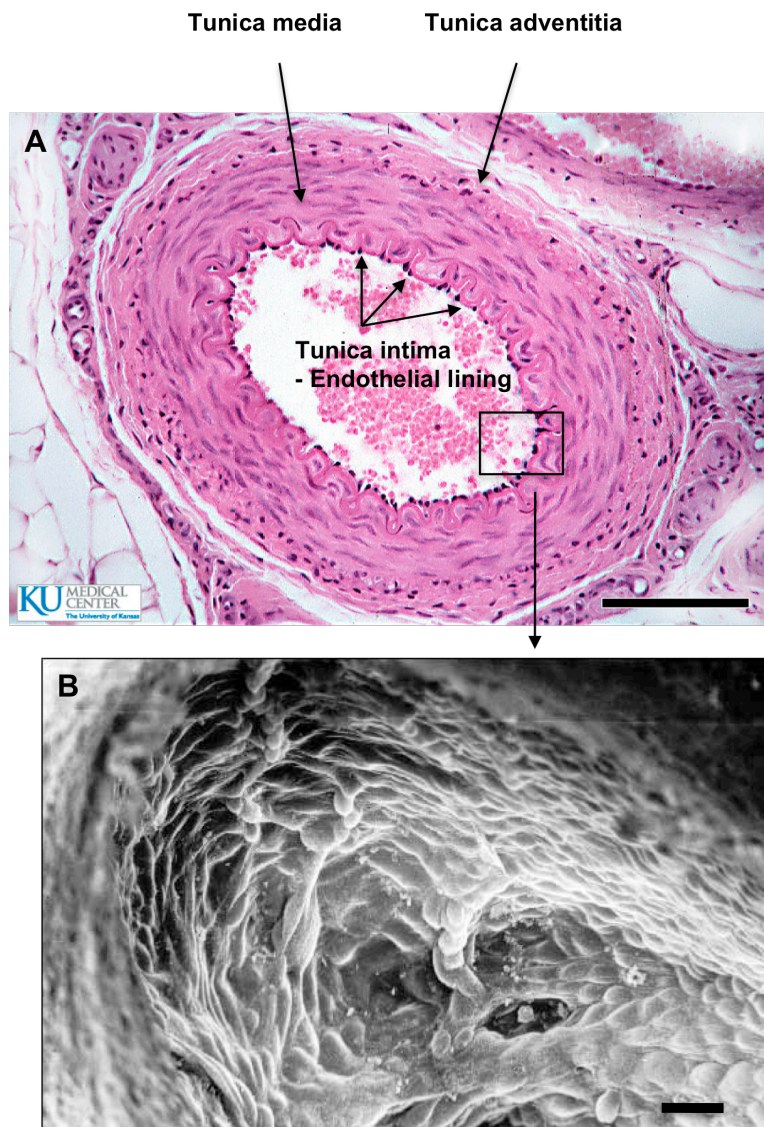


Figure 1.3 The longitudinal view of the arterial wall. (A) Micrograph of the tunica intima, media and adventitia (Adapted and redrawn from [10]), bar=100 μm ; (B) Micrograph of the endothelial cell lining (Retrieved and redrawn from [11]), bar=10 μm .

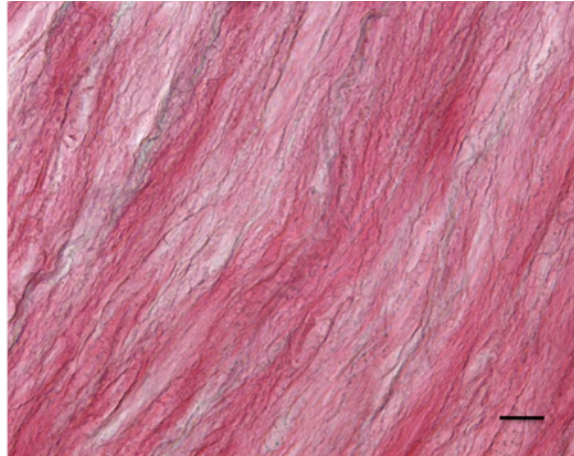


Figure 1.4 Collagen type I and III in the tunica media (Adapted and redrawn from [12]) bar=10 μm .

1.1.2.3. The Tunica Adventitia

The adventitia tunica is the outermost layer of the arterial wall. It is mainly composed of connective tissue, with collagen fibers (type I) and elastic fibers (Figure 1.5) [1, 2]. Collagen is responsible for strengthening the vessel and providing anchorage of the vessel to surrounding organs. Another important part of the tunica adventitia is the vasa vasorum (“vessels of the vessel”), the vasculature which supplies the tunica adventitia with nutrition and oxygen (Figure 1.5) [1, 2].

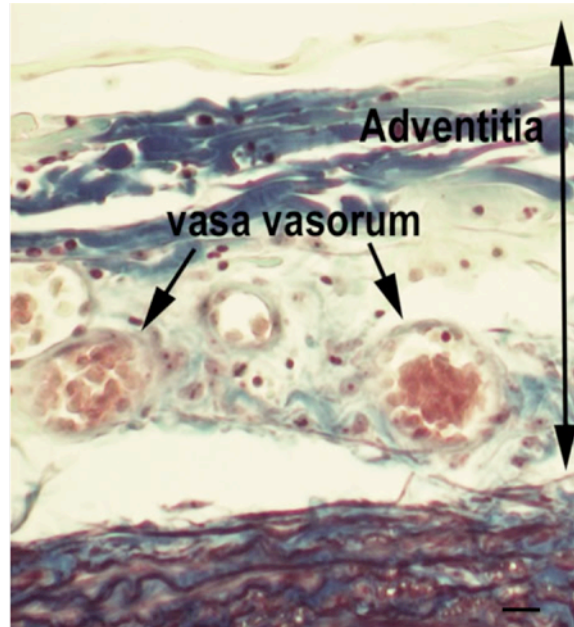


Figure 1.5 The tunica adventitia of the artery (Adapted and redrawn from [13]), bar=10 μ m.

1.1.3. Arterial Cells

1.1.3.1. Endothelial Cells

Vascular endothelial cells (Figure 1.6) are flat, thin and elongated cells resting on a basement membrane basically composed of collagen type IV, which is produced by the cells themselves. Although endothelial cells have the same organelles as any other epithelial cells, such as prominent nuclei, extensive endoplasmic reticulum and mitochondria, they also possess a large number of small plasmalemmal vesicles (about 70 nm in diameter; 10,000 to 15,000 per cell) [2]. Among the endothelial cells, about 70 % open toward the blood front, which increases the exposed area of the cell surface by two-fold to maximize nutrient exchange.

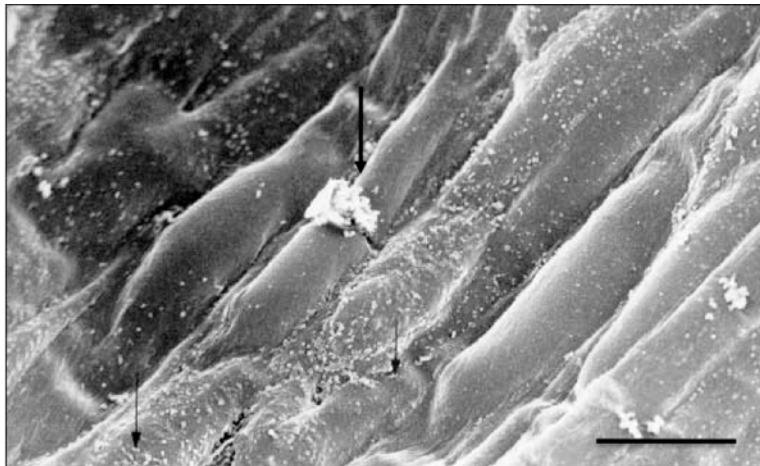


Figure 1.6 Surface of the endothelial cell layer covered with atherosclerotic plaques (indicated with arrows) (Adapted and redrawn from [11]), bar=100 μm .

A monolayer of vascular endothelial cells (called the endothelium) aligns with each other inside vessel walls. The endothelium works as a biocompatible protector between the vessel tissue and blood. As a squamous epithelium, its permeability and hydraulic conductivity coefficients (the ability of water to move through cell membranes) are two to three times higher than any other epithelium (such as the cuboidal epithelium and the columnar epithelium) in the body. The endothelium controls the bi-directional transport of molecules between the vessel tissue and blood, secretes all kinds of compounds (such as nitric oxide and platelet activating factor; Table 1.1), and contains various cell surface receptors (such as CD 40) to adjust vessel morphology and modulate the activity of other cells [2, 14-18]. All of these endothelium functions provide critical roles in maintaining cardiovascular haemostasis [14-18]. The main

compounds secreted by the endothelium and their associated activities are listed in Table 1.1. Among them, nitric oxide is critical in preventing platelet aggregation and adhesion to the endothelium and controlling vascular tone as a vasodilator.

Table 1-1 Compounds secreted by the endothelium and their respective activities.

Secretion Products of the Endothelium	Activities of the Endothelium
Prostacyclin [18, 19]	✓ Inhibition of platelet aggregation ✓ Vasodilation
Nitric oxide [18]	✓ Inhibition of platelet aggregation and adhesion ✓ Vasodilation
Ectonucleotidase	✓ Inhibition of platelet aggregation
Platelet activating factor (PAF) [16]	✓ Activation of platelet and neutrophil adhesion to the endothelium
Tissue factor [17, 19]	✓ Promotion of blood coagulation
Tissue factor pathway inhibitor [17, 19]	✓ Inhibition of tissue factor expression
Thromboplastin [17, 19]	✓ Promotion of blood coagulation
Thrombomodulin [17, 19]	✓ Anticoagulant activity
von Willebrand Factor [18]	✓ Promotion of platelet adhesion ✓ Activation of blood coagulation

1.1.3.2. Smooth Muscle Cells

Vascular smooth muscle cells are generally characterized by their elongated (flattened-spindle) shape and fusiform (spindle-like shape)

nuclei [2, 20]. They are located in the tunica media. Vascular smooth muscle cells play an important role in regulating the performance of the vasculature. For example, by changing the vessel diameter through contraction and relaxation by the sliding of myosin (motor proteins) and actin filaments over each other, they enable the blood vessel to maintain an appropriate blood pressure [2, 21-23]. Besides short-term functions, such as luminal diameter regulation, smooth muscle cells also change cell growth rates and connective tissue composition during vessel remodeling in different physiological conditions, which is called long-term adaption. For example, in cases of pregnancy and vascular injury, smooth muscle cells increase proliferation and migration rates, as well as secrete large amounts of extracellular matrix (ECM) components such as collagen and elastin and several types of proteoglycans [2, 21-23].

Because of the different functions vascular smooth muscle cells perform, they are often differentiated into a variety of phenotypes, ranging from contractile to synthetic (Figure 1.7) [2, 22]. The contractile and synthetic smooth muscle cells are at the two ends of the smooth muscle cell groups. They have distinct morphologies, marker gene expression and proliferation properties. Briefly, contractile smooth muscle cells are more spindle-shaped, have extensive cytoplasmic filaments composed of actin and myosin, while the synthetic smooth muscle cells are less elongated (Figure 1.7), have less thick (contractile) filaments and myosin, and lose

the ability to contract [2, 22]. Synthetic smooth muscle cells are capable of synthesizing more proteins since they have a large number of organelles, but for contractile smooth muscle cells, most of the organelles are replaced by contractile filaments. Synthetic cells also have greater proliferation and migration rates than contractile phenotypes [2, 22].

Several smooth muscle cell protein markers have been used to define the two phenotypes. For example, cellular retinol binding protein (CRBP)-1, a protein involved in retinoid transport and metabolism, is an important marker for synthetic smooth muscle cells [24], while smoothelin, a cytoskeleton protein specific for smooth muscle cells, and smooth muscle myosin heavy chain (SM-MHC) represent mature contractile smooth muscle cells [25, 26].

A number of factors are involved in regulating smooth muscle differentiation between synthetic and contractile phenotypes, including: (i) mechanical stimuli such as endothelium-regulated shear stress and stretching of blood vessels, (ii) chemical factors such as cytokines secreted by macrophages, transforming growth factor (TGF- β) and nitric oxide [23, 27-30]. Extracellular matrix proteins also affect smooth muscle cell differentiation. For example, fibrillar collagen type I / IV, proteoglycan heparin and laminin have all been reported to promote the smooth muscle cell contractile phenotype [31-33].

Of course, the structure of the artery and the function of the arterial cells may change under certain stimuli. This will be discussed next.

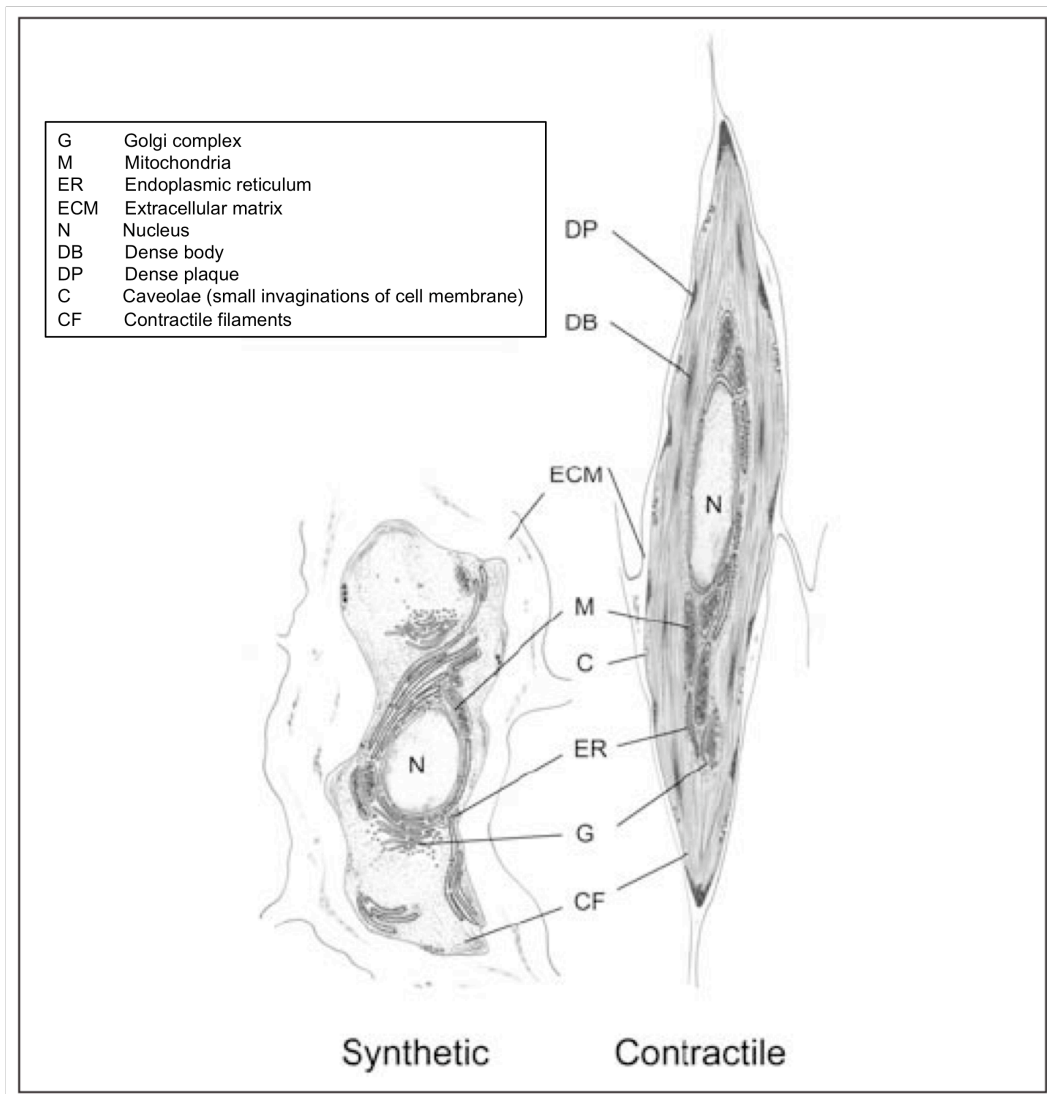


Figure 1.7 Characteristics of the vascular smooth muscle cell phenotype (Adapted and redrawn from [22]).

1.1.4. Function of the Normal Artery

1.1.4.1. Vascular Remodelling

Vascular remodeling is a process during which the normal artery can adjust its mass and dimension in response to external stimuli (such as modified blood flow, hypertension or vascular injury). During the remodeling process, the mechanical signals due to the stimuli is detected by sensors on the endothelium, which transfer such signals within cells or to adjacent cells and induce the synthesis and release of factors influencing vascular cell growth, proliferation and migration as well as extracellular matrix composition. This process finally leads to structural modification of the vessel wall (Figure 1.8).

As shown in Figure 1.8, both large and small blood vessels increase their luminal area as a compensatory mechanism to increased blood velocity (Figure 1.8 A and B). For example, when a vessel undergoing stenosis experiences (abnormal narrowing in a blood vessel) intimal thickening or an atherosclerotic plaque, which leads to an increase in blood velocity, the vessel dilates at the site of the lesion. Or even when the vessel has an intimal plaque, it can still regulate its luminal dimension properly if the size of the plaque is small (less than 40% of the internal elastic lamina area) [34].

Blood vessels also exhibit medial or intimal thickening and rearrangement in response to several stimuli (Figure 8 C and D). For this, proliferation of smooth muscle cells, accumulation of extracellular matrix proteins, as well as accumulation of lipid and lipid-filled macrophages are important components. In the normal artery, smooth muscle cells proliferate during growth and development, and then enter a quiescent state in adult arteries. However, under certain circumstances (such as denudation of the endothelium), smooth muscle cell proliferation is stimulated again. For example, platelets start to adhere to the vessel wall, spread and granulate. Granule proteins secreted by the platelets, including platelet-derived growth factor (PDGF) and transforming growth factor β (TGF- β), accumulate in the artery wall and stimulate subsequent smooth muscle cell growth [2, 35]. But this growth inside the tunica media does not lead to artery wall thickening until smooth muscle cells migrate from the tunica media and proliferate in the intimal region [2, 35].

Although the exact mechanism of vascular remodeling is unknown, it is clear that the endothelium plays a critical role in the remodeling process. During its direct exposure to external stimuli, the ion channels (flow-sensitive potassium channels or stretch-responsive cationic channels) are activated and change calcium concentrations, which leads to modification of vascular structure and functions, including control of the contractile state of smooth muscle cells in the tunica media [36-38]. One of the

factors that are involved in the relaxation of smooth muscle cells is EDRF (endothelial-dependent relaxing factor) [2].

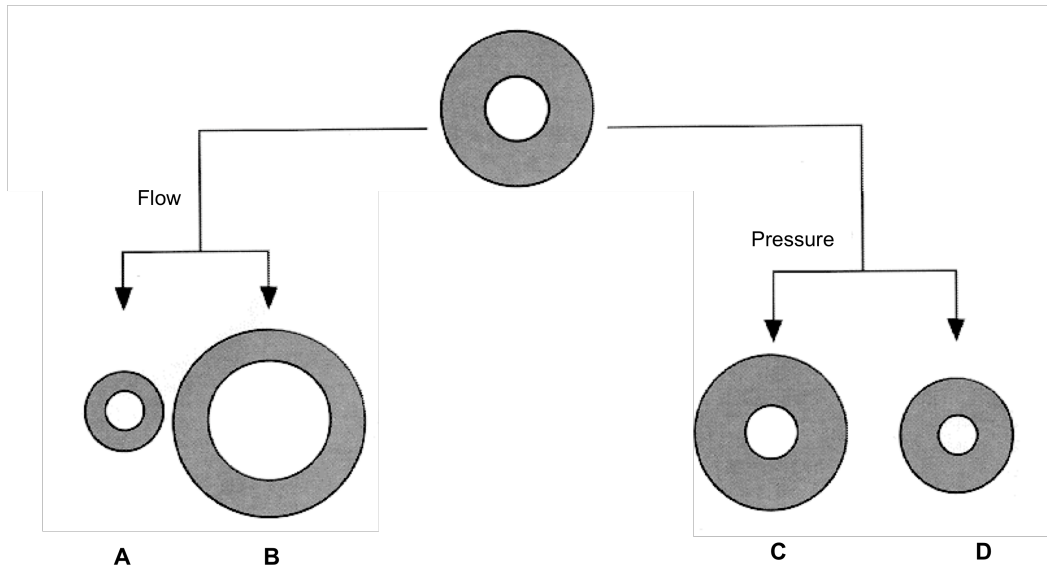


Figure 1.8 The classification of vascular remodeling (circles represent top view of blood vessels). A) Decreased vessel luminal dimensions in response to a decreased blood flow velocity; B) Increased vessel luminal dimensions in response to an increased flow velocity; C) Increased medial thickness in response to vascular hypertension and vascular hypertrophy; and D) Unchanged medial thickness in response to vascular hypertension (Adapted and redrawn from [35]).

1.1.4.2. Hemodynamics of Blood Flow

Under normal conditions, blood flow is laminar throughout most of the circulatory system. Laminar flow is composed of concentric layers of blood in a direction parallel to the length of a blood vessel. It has parabolic velocity profiles, with the highest velocity in the center the vessel and the lowest one near the vessel wall as shown in Figure 1.9. The equation to describe the laminar, parabolic blood flow is shown as follows: (assuming

that: (i) the vessel is rigid; (ii) blood is a homogeneous, Newtonian fluid whose stress is linearly proportional to strain rate; and (iii) slip existing at the vessel wall is neglected.)

$$v(a) = \frac{\Delta P}{4\mu l}(r^2 - a^2) \quad (1.1)$$

where r is the vessel radius, a is a radial location within the vessel, μ is the viscosity of blood, l is the length of the vessel, Δp is pressure difference between the inlet and outlet of the vessel, and where

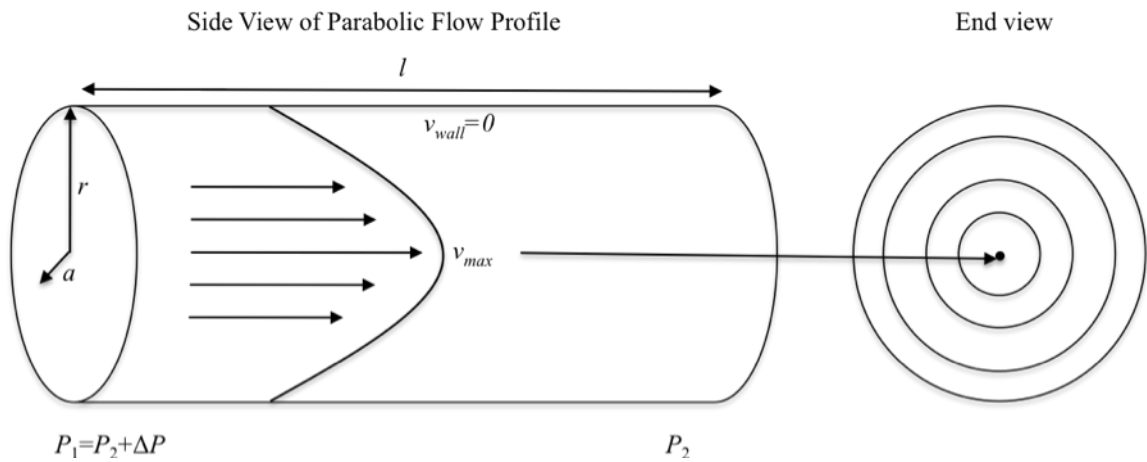


Figure 1.9 Schematic of parabolic flow through a blood vessel.

$$\Delta P = P_1 - P_2 \quad (1.2)$$

where P_1 is the inlet pressure and P_2 is the outlet pressure. Based on this equation, it is clear that the highest velocity in the center of the blood flow is

$$v_{\max} = \frac{\Delta P}{4\mu l} r^2 \quad (1.3)$$

while the velocity at the vessel wall is zero ($v_{\text{wall}} = 0$).

The maximum blood velocity in the arteries is about 27 to 28 cm/s [39].

The volumetric flow rate (Q) is defined as the volume of blood passing through the cross-sectional area of the vessel ($A = \pi r^2$) during a unit time:

$$Q = v_{\text{average}} A \quad (1.4)$$

The average blood velocity is given by:

$$v_{\text{average}} = \frac{\Delta P}{8\mu l} r^2 \quad (1.5)$$

Therefore, the final formula of the volumetric flow rate is:

$$Q = \frac{\pi \Delta P}{8\mu l} r^4 \quad (1.6)$$

Wall shear stress τ (dynes/cm²), another important parameter in the mathematical analysis of the circulatory system, is defined as:

$$\tau = \mu \frac{dv}{dr} \quad (1.7)$$

If substituting the equation of the volumetric flow rate into this equation, this equation becomes:

$$\tau = \frac{4\mu Q}{\pi r^3} \quad (1.8)$$

This equation indicates the linear relationship between the shear stress and the velocity/volumetric flow rate. That is, as the velocity or the flow rate increase, the shear stress increases as well. While, as the vessel radius increases, the shear stress decreases. Normally, shear stress in the arteries ranges from 10 to 70 dynes/cm² [40], but 15 dynes/cm² is often used as a typical shear stress value for arteries of different sizes [41]. In diseased arteries such as atherosclerosis, shear stress values range from -4 to 4 dynes/cm².

1.2. Atherosclerosis

1.2.1. An Overview of Atherosclerosis

Atherosclerosis is conventionally defined as a disease of the artery characterized by the accumulation of smooth muscle cells and lipids on the intimal surface. Nowadays, studies have indicated that atherosclerosis also involves an ongoing inflammatory response, implicating that atherosclerosis is an inflammatory disease [42].

Generally, atherosclerosis affects vessel walls through the accumulation of atheromas (or atherosclerotic plaques) which involves endothelial cell dysfunction, inflammatory responses, vascular cell (vascular endothelial cells and smooth muscle cells) proliferation, etc. [42-44]. A brief development of atherosclerosis is illustrated in Figure 1.10, where Figure 1.10 A shows a healthy artery wall without atherosclerotic lesions (similar to Figure 1.2). Atherosclerosis begins with a series of events including recruitment of monocytes from the blood stream by MCP-1, oxidation of low-density lipoproteins (LDL) with help of reactive oxygen species, and consumption of oxidized LDL by monocyte-derived macrophages, which leads to the formation of foam cells (Figure 1.10 B). Accumulation of elastin fibers and proliferation of smooth muscle cells, along with the further recruitment of monocytes into the plasma, result in the formation of fatty streak lesions (Figure 1.10 C). As the lesion develops from the tunica intima into the lumen, smooth muscle cells migrate into broken internal elastic lamina. Lipid droplets accumulate at this time (Figure 1.10 D). The whole process leads to the formation of an atherosclerotic plaque (called atheromas) inside the artery channel (Figure 12 (E)). Clearly, such events block arteries and create severe blood flow complications including thrombus formation, increased cellular debris and the appearance of cholesterol crystals [45].

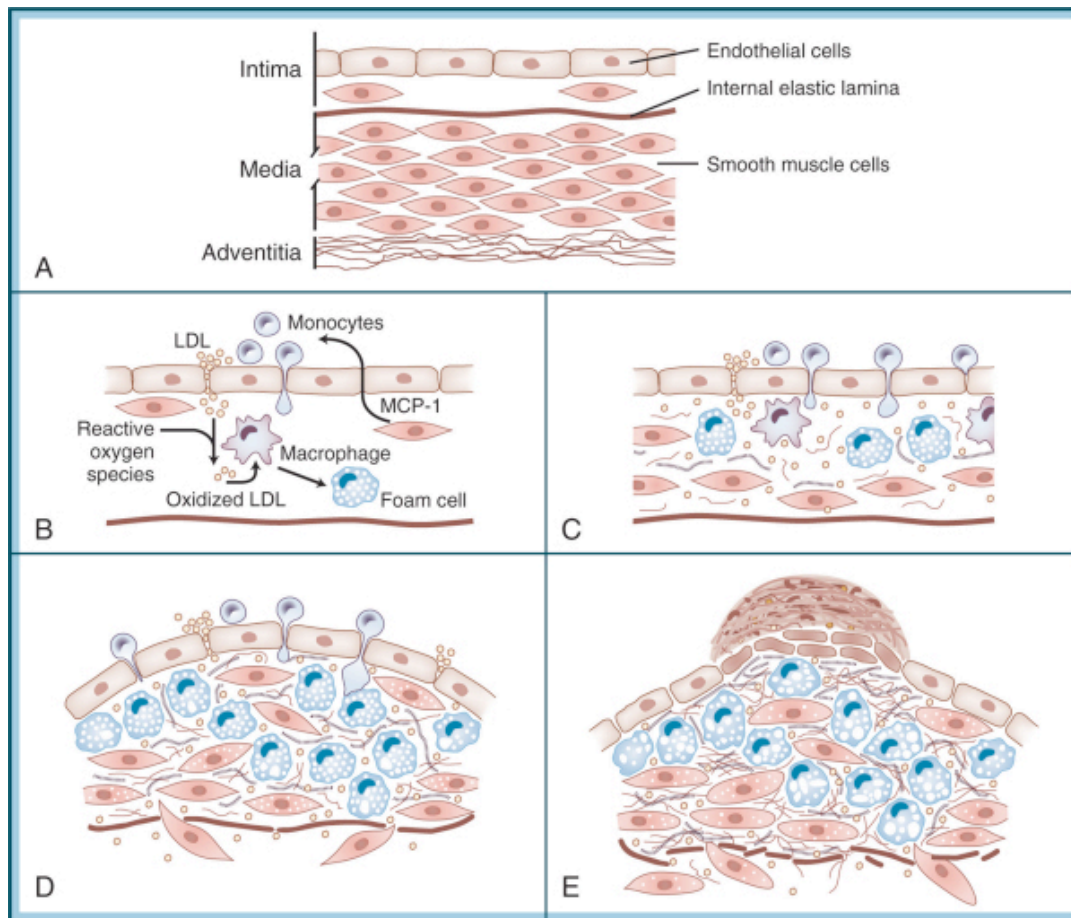


Figure 1.10 Atherosclerosis development process: (A). Structure of the normal arterial wall; (B). The initial event in atherosclerosis development; (C). Formation of fatty streak lesions; (D). Formation of proliferative or fibrous lesions; (E). Formation of a complicated lesion (Adapted from [45]).

1.2.2. Atherosclerosis Initiation

Numerous studies have indicated that the response-to-injury hypothesis is one of the most important mechanisms to atherosclerosis formation [2, 42]. In this theory, the first step in atherosclerosis is endothelial cell dysfunction/denudation. The dysfunction results in altered endothelium homeostatic properties. For example, different injuries to arteries increase the capacity of the endothelium to adhere monocytes (a premature form of

macrophages) and platelets (or thrombocytes, a type of blood cell that maintains homeostasis and formation of blood clots) as well as increase permeability. Following that, the inflammatory response, which is mediated by adherent immune cells (i.e., platelets, monocyte-derived macrophages and specific subtypes of T lymphocytes), stimulate smooth muscle cell proliferation and migration from the tunica media to form an intermediate lesion.

The longer the inflammation continues, the more monocyte-derived macrophages and lymphocytes migrate and proliferate to and into the lesion. During development, the activated immune cells as well as the vascular cells secrete and synthesize cytokines, chemokines and growth factors. Specifically, the released cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF) modify the growth and metabolism of endothelial cells. Moreover, at this time, the anticoagulation-procoagulation balance in the endothelium is shifted away from anticoagulation to procoagulation. Thus, all of these events lead to the thickening and remodeling of the diseased artery wall and further development of the complicated lesions characteristic of late stage atherosclerosis, which usually consist of fibrous plaques covered by a thick fibrous cap containing smooth muscle cells and leukocytes overlaying a central core of necrotic debris and lipids (termed atheroma) [2, 42].

1.2.3. Previous Atherosclerosis Theories

Besides the “reaction to injury hypothesis” as presented above, there are a number of other atherosclerosis initiation and maintenance theories developed since the 19th century. These theories try to explain the evolution of atherosclerosis from one or multiple aspects. Among them, the oldest one is “lipid insudation”, in which it states that the lipids in the atherosclerotic lesion come from lipoproteins circulating in the blood. This hypothesis emphasizes the importance of hypercholesterolemia in atherosclerosis but doesn’t explain other factors in lesion development such as the accumulation of smooth muscle cells [2].

Along this line, a second “encrustation hypothesis” focuses on the initiation of atherosclerosis, which proposes that atherosclerosis is initiated from repeated cycles of thrombosis (blood clotting formation) and remodeling. On the other side, another hypothesis, the “monoclonal hypothesis”, pays more attention to the contribution of smooth muscle cells accumulating in atherosclerotic development. This hypothesis states that a single progenitor smooth muscle cell can be stimulated to proliferate to form a monoclonal lesion. The “intimal cell mass hypothesis” states that atherosclerosis lesions are the consequence of small accumulations of intimal smooth muscle cells [2].

1.2.4. Roles of Cells Involved in Atherosclerotic Lesions

Regardless of the above mentioned mechanisms, it is clear that both arterial cells and immune cells are involved in atherosclerotic lesions. In the next few sections, their respective roles in atherosclerosis will be discussed.

1.2.4.1. The Functions of the Endothelium in Vascular Disease

As previously described, the endothelium is not only a single-cell layer but also an important organ in maintaining the health of normal arteries.

Empirical clinical observations show clearly that the endothelium is resistant to thrombosis formation[2] . However, it also plays a critical role in the pathogenesis of vascular tissue based on the fact that its structural and functional properties are responsive to a variety of local and systemic stimuli (such as modified blood flow, hypertension or vascular injury) [36-38]. The subsequent modulation of the endothelial cell phenotype might become a pathogenic factor for disease (such as atherosclerosis) progression.

It has been proven that the endothelium has a balance of anticoagulation-procoagulation properties. On the anticoagulant side, a membrane-associated heparin sulfate synthesized by the endothelium, when binding with antithrombin III, could rapidly inactivate the blood clotting cascade and inhibit smooth muscle cell proliferation [2, 46]. The endothelium also

releases thrombomodulin, a protein that is bound to a surface receptor to bind thrombin so that the complex inactivates the proteolytic activity for fibrinogen (a major protein for vertebrate blood clotting) [2]. Furthermore, the protein-C-protein-S complex can inhibit the clotting cascade as well after activation by the thrombomodulin-thrombin complex. Lastly, it is proven that the endothelium activates plasminogen to plasmin to degrade fibrin and synthesize prostaglandin I₂ (PGI₂) [2].

While on the other side, the endothelium changes the balance of anticoagulation-procoagulation towards procoagulation when exposed to inflammatory factors such as IL-1 and TNF released by monocyte-derived macrophages and other cells. At this moment, endothelial cells secrete various factors such as platelet activating factor, plasminogen activator inhibitor as well as a number of receptors to favor the clotting cascade [2].

1.2.4.2. Factors that Induce Endothelium Dysfunction

There are several factors that induce endothelium dysfunction, including elevated or modified LDL, free radicals caused by hypertension, elevated plasma homocysteine concentration and infectious microorganisms [42]. Among them, modified LDL is one of the major causes for endothelial and smooth muscle cell injury. After being trapped in the artery, LDL particles are oxidized progressively and finally internalized by macrophages via scavenger receptors. This internalization stimulates lipid peroxide

formation and cholesterol ester accumulation, resulting in foam cell formation and endothelial cell dysfunction [42, 47, 48]. Besides, oxidized LDL may expand inflammatory responses after endothelial dysfunction by stimulating the proliferation of monocyte-derived macrophages [42]. The binding of LDL to endothelial cells and smooth muscle cells can also be enhanced by the release of TNF- α , IL-1 and macrophage colony-stimulating factors released by the macrophages [42, 49].

Another factor, homocysteine, is an amino acid in the blood synthesized by methionine. High level of homocysteine (called hyperhomocysteinemia) is a major risk factor for cardiovascular diseases. Hyperhomocysteinemia decreases the production of nitric oxide and several of the main components of extracellular matrix proteins (collagen, elastin and proteoglycan), which lead to the dysfunction of the endothelium [42, 50, 51]. Although folic acid has been found to decrease plasma homocysteine concentrations, clinical studies indicated that folic acid treatment to patients with cardiovascular diseases do not reduce the risk of death [50, 52].

Hypertension, defined as high blood pressure in the arterial system, is another form of cardiovascular diseases. It can induce the injury of the endothelium and the development of atherosclerosis lesions by stimulating the growth of smooth muscle, increasing the oxidation of LDL or reducing

the availability of nitric oxide [53]. Lastly, clinical results showed that some infectious microorganisms, such as herpes viruses or *Chlamydia pneumoniae*, when combined with other factors previously described, might be responsible for the initiation of atherosclerosis [54, 55].

1.2.4.3. Smooth Muscle Cells

Only a few *in vivo* studies have investigated the role of the smooth muscle cells in the progression of atherosclerosis because it is difficult to find a single gene that marks the smooth muscle cell lineage [56]. However, histological studies of human coronary arteries indicated that there are intimal-abundant smooth muscle cells in atherosclerotic lesions [57, 58]. These intimal smooth muscle cells play important roles in not only late stage atherosclerosis but also in its initiation and early progression [56-58].

As previously presented, smooth muscle cells have two phenotypes: contractile and synthetic. Medial smooth muscle cells predominantly show a contractile capacity as they express proteins such as smooth muscle myosin heavy chain (SM-MHC) and smooth muscle α -actin (SM- α A), whereas smooth muscle cells in the intimal atherosclerotic lesions are characterized by their synthetic properties, that is, enhanced proliferation rates and production of extracellular matrix proteins (collagen type I, proteoglycans and fibronectin) [56, 59-61] and cytokines [61].

It is widely known that the major function of smooth muscle cells in atherosclerosis development is to produce extracellular matrix proteins (such as proteoglycans and fibronectin) that are the main components in atherosclerotic lesions. In addition, smooth muscle cells express a number of receptors including LDL receptors, type I and type II scavenger receptors with the stimulation of atherogenic cytokines (IL-1 β or TNF- α), as determined by *in vivo* studies in rats and rabbits [56, 59, 62]. All of these results have lead to a documented increased uptake of LDL by smooth muscle cells and enhanced foam cell formation.

Like endothelial cells, T cells and macrophages, smooth muscle cells also produce many cytokines under atherogenic conditions to favor endothelial cell dysfunction, smooth muscle cell proliferation, leukocyte activation and adhesion onto the endothelium [56, 59]. Some of the most important cytokines that smooth muscle cells release include: platelet derived growth factor (PDGF), macrophage inhibitory factor (MIF), interferon gamma (IFN γ) as well as monocyte chemoattractant protein (MCP-1) [56, 59, 61].

Another role of smooth muscle cells in the progress of atherosclerosis is their regulation of monocyte/macrophage functions (such as their retention in atherosclerotic lesions and apoptosis). Endothelial cells as well as smooth muscle cells directly interact with monocyte-derived macrophages.

The interaction is regulated by a variety of adhesion molecules secreted by synthetic smooth muscle cells, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which enables the interaction between endothelial cells and macrophages or lymphocytes, and fractalkine (CX3CL1) that enables the binding between smooth muscle cells and monocytes [56, 63, 64]. In addition, smooth muscle cells have been shown to protect monocytes/macrophages from apoptosis [65].

1.2.4.4. Macrophages

The morphology of macrophages is characterized by its complex and abundant surface folds (microvilli), indented nuclei, a well-developed Golgi apparatus, and abundant lysosomes and endoplasmic reticulum [2].

Monocyte/lymphocyte adhesion to the endothelium (i.e., activated endothelial cells) and subendothelial migration of monocytes have been now widely accepted as another major cause for the initiation of atherosclerosis [2, 3]. Macrophages, which are derived from monocytes, have an enhanced capacity for phagocytosis and intracellular hydrolysis of infective microorganisms, modified biological substrates and implanted surfaces [2, 66].

Macrophages are the main source of cytokine production in atherosclerosis. They secrete proinflammatory cytokines (such as

interleukins (IL)-1, IL-6, IL-12 and tumor necrosis factors (TNF)- α), while they secrete anti-inflammatory cytokines (IL-10, IL-32 and TGF- β) as well (shown in Table 1). Besides, they can also release a large number of other biologically active products, including enzymes (and inhibitors), plasma proteins, chemotactic factors, bioactive lipids (arachidonic acid metabolites and platelet activating factor (PAF)), oxygen metabolites, and activating and inhibiting factors regulating the proliferation and function of other cells [2].

1.2.4.5. Overview of Cytokines

According to Balkwill and Burke, cytokines are defined as “one term for a group of protein cell regulators, variously called lymphokines, monokines, interleukins, interferons and chemokines which are produced by all the cells involved in the development of atherosclerosis (monocytes, endothelial cells, smooth muscle cells and platelets), play an important role in many physiological responses, are involved in the pathophysiology of a range of diseases, and have therapeutic potential.” [67]. There are several classes of cytokines including more than 50 secreted factors: interleukins (IL), tumor necrosis factors (TNF), interferons (TNF), colony stimulating factors (CSF), transforming growth factors (TGF) and chemokines. They are categorized by the structural homology of their receptors. The main families of cytokines include: class I and class II cytokines, the IL-1 family, TNF family and TNF- β superfamily. According to

their pro- and anti-inflammatory activities, cytokines are also classified as pro- and anti-atherogenic cytokines [66]. Their main classifications and specific roles in mediating inflammatory responses are shown in Table 1.2.

Table 1-2 List of important pro- and anti-inflammatory cytokines (Adapted from [66]).

Pro-inflammatory Cytokines	Anti-inflammatory Cytokines
<u>Class I Cytokines</u>	
IL-2	
IL-4	
IL-6	IL-6
IL-12	
<u>Class II Cytokines</u>	
IFN- γ	IL-10
<u>The IL-1 Family</u>	
IL-1	IL-1Ra
IL-18	IL-18BP
<u>TNF Family</u>	
TNF- α	
<u>TGF-β superfamily</u>	TGF- β

In the development of atherosclerosis, cytokines (once their production is triggered by bioactive lipid mediators such as oxidized LDL [66]) are involved in all kinds of intercellular communication to controlling fundamental cell functions. They induce different cellular responses of various cells involved in atherosclerosis. Figure 1.11 shows the source and target cells for specific cytokines involved in atherosclerogenesis. For example, oxidized LDL induces the production of cytokines from

endothelial cells (IL-1, MIF, M-CSF and GM-CSF) and from smooth muscle cells (M-CSF) [66, 68]. Among them, MIF, GM-CSF and M-CSF upregulate macrophage proliferation and differentiation. IL-2, IL-3, IL-12, TNF and IFN γ released by T helper cells can also enhance macrophage proliferation [66, 68]. Besides, IL-2 is a T-cell growth factor, while IFN γ inhibits smooth muscle cell functions. Smooth muscle cells are targets of many other cytokines (IL-1, IL-6 and TNF- α), while smooth muscle cells themselves secrete various cytokines (IL-1, IL-6, M-CSF and IFN γ) which activate macrophage function [66, 68]. Specific cytokines important during atherosclerosis development and maintenance are elaborated on below.

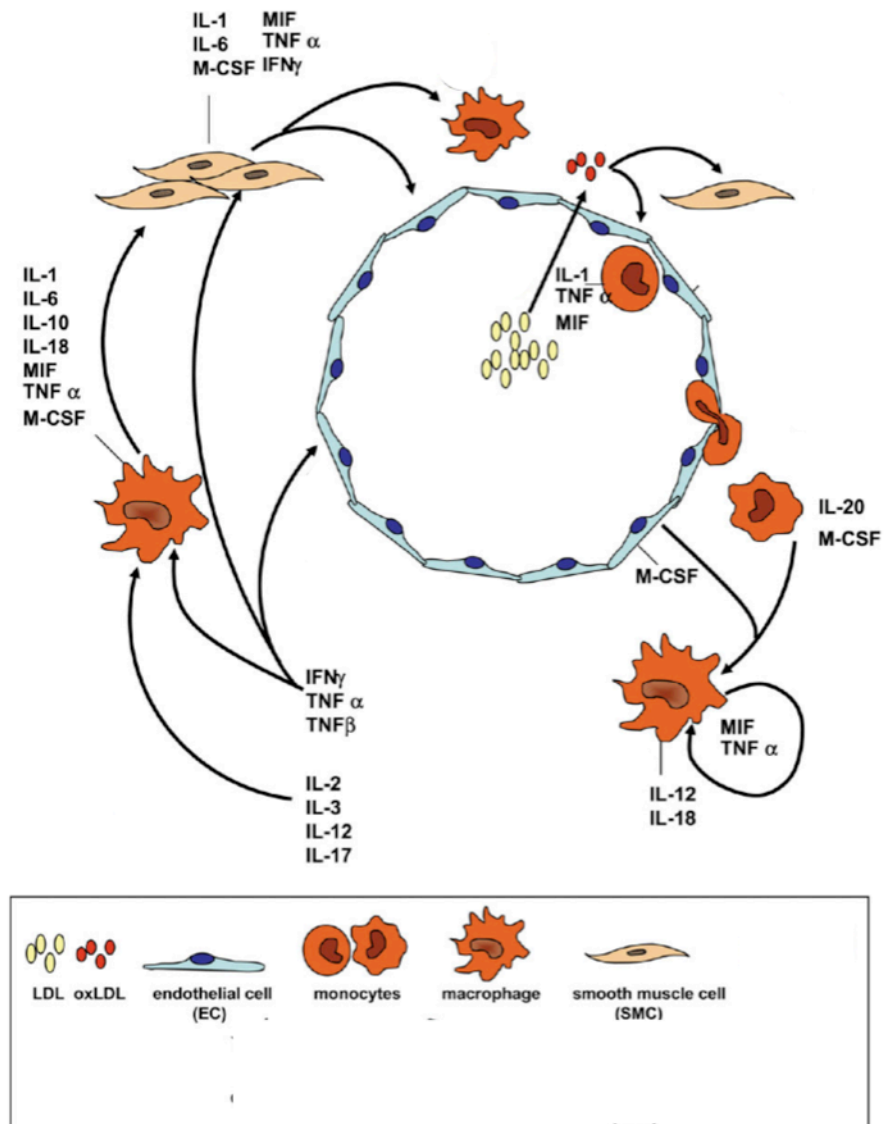


Figure 1.11 Cytokines involved in atherosclerosis and their cellular source and targets (adapted and redrawn from [68]).

1.2.4.6. TNF- α

TNF- α is mainly synthesized by monocytes / macrophages. It is present in human atherosclerotic lesions as well as in the blood stream. TNF- α is a multifunctional cytokine that not only has potent proinflammatory effects in atherosclerosis but also in other metabolic and inflammatory disorders that are also related to cardiovascular diseases. In addition, it is a risk factor for recurrent myocardial infarction, atherosclerotic thickening of carotid intima-media, and disturbance in glucose homeostasis [42, 66, 68, 69].

1.2.4.7. IL-1

IL-1 has two forms: IL-1 α and IL-1 β , both of which are prototypic pro-inflammatory cytokines with slightly different biological functions mediated through the IL-1 receptor (IL-1R). After binding to IL-1R, IL-1 induces the recruitment of inflammatory cells via the production of various cytokines, chemokines as well as endothelial cell adhesion molecules [68, 70]. Moreover, IL-1 releases matrix-degrading enzymes to enhance the development of vascular damage. While IL-1R is an antagonist (IL-1Ra), a structural homologue of IL-1, also binds to IL-1R but is an IL-1 inhibitor [68, 70].

There are quite a few studies investigating the role of IL-1 in the development of atherosclerosis. Kirii *et al.* showed that compared with IL-

IL-1 β -expressing-ApoE^{-/-} mice, atherosclerosis decreased by 30% in IL-1/ApoE^{-/-} mice at 12 and 24 weeks of age [71]. Kamari *et al.* found that the genetic deletion of IL-1 α also decreased the lesion area by 56% [72]. Similarly, IL-1R absence has led to a significant reduction in the progression of atherosclerosis [73]. In all, IL-1 signaling plays an important role in atherosclerotic development [73].

1.2.4.8. IL-6

IL-6, induced by IL-1, is considered to be a multifunctional cytokine involved in various aspects of the inflammatory response. *In vivo* experiments have shown it to be one of the most important factors regulating the development of atherosclerosis as it directly regulates atherosclerotic lesion development [66, 68]. In IL-6^{-/-}/ApoE^{-/-} mice, fatty streaks decreased by less than 1 year [74]. However, in another study by Schieffer *et al.* enhanced atherosclerotic lesions with higher levels of total cholesterol and LDL were observed in male IL-6^{-/-}/ApoE^{-/-} double knockout mice [75].

Together with other experiments, it has been indicated that IL-6 has both a positive and negative role in atherosclerotic lesions. As a proinflammatory cytokine, IL-6 has shown to increase the size of fatty streaks in atherosclerosis. While as an anti-inflammatory cytokine, it can deactivate

other pro-inflammatory cytokines (specifically, IL-6 decreases the activity of IL-1 by inducing the synthesis of IL-Ra [66]).

1.3. Treatments to Atherosclerosis

The above sections reviewed the physiology of artery and discussed general mechanisms of atherosclerosis. In fact, atherosclerosis poses serious threat to many patients and, thus, usually requires prompt medical therapies. These treatments include grafting, balloon angioplasty, and vascular stenting. The application and limitation of each of the treatments will be discussed in the following sections.

1.3.1. The Need for More Efficient Atherosclerosis Treatments

Atherosclerosis is the main cause of coronary artery diseases (CAD) and peripheral arterial disease (PAD) and accounts for about three fourths of all deaths from these diseases [76]. According to an American Heart Association (AHA) Report updated in 2008 [77], both the absolute number and the prevalence of atherosclerosis are quickly increasing. For example, coronary heart disease, which is atherosclerosis of heart arteries, has become the single leading cause of death in the United States today (Figure 1.12) [77]. In 2004, 15,800,000 Americans suffer from all kinds of coronary heart diseases causing about 452,300 deaths [77]. This translates into a coronary event for an American every 26 seconds and a death every minute. Based on the AHA computation, “About 38% of the people who experience a coronary attack in a given year will die from it.”

[77]. As a result, atherosclerosis and its related atherothrombotic diseases (CAD or PAD) are currently considered to be or are projected to be the leading cause of death all over the world by 2020 [76, 77]. As for their treatment, in 2004 more than half of the hospital stays for atherosclerosis was for patients receiving Coronary artery bypass grafting (CABG) and coronary artery angioplasty (PTCA) [77, 78]. Treatments for atherosclerosis led to 1.2 million hospital stays and cost 44 billiond dollars, which was 5.6% of the total American hospital bill and was the most expensive condition treated [77, 78].

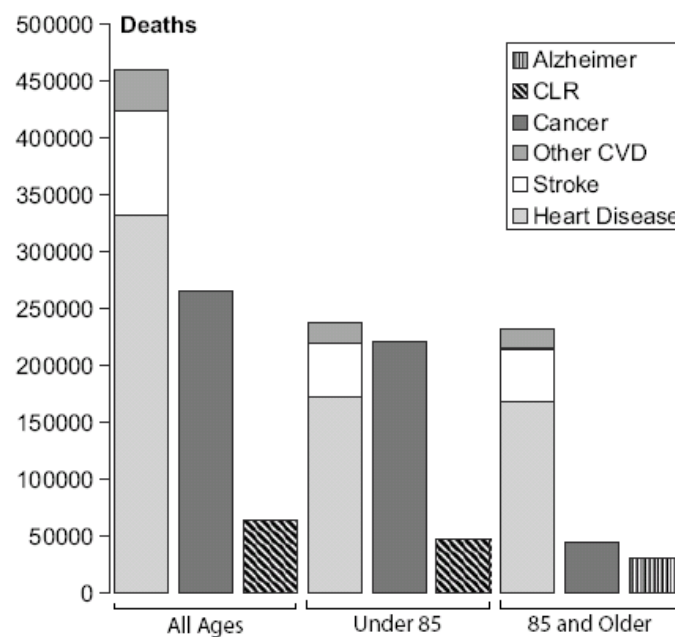


Figure 1.12 Leading causes of death in the United States in 2004 [77]
(CLR: Chronic Lower Respiration; CVD: Cardiovascular Disease).

1.3.2. Coronary Artery Bypass Grafting

Coronary artery bypass grafting (CABG), also called revascularization, was first introduced in the 1960s. According to the American Heart Association (AHA), as one of the most common open-heart surgeries, there are more than 500,000 CABG surgeries performed per year in the United States [79]. CABG begins with a median (middle) sternotomy, during which an incision is made down in the middle of chest. A piece of healthy artery from elsewhere in the patient's body or a synthetic artery is then grafted to bypass the blocked site. The most widely used autologous arteries are the saphenous vein from the leg or the left internal mammary artery from the chest wall [2]. CABG is an expensive surgery with high risks. Usually, only patients with severe narrowing or blockage of the left main coronary artery or those with disease involving two or three coronary arteries are considered for bypass surgery. This approximately accounts for 10% of those with coronary artery disease.

1.3.3. Balloon Angioplasty and Restenosis

Roughly one-third of patients, whose atherosclerosis does not involve multiple coronary arteries, will undergo coronary angioplasty and stenting instead [77]. Vascular stenting originated from balloon angioplasty. Coronary angioplasty, now referred to as percutaneous coronary intervention (PCI), is a medical procedure to re-open blocked arteries. It was introduced in the 1970s as an alternative to CABG. In angioplasty, a

balloon-tipped catheter is inserted into the blocked site with the help of guide wires. The balloon is inflated at that site to compress atheromatous plaques and push them away from the lumen. The balloon is then deflated and removed from the artery (Figure 1.13). About 1 million angioplasty procedures are performed in the United States every year [80].

Angioplasty has many benefits over conventional bypass surgery including high procedural success and relative ease and low-risk of operation (that is, the angioplasty process doesn't require large incisions) [81].

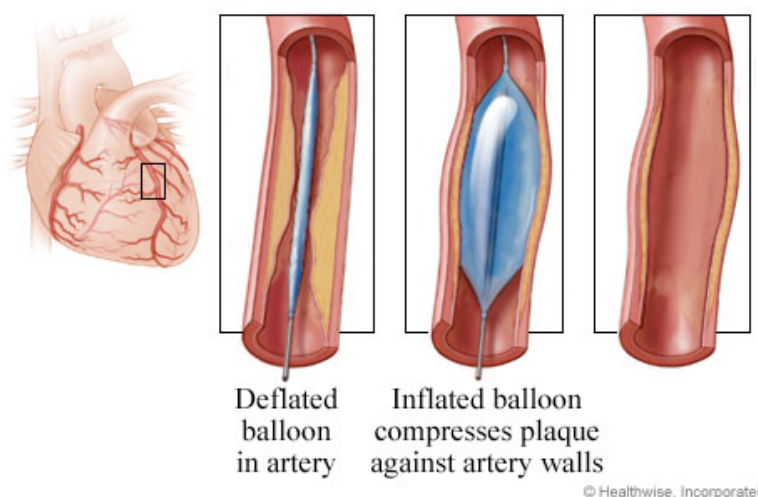


Figure 1.13 Schematic of balloon angioplasty (Adapted from [82]).

The main limitation of balloon angioplasty is restenosis [83]. Restenosis is a phenomenon in which the treated artery becomes narrowed again due to injury and damage induced on the artery intimal by the angioplasty procedure itself. Intimal hyperplasia plays a central role in restenosis. After angioplasty, the dilated balloon may cause atheroma damage or rupture,

inducing intimal hyperplasia, which happens when a mural thrombus is formed at the injured site [83, 84]. Besides intimal hyperplasia, many other factors contribute to restenosis after balloon angioplasty, such as vascular remodeling (elastic recoil and chronic constriction) and vascular smooth muscle cell hyper-proliferation and excessive extracellular matrix production at the injured site [83-85]. In 6 months to 1 year after an angioplasty procedure, 50% of patients suffer from restenosis [84].

1.3.4. Vascular Stenting and In-stent Restenosis

As the problems of restenosis became more severe in patients after PCI, which was relatively inevitable due balloon technology, vascular stenting was introduced [84]. Vascular stenting is the procedure of implanting a thin expandable tube composed of a metal wire mesh into a narrowed artery that is blocked as a result of atherosclerosis. It is often performed simultaneously with balloon angioplasty, where stents are placed over a balloon-tipped catheter. When the catheter is inflated at the diseased site, the stent expands to stretch the artery wall, and to push and hold atheromas against the artery wall. The balloon is then deflated and removed from the artery while leaving the stent in place as a permanent intravascular prosthesis (Figure 1.14). Fischman *et al.* and Serruys *et al.* both compared coronary stent implantation to balloon angioplasty in the treatment of coronary artery disease. Both studies confirmed that coronary stenting could reduce restenosis rates (from 50% to 25%) compared with balloon angioplasty [86, 87].

The metals used in vascular stents should possess excellent mechanical properties such as good flexibility, dilatability, strength, and the most importantly, biocompatibility [88, 89]. Currently the most widely used stent materials are titanium, cobalt chromium alloys, stainless steel and Nitinol (titanium-nickel alloys) [88, 90, 91]. Among them, stainless steel lacks sufficient biocompatibility. Stainless steel can corrode and, thus, can induce severe thrombosis and restenosis [88]. Nitinol has excellent shape-memory properties and biocompatibility as well, but it is difficult to fabricate [88].

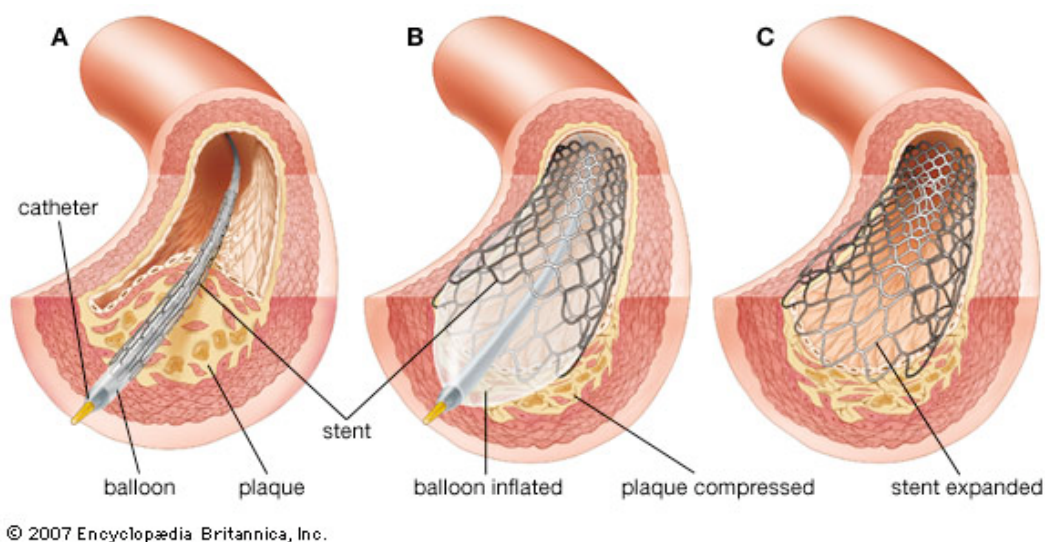


Figure 1.14 Schematic of a vascular stenting procedure. A) Implantation of a cardiac catheter in an inflatable balloon and wire-mesh stent; B) Inflation of the balloon to expand the stent and dilate the artery; and C) Deflation and withdrawn of the balloon with the catheter to leave the stent expanded against the arterial wall. (Adapted from [92])

Current vascular stent metals consist of conventional micron particle sizes (larger than 100 nm both vertically and horizontally) which have resulted in

several problems including in-stent restenosis [93-95]. Although stent implantation successfully inhibits restenosis induced by vascular recoil and elasty artery remodeling, it allows for neointimal tissue formation and thrombus inflammation, which will lead to in-stent restenosis [93-97].

Histological studies have revealed hyperproliferation of stellate-shaped smooth muscle cells, proteoglycan matrix abundance and paucity, and the absence of endothelial cells during in-stent restenosis [96, 97].

In-stent restenosis usually occurs in more than 25% of patients within 3-6 months postprocedure [93], which is less frequent than post-balloon angioplasty restenosis but still problematic. Some treatments for in-stent restenosis exist now, including: 1) implantation of a new bare metal stent; 2) “cutting” balloon angioplasty through implanting a blade-coated balloon catheter to disperse in-stent restenosis; 3) directional coronary atherectomy (DCA) through immediately removing neointimal tissue; and 4) high-speed rotational atherectomy involving mechanical rotation prior to percutaneous transluminal coronary angioplasty/stent to reduce the occurrence of neointimal proliferation [98]. However, none of these methods are effective in the reduction in the reoccurrence of in-stent restenosis [98].

1.3.5. Improvements on Current Vascular Stents

1.3.5.1. Drug-eluting Stents

Numerous studies have focused on two strategies to inhibit or limit in-stent restenosis: inhibition of vascular smooth muscle cell proliferation and/or acceleration of endothelialization. For smooth muscle cells, recently numerous approaches have centered on drug-eluting stents (DES).

Currently there are four DES systems approved by FDA:

1. Cypher® from Johnson&Johnson which uses 316L stainless steel stent struts and a 3-layer polymer (Parylene C and polyethylene-co-vinyl acetate (PEVA)/poly n-butyl methacrylate (PBMA) copolymer) coating embedded with sirolimus (an immunosuppressant drug inhibiting T-cell activation) [81]. The eluting period of the sirolimus is about 30 days [99];
2. Taxus® from Boston Scientific which also uses 316L stainless steel stent struts and a single layer of Translute SIBS copolymer coating embedded with paclitaxel (an anti-proliferative drug limiting smooth muscle cell proliferation) [81]. The eluting period of the paclitaxel is about 90 days [99];
3. Endeavour® from Medtronic which uses cobalt chrome struts and single layer of a phosphorocholine based polymer (non-thrombogenic) embedded with zotarolimus (an anti-proliferative drug limiting smooth muscle cell proliferation) [100]. 95% of the zotarolimus is

released within 15 days after implantation [100]; and

4. Xience V® from Abbott which uses cobalt chrome struts and a fluoropolymer multilayer coating embedded with everolimus (a derivative of sirolimus) [101]. The eluting period of the everolimus is about 4 months [101].

Among these drug-eluting stents, the release of the drugs has proven to substantially reduce in-stent restenosis by inhibiting neointimal proliferation [100-103]. The main advantages of drug-eluting stents over bare metal stents lie in local drug delivery to inhibit intimal re-thickening: i.e., the drugs embedded in polymer coatings on bare metal stents interfere with inflammation, migration, and proliferation of smooth muscle cells and/or secretion of the extracellular matrix proteins by autocrine or paracrine mechanisms [81].

However, despite of the early success of DES, recent studies have begun to question the wisdom of a therapeutic strategy effectively based on the inhibition of healing [81, 104-110]. In particular, there is some evidence that the incidence of stent-related thrombosis (i.e., clotting) is higher in DES than bare-metal stents (Figure 1.14) [104-110]. Moreover, the incidence of stent-related thrombosis increases considerably when there is cessation of adjunctive anti-platelet therapy, even after the 6 to 12 month period commonly prescribed for such therapies [108]. While overall incidences of stent-related thrombosis in DES is low (<1.5%), these

results provide significant concerns because the fatalities in such cases can be as high as 45% [108]. Although the mechanism of such stent-related thrombosis has yet to be fully understood, a causal relationship between DES and delayed healing has been established [104-110]. Most significantly, drug-induced inhibition of hyperplasia also inhibits the reestablishment of a healthy endothelium, thus, such approaches increase the potential for thrombogenic stimuli [106, 109]. This, therefore, provides the impetus for the exploration of alternative therapeutic strategies that enhance, rather than inhibit, endothelialization of bare metallic stent surfaces.

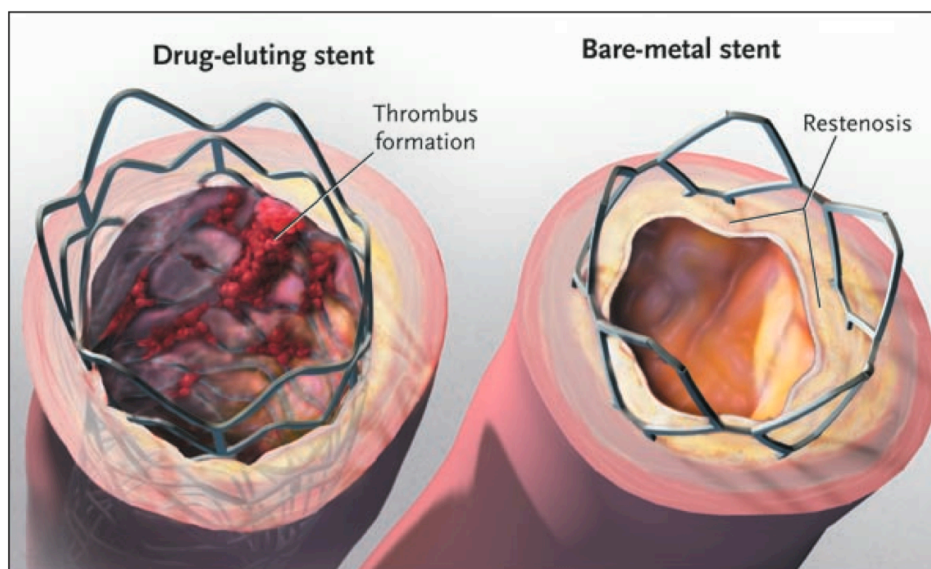


Figure 1.15 Risks associated with drug-eluting stents and bare-metal stents: Drug-eluting stents can inhibit in-stent restenosis in bare-metal stents, however, uncovered struts may be prone to late thrombosis after the discontinuation of drug release. (Adapted from [111])

1.3.5.2. Other Therapies

Considering the side effects of drug-eluting stents, coating bare metal stents with highly biocompatible materials not containing drugs is another method to enhance the biocompatibility of stents. Currently, ceramics (such as silicon carbide and tantalum oxide) [112, 113] or polymers (such as polyhydroxyalkanoates (PHA) or polyurethane) [114, 115] have been used as coating materials. Among them, PHA-coated stents tended to decrease intimal thickness compared with conventional metal stents. However, those coatings might be easily damaged due to shear stresses created by circulating blood and their fragments might cause severe blood diseases [116].

As mentioned, to improve the post-procedure success of vascular stenting, another therapy is to accelerate endothelialization (or coverage by endothelial cells) of vascular stents. In this case, the applications of nanotechnology in vascular stents have shown great potential. For example, Nakano *et al.* have developed a biodegradable polymer nanoparticle-eluting stent system by a novel cationic electrodeposition coating technology. *In vitro* studies demonstrated that such drug nanoparticles were stable and efficiently internalized in vascular smooth muscle cells. In addition, *in vivo* studies indicated that the nanoparticles were distributed homogenously throughout the implanted area [117]. Besides using nanoparticles, another nanotechnology approach that

seems promising for current vascular stents is the creation of nanoscale features on vascular metallic stent surfaces which mimic the natural structure of the healthy vessel wall. Recently, Choudhary *et al.* created random nanostructured titanium surface features by using a uniaxial hydraulic press to press titanium nanoparticles (250-750 nm) into titanium compacts. *In vitro* studies indicated that vascular stents with random nanostructured surface features invoked vascular cell responses promising for improving vascular stent applications [116]. *In vitro* studies of Peng *et al.* and Karla *et al.* showed significant enhancement of vascular endothelial cell proliferation and mobility on TiO₂ nanotubes prepared by anodization compared with flat titanium [118, 119]. However, compared to biocompatible coatings and drug-eluting stents, such surface modification has received much less attention to date.

1.4. Titanium Used for Cardiovascular Applications

Currently, there are three different kinds of materials used for vascular stent implants in the market. They are stainless steel, cobalt-chrome, and titanium. Titanium and its alloys show great potential due to their excellent mechanical and biomedical properties. In the following sections, such attractive properties of titanium will be discussed and methods to fabricate titanium-based vascular prosthesis will be reviewed.

1.4.1. Titanium Chemistry and Crystal Structure

Titanium is an abundant element in minerals with alpha and beta allotropes. Alpha phase titanium has a hexagonal closed-packed (hcp) crystal structure (Figure 1.16 a) and exists at room temperature. Beta phase is a body-centered cubic (bcc) structure (Figure 1.16 b) and appears when titanium solidifies to equilibrium from liquid when titanium is heated to over 883 °C.

At room temperature, titanium can be alloyed with other elements to form stable crystal structures. Common alpha-stabilizing elements include aluminum, tin and oxygen and common beta-stabilizing elements include niobium, molybdenum, chromium, iron and vanadium. Such kinds of alloys are called “alpha” and “beta” titanium alloys respectively. Many alloys contain a combination of the above elements and are classified as “alpha-beta” alloys. A highly biocompatible alloy used in vascular stent applications, Nitinol, is a nickel-titanium alloy with shape memory properties (Nickel Titanium Naval Ordnance Laboratory).

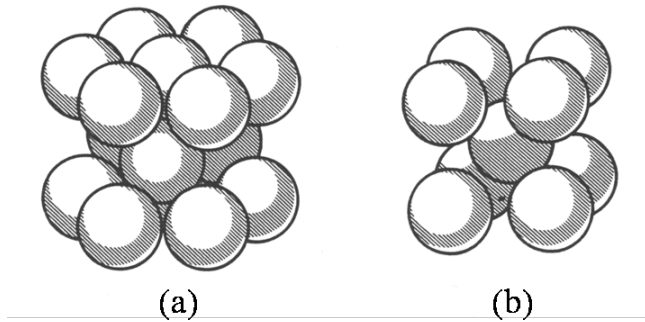


Figure 1.16 Crystal structures of (a) alpha and (b) beta phase titanium. Lattice parameters for hcp alpha titanium are: $a = 2.95 \text{ \AA}$, $c = 4.68 \text{ \AA}$ [120] (Adapted from [121]).

1.4.2. Physical and Mechanical Properties

The physical and mechanical properties of elemental titanium are shown in Table 1.3, which indicates that the density of titanium is low (about 4.51 g/cm^3) but with excellent mechanical properties [120] (Table 1.4).

Depending on different crystalline phases, titanium exhibits different properties. Table 1.3 shows the mechanical properties of pure and alpha, beta, and alpha-beta titanium alloys. Unalloyed and alpha titanium alloys exhibit high strength and high hardness, while beta alloys are more ductile and have a lower modulus but higher strength compared with the other two forms of titanium. However, these mechanical properties can be modified by heat treatment and mechanical processing on titanium, which changes its microstructure. For example, mechanical processing lead to increased aspect ratios of grain sizes in the working direction of applied forces and the following heat treatment results in recrystallization of these

elongated grains into an equiaxed structure. As for their mechanical properties, the equiaxed structures exhibit lower non-directional strength and higher ductility than elongated structures, which have higher anisotropic tensile strength. The properties of titanium materials are also determined by its microstructure, which can be manipulated through appropriate mechanical processing and thermal treatments.

Table 1-3 Physical properties of titanium.

Atomic number	22
Atomic weight	47.90
Covalent radius	1.32Å
Color	Dark grey
Density	4.51 g/cm ³
Melting point	1668 ± 10 °C
Boiling point	3260 °C
Thermal conductivity	97 BTU/hr m ² °F
Coefficient of thermal expansion	8.64 x 10 ⁻⁶ °C
Electrical conductivity	3 % IACS (copper 100 %)
Hardness	HRB 70 to 74
Tensile strength	240 MPa min
Young's modulus of elasticity	102.7 GPa
Poisson's ratio	0.41
Coefficient of friction	0.8 at 40 m/min

Table 1-4 Mechanical properties of selected titanium, titanium alloys
(Adapted from [122]).

Material and Designation (or Condition)	Young's modulus (GPa)	Tensile strength (MPa)	Yield strength (MPa)
Ti ASTM Grade 1 (alpha)	102.7	240	170
Ti ASTM Grade 2 (alpha)	102.7	345	275
Ti ASTM Grade 3 (alpha)	103.4	450	380
Ti ASTM Grade 4 (alpha)	104.1	550	485
Ti-6Al-4V (alpha/beta; annealed)	110-114	895-930	825-869
Ti-6Al-4V ELI (alpha/beta)	101-110	860-965	795-875
Ti-13 Nb-13Zr (beta; aged)	79-84	973-1073	836-908
Ti-12Mo-6Zr-2Fe (beta; annealed)	74-85	1060-1100	1000-1060

1.4.3. Chemical Properties

Titanium is a very reactive metal. When exposed to air, water and other media (except under certain extreme conditions such as ultra-high vacuum), the surface of titanium always forms a passivation oxide layer composed of titanium dioxide (or titania). Titania has three different crystalline structures: rutile, anatase and brookite, in which both rutile and anatase have the same structures: tetragonal crystal structure (Figure 1.17 a: titanium atoms are coordinated to six oxygen anions) and the temperature of anatase-rutile transformation is between 700 °C to 900 °C [123]. Numerous in vitro and in vivo studies confirmed the biocompatibility of titanium and titanium alloys [122].

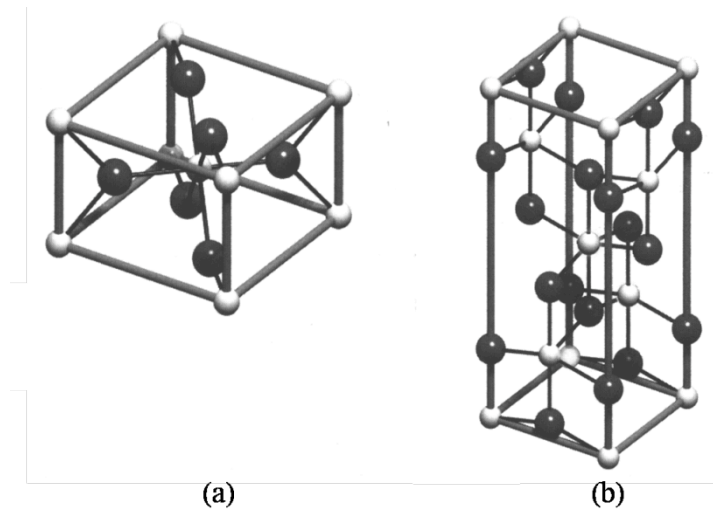


Figure 1.17 Crystallographic unit cell of rutile and anatase titanium dioxide: (a) rutile; (b) anatase. White small balls represent titanium cations while dark large balls represent oxygen anions (Adapted from [124]).

1.5. Nano/microtextured Template Techniques in Tissue Engineering and Cardiovascular Device Design

Although titanium is generally considered to be biocompatible, it is still necessary to modify its surface properties, such as surface roughness and feature size, to improve their performance as vascular stent materials. In this section, methods particularly used to fabricate nanostructured features on titanium will be discussed.

1.5.1. Review of Nano/microtextured Template

Khademhosseini *et al.* defined tissue engineering as an interdisciplinary field that involves the use of biological sciences and engineering to develop tissues that restore, maintain, or enhance tissue function [125]. Generally, tissue engineering includes three developing methods [125]:

1. Using isolated cells or cell substitutes as cellular replacement parts.
2. Using acellular biomaterials capable of inducing tissue regeneration.
3. Using a combination of cells and materials.

Nano/microtextured scaffold templates have been widely applied in the second and third approaches, as numerous studies have demonstrated that changes in the alignment and shape of cells (controlled by the underlying surface features) improve vascular and other cell functions [126-128] and that microtextured topographies control their morphology and orientation [129]. This topographical effect is thought to be distinct from a biochemical effect, but still plays an important part in modulating cell function. For example, Palmaz *et al.* have reported higher endothelial cell migration rates (up to 64%) on Nitinol surfaces consisting of micrometer-scale grooves compared to smooth metal surfaces [130]. They hypothesized that such patterns may reduce the time of complete endothelialization of vascular stents, thus, reducing the risk of in-stent restenosis and late stage thrombosis [130]. Other studies confirmed that cell shape and orientation controlled by underlying surface features were related to protein and gene expression [126, 127, 131, 132]. Physical expansion of cells may partially control DNA synthesis. Namely, more spread cells exhibit higher DNA synthetic rates [126].

Textured features have also been applied to cardiovascular devices in as early as the 1960s [133]. The surfaces, consisting of randomly positioned fibers and cavities, proved to decrease thromboembolic events by forming a stable blood-contacting substrate (Figure 1.18) [133]. However, considering the specific cell organization properties of the endothelium, scaffolds with highly controllable surface features are desirable. Various fabrication methods have been developed to create highly controllable surface features. For example, with the help of polystyrene nanospheres, Miller *et al.* succeeded in casting nano-rough PLGA (poly(lactic-co-glycolic acid)) surface features for a new-generation synthetic vascular graft design. Specifically polystyrene nanospheres (100 nm, 200 nm and 500 nm in diameter: Figure 1.19) were used to imprint the nanostructure onto PLGA. Vascular endothelial cell adhesion experiments showed greater endothelial cell density and fibronectin (a key protein for mediating endothelial cell adhesion) adsorption on nano-rough PLGA made from 200 nm nanospheres than smooth PLGA surfaces [134].

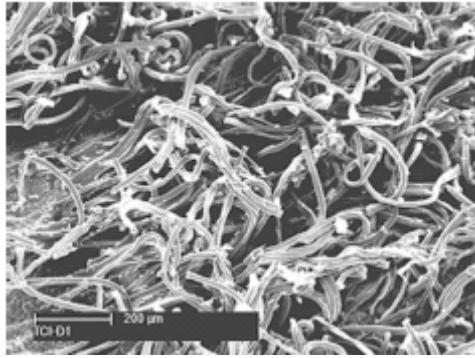


Figure 1.18 Scanning electron micrograph of the HeartMate® integrally textured fiber surface. Bar = 200 μm (Adapted from [133])

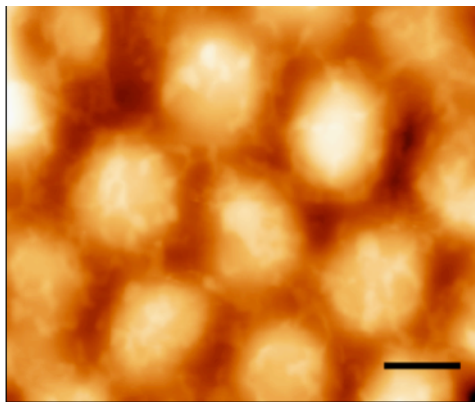


Figure 1.19 Atomic force microscope (AFM) images of a PLGA cast nanosphere (200 nm) surface. Bar = 150 nm (Adapted and redrawn from [134])

1.5.2. Micro/nano Fabrication in Tissue Engineering

There are several established ways to create materials with nanostructured surface features, from highly expensive photolithography methods to some relatively inexpensive physical deposition methods.

1.5.2.1. Photolithography

Micro/nano fabrication can be defined as a process used to construct physical objects with dimensions at the micro/nano-meter to millimeter range [135, 136]. Currently, a combination of micro/nano fabrication processes with cell culture has been widely used for fabricating BioMEMS/BioNEMS (micro-electromechanical systems/nano-electromechanical systems for biological applications) [135, 136]. Among microfabrication techniques, the most commonly used is photolithography [136, 137]. The photolithography process is shown in Figure 1.20. A substrate with a photoresist, which is made of a thin polymeric film, is exposed to light through a mask containing a desired opaque pattern. A photochemical reaction occurs in the exposed area to make it soluble in a developer solution, which leads to a patterned substrate.

All kinds of feature types can be created by this method including: grooves, needles, pores or ridges. One of the first research groups investigating cell function on photolithographically-made patterned surfaces revealed that microgrooved titanium-coated surfaces increased bone like tissue formation and controlled tissue configuration as expected [138]. Lithography techniques have also been applied to fabricate cardiovascular device tissue-substrate interfaces. Among them, poly(dimethyl siloxane) (PDMS) micropatterns made by replica-molding

from lithography-created silicon wafers have been used to promote endothelial cell and smooth muscle cell co-culture layers [139, 140].

Another common method in micro/nano fabrication is microcontact printing [137]. The patterning of cells and proteins often requires the use of self-assembled monolayers of alkanethiols on gold. Briefly, a PDMS stamp with a pattern is prepared from a mask of silicon by photolithography. Alkanethiol layers are then coated on the PDMS stamp and transferred onto the substrate. The untouched areas can also be coated with another layer of alkanethiols [137]. These self-assembled layers can be functionalized to have different properties such as hydrophobicity/hydrophilicity or chemically conjugated with peptides which favor protein and cell attachment [137].

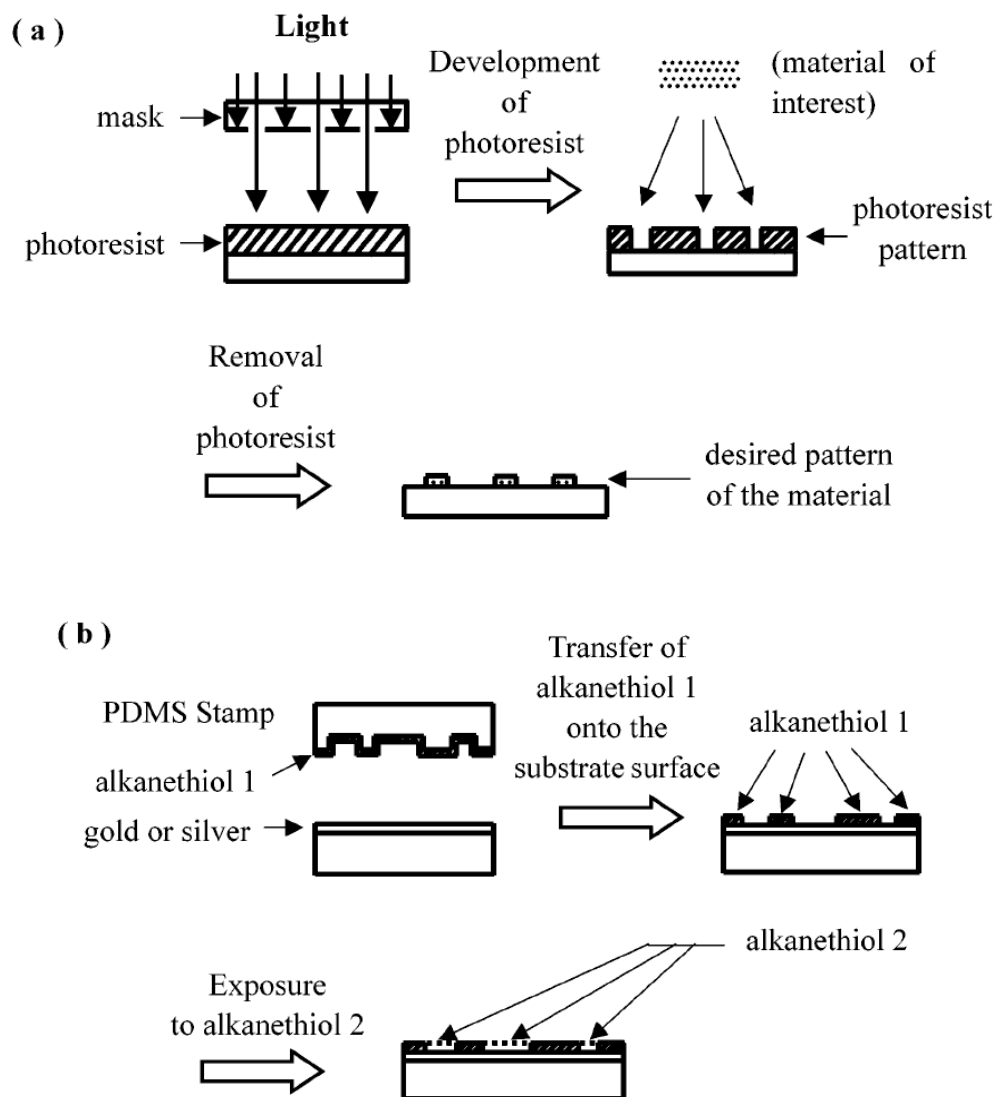


Figure 1.20 Schematic of micro/nanopatterning by (a) photolithography; and (b) microcontact printing (Adapted and redrawn from [137]).

1.5.2.2. Electron Beam Evaporation

The process of electron beam evaporation, or electron beam physical vapor deposition (EBPVD), is similar to other physical vapor deposition (PVD) processes. As shown in Figure 1.21, an electron beam generated

by a charged tungsten filament bombards the target anode with a desired deposition material in a water-cooled crucible. The materials are then transformed into vapor phase and form a thin layer on a substrate on top of the vacuum chamber, where they return back to solid form. Compared with other PVD methods, electron beam evaporation has relatively high deposition rates so that the whole process can be controlled in a short time. The substrate can be maintained at low temperature and protected from contamination.

Different PVD methods have been applied to coat all kinds of implants. Lee *et al.* used electron beam evaporation to coat a 1 μm thick layer of hydroxyapatite (HA)-titania (TiO_2) on titanium implants to improve their biocompatibility [141]. As a result, the alkaline phosphate (ALP) activity of the osteoblast-like cells increased compared to pure titanium. In the area of stents, with the application of PVD, stainless steel vascular stents (BeStent, Medtronic, Inc.) have been coated with titanium-nitride-oxide alloys [142]. Long-term experiments with porcine coronary arteries showed that fibrinogen adsorption and platelet adhesion significantly decreased on the coated substrate compared with bare metal stents.

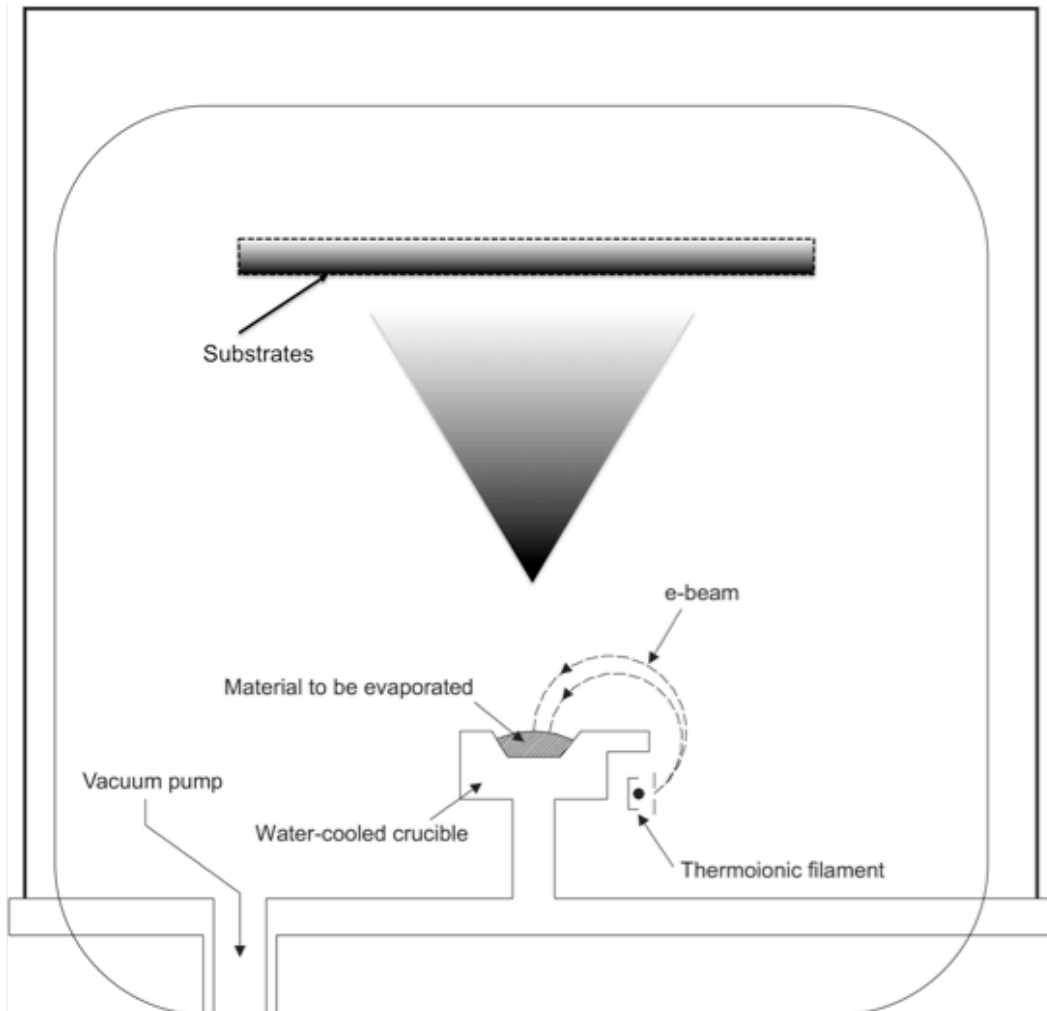


Figure 1.21 Schematic of E-beam evaporation process (Adapted and redrawn from [143]).

1.6. Tissue-Implant Interactions

When titanium stents are inserted into the human artery, a complex series of events involving instantaneous interactions of the biomaterial surface with proteins and then subsequently cells in the biological fluid ensues. It is this initial interaction of proteins and then cells that will determine

eventual biomaterial success or failure. In this section, the interactions between proteins, cells, and the prosthesis will be discussed.

1.6.1. Protein-Implant Interactions

It is well known that proteins interact with implant surfaces before cells adhere to them. For example, oxide-covered titanium surfaces (a popular vascular stent material), proteins adsorb within seconds of exposure to body fluids (blood plasma). The amphoteric oxide surface binds structural water in the outermost atomic layers (forming O^- , OH^- and OH_2^+ sites) and possesses a weak negative charge at physiological pH. After that, a primary strongly-adsorbed protein layer and a secondary loosely-held protein layer are formed at this interface. This is called the electrochemical double layer (Figure 1.22).

The composition of the initially adsorbed proteins varies according to properties of underlying substrates. Among them, some soluble plasma proteins (specifically, vitronectin, fibronectin and laminin) mediate desirable cellular events (adhesion or proliferation) [144, 145]. Therefore, the investigation of protein adsorption coupled to cell adhesion provides a way to explain why cells respond differently to various implant materials.

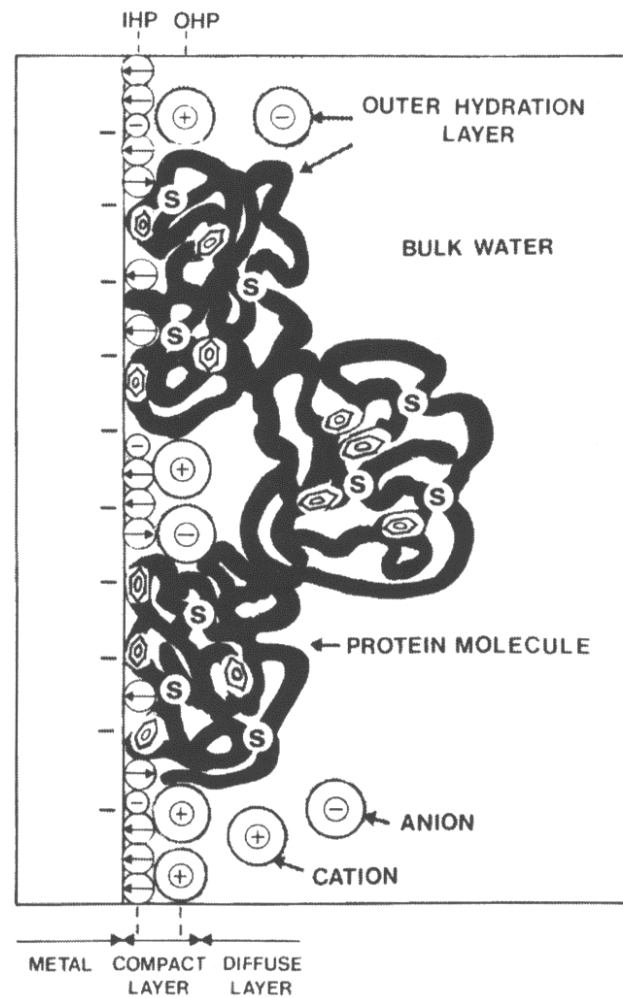


Figure 1.22 Schematic illustration of the electrochemical double layer formed at the titanium/electrolyte interface. IHP and OHP, inner and outer Helmholtz planes, respectively. (Adapted and redrawn from [146]).

1.6.2. Cell-Implant Interactions

Immediately after proteins adsorbed to implants, cells adhere to the proteins, which clearly plays a critical role in controlling cellular responses. After stent implantation, endothelial cells and smooth muscle cells are exposed to the stent surface simultaneously, and may spread, grow and

secret an extracellular matrix depending on the surface properties of that material. Endothelial cell-produced extracellular matrix is a laminin-rich base membrane matrix containing vitronectin, fibronectin, fibrin/fibrinogen and collagen. Endothelial cells attach to proteins adsorbed onto surfaces via integrins [147], which are receptors that recognize specific attachment sites on proteins (for example, Arg-Gly-Asp (RGD) sequences in fibronectin and vitronectin, Figure 1.23). The list of integrins on endothelial cells and their associated proteins are shown as follows [148-150]:

- Laminin: $\alpha_6\beta_1$, $\alpha_2\beta_1$, $\alpha_1\beta_1$, $\alpha_3\beta_1$
- Vitronectin: $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_1$ and $\alpha_{II}\beta_3$
- Collagen/fibronectin/fibrinogen: $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_1\beta_1$, $\alpha_3\beta_1$

As a result, to study cell functions, it is worthwhile to investigate the adhesion and proliferation of individual cells as well as the competitive adhesion of both vascular cells simultaneously to vascular implant materials.

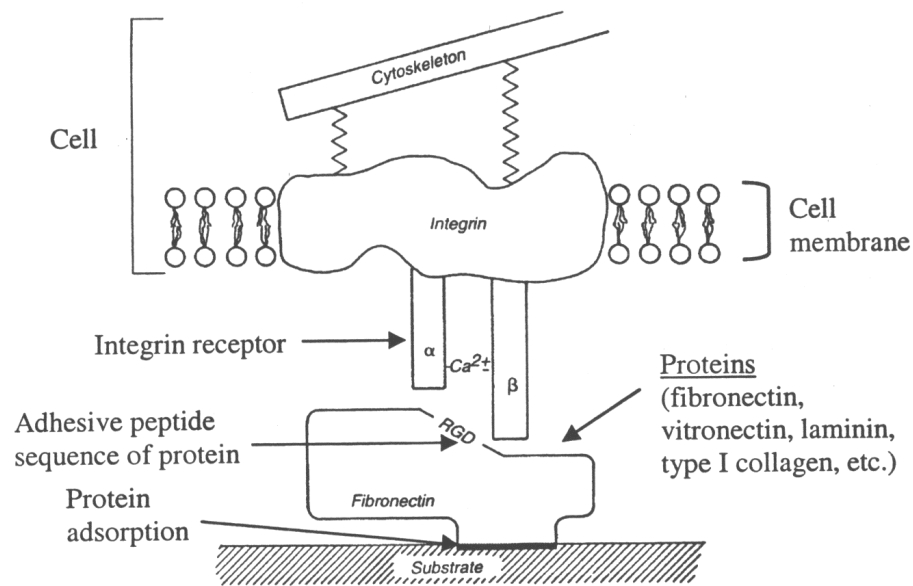


Figure 1.23 Schematic of the interaction of cells to an implanted surface as mediated by proteins (Adapted and redrawn from [151]).

1.6.3. Cell-Implant Interactions Under Shear Stress

In the natural vessel, vascular endothelial cells are exposed directly or indirectly to shear stress which results from the friction between circulating blood flow and the vessel wall. Shear stress (between 10 and 70 dyne/cm² in normal arteries) plays an important role in vessel homeostasis modulation and vascular cell function.

Endothelial cells align and elongate in a direction parallel to that of flowing blood when exposed to laminar shear stress [152, 153]. Moreover, endothelial cell proliferation, gene and protein expression are also greatly influenced by shear stress. Previous studies [154] revealed that the

proliferation of bovine aortic endothelial cells significantly decrease after 4 hours of exposure to laminar shear stress of 5 and 30 dynes/cm², suggesting that shear stress could inhibit endothelial cell proliferation when reaching certain values. When human umbilical vein endothelial cells were exposed to a laminar shear stress of 8 dynes/cm², the expression of eNOS (nitric oxide secretion mediator; nitric oxide is known as a vasodilator and platelet-aggregation inhibitor) was up-regulated [155].

In diseased vessels, the profile of shear stress changes into a non-laminar flow mode, which includes turbulent flow, oscillatory flow, stagnation zones and so on. Under these circumstances, endothelial cells function quite differently from normal laminar flow. In stagnation zones for example, the shear stress is lower than normal (around 2 dynes/cm²). At this time, the expression of several mediator factors such as endothelin-1 (a vasoconstrictor contrast to nitric oxide and platelet-aggregation promoter), MCP-1 (monocyte chemoattractant protein for monocyte recruitment and activation during inflammation) and VCAM-1 (vascular cell adhesion molecule-1 to induce monocyte/macrophage adhesion onto vascular endothelium) increased in stagnation zones so that an easier trigger of inflammation and smooth muscle cell proliferation could result [156-158].

In oscillatory flow, which accompanies atherosclerosis initiated lesions, or in spatial and temporal flow variations, which are implicated in atherosclerosis development, endothelial cell morphologies (i.e., changes to round morphologies in oscillatory flow) [159], proliferation, release of various factors affecting vessel dilation (endothelin-1) and immune cell responses (VCAM-1 and MCP-1) are all influenced [160, 161].

When the endothelium is destroyed through atherosclerosis, smooth muscle cells are exposed directly to shear stress. Thus, under shear stress, smooth muscle cells also respond in a similar way to endothelial cells (specifically changes in cell morphology, function and gene expression) [162-164]. For example, decreased smooth muscle cell proliferation was observed when they were exposed to laminar blood flow. Moreover, the expression of PGI₂ (vasodilator and smooth muscle cell proliferation inhibitor) [165] and PDGF (platelet derived growth factor; cell growth regulator) increased in response to shear stress [166], indicating that such smooth muscle cell related factors are shear stress controlled.

Based on the differences of cell responses among static and normal laminar flow and abnormal non-laminar flow conditions, it is important to investigate vascular cell functions in all of these three different conditions. For this, many have built a parallel plate flow chamber system to mimic in vivo vessels for both laminar and non-laminar flow models [162].

1.7. Objectives

As discussed above, the limits of current drug-eluting vascular stents lie in late-stage thrombosis, which usually occurs before endothelialization has been completed, because drugs released are toxic not only to wound healing cells (which cause restenosis), but also to endothelial cells, which serve as a healthy boundary between blood and arterial walls. Therefore, numerous studies focus on methods to accelerate endothelialization (or coverage by endothelial cells) of vascular stents, as well as to limit development of atherosclerotic plaques without the use of drugs. In this case, modifying vascular metal stent surface properties without coatings becomes important. Therefore, the objective of this study is to develop optimal surface properties for titanium bare-metal vascular stents. It is expected that the novel bare-metal stents could limit or inhibit in-stent restenosis by accelerating endothelialization as well as mediating vascular smooth muscle cell and immune cell activities at the same time. The specific aims of this study are, thus, to:

Part 1: Fabricate titanium and NiTi substrates with various nanostructured features:

1. Create highly controllable nanostructured (nano/micropatterned) titanium surface features by the titanium inductively coupled plasma deep etch (TIDE) process.

2. Create nano and sub-micron titanium surface roughness on borosilicate glass coverslips by e-beam deposition.
3. Create highly aligned patterns of flat, nano and sub-micron surface roughness e-beam deposition.
4. Create random nanostructured nitinol (NiTi) by cold compaction.
5. Characterize surface properties (such as surface topography, roughness and energy) of all the substrates.

Part 2: Investigate vascular cell and immune cell responses to titanium or NiTi substrates with various nanostructured features:

1. Characterize the in vitro vascular endothelial cell and smooth muscle cell responses on highly controllable nanostructured (nano/micropatterned) titanium surfaces created by the TIDE process, bulk and patterned nano and sub-micron titanium surface roughness created by e-beam deposition, and nanostructured nitinol (NiTi) created by cold compaction.
2. Characterize the in vitro monocyte/macrophage responses on bulk and patterned nano and sub-micron titanium surface roughness created by e-beam deposition.
3. Characterize the in vitro osteoblast cell responses on bulk and patterned nano and sub-micron titanium surface roughness created by e-beam deposition.

4. Characterize initial extracellular protein adsorption to bulk and patterned nano and sub-micron titanium surface roughness created by e-beam deposition.

Part 3: Investigate vascular cell and immune cell responses to titanium with various nanostructured features under flow:

1. Test the stability of titanium coatings under flow on borosilicate glass coverslips created by e-beam deposition.
2. Characterize the in vitro vascular endothelial cell responses under flow on nano and sub-micron titanium surface roughness created by e-beam deposition.
3. Characterize the in vitro monocyte/macrophage responses on nano and sub-micron titanium surface roughness created by e-beam deposition.

CHAPTER 2. MATERIALS AND METHODS

2.1. Substrate Preparation

2.1.1. Etched Glass

Optical borosilicate glass coverslips (diameters: 12mm, 18mm and 22mm; Fisherbrand) and microslides (75 x 38mm; Fisherbrand) were degreased by soaking in acetone (Fisher) for 10 minutes and sonicated in acetone for 10 minutes. They were then immersed in 70% ethanol (Brown University Chemical Store) for 10 minutes, sonicated in 70% ethanol for another 10 minutes and finally rinsed in deionized water (dH₂O; Milli-Q® Advantage A10 Ultrapure Water Purification System; Milipore). The glass was then etched in 1 N NaOH (Sigma) for 1 hour, and rinsed again in dH₂O. They were dried in an oven at 80°C and sterilized by autoclaving at 120°C for 30 minutes. These sterile, etched glass coverslips were used as reference substrates in all *in vitro* cell experiments and the microslides were used as reference substrates in all parallel plate flow chamber experiments.

2.1.2. Random Nanostructured Titanium

The random nanostructured titanium substrates were thin discs (12 mm diameter, 0.5 mm thickness) with non-homogenous nanostructured

surface features created by the cold compaction of 99.38% commercially pure Ti nanopowders (average particle size: 100 nm, Reade International Inc.). Considering the possibility of surface chemistry alterations caused by exposing fine particles of reactive metals to unprotective, oxidizing, or contaminated atmospheres at elevated temperatures, the powder metallurgy prepared compacts were created at room temperature in the absence of heat. Briefly, nanopowders were loaded into a hardened tool steel die and compacted using a uniaxial, single-ended hydraulic press (Carver Inc.; compaction conditions: 10 GPa pressure, 10 minutes at room temperature in an ambient environment).

2.1.3. Random Nanostructured Nitinol (NiTi)

Nitinol powder was purchased from the Special Metals Company (New Hartford, NY) with a weight composition of Ni = 55.5% and Ti = 44.5% and with quoted particle sizes ranging from 10 microns to -80 mesh (that is, 90% or more particles could pass through a 177 μm porous diameter sieve). Different dimensions of NiTi powder were obtained by sifting the stock powder through a 60 micron diameter opening stainless steel wire cloth. Fine grain samples ($<60\ \mu\text{m}$) and coarse grain stock samples ($<100\ \mu\text{m}$) were derived. Powders were loaded into a steel-tool die and all compact discs (diameter: 12 mm, thickness: 1–2 mm) were produced using a uniaxial, single-ended compacting hydraulic press. The pressure increased to 2–3 GPa at intervals of 2 minutes to a maximum pressure of 8 GPa over 7 minutes. Permanent deformation of Nitinol in the martensitic

(low-temperature) phase was achieved by compression in a liquid nitrogen bath. Compacts were synthesized by systematically increasing the pressure followed by cooling in liquid nitrogen to emulate the Nitinol stress-strain relation. Before cell experiments, all Nitinol compacts were placed in a phosphate buffer saline (PBS) solution for 1 day and sterilized by ultra violet light exposure for 30 min on both sides.

2.1.4. Highly Controllable Nanostructured (Nano/micropatterned) Titanium Surface Features

Plasma dry etching-based micromachining techniques were used to fabricate uniform, high-precision patterns on the surface of titanium substrates in the present study. Wafer-based substrates composed of polished grade 1 titanium (99.6% Ti, Tokyo Stainless Grinding Co., Ltd., Japan) were first cleaned in subsequent ultrasonic baths of acetone and isopropanol, followed by rinsing with dH₂O and drying with N₂ gas. The wafers were then dehydration baked on a hotplate at 150 °C for 3 minutes. The wafers were primed with hexamethyldisilazane (an adhesion promoter) and a layer photoresist (Shipley SPR 955) was applied by spin-coating. After lithographic exposure (GCA 6300 i-line wafer stepper), the wafers were baked at 95 °C for 60 seconds, then developed (Shipley MF 701) for 60 seconds, rinsed in dH₂O, and dried with N₂ gas.

The lithographically defined patterns in the present study were composed of nine 5 x 5 mm sub-regions, each of which was composed of a periodic

array of lines of equal width and spacing ranging from 750 nm to 100 μm (Figure 2.1). A different line width was used for each sub-pattern, thus enabling the definition of nine unique topographical regions within the same die.

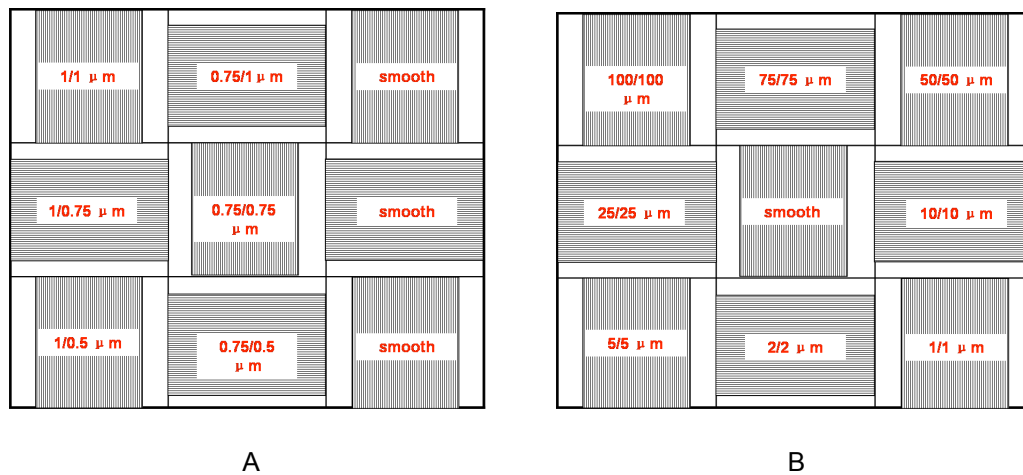


Figure 2.1 Pattern dimension distribution in the two kinds of multiplexed chip design. (A) Pattern dimensions from 750 nm to 1 μm ; and (B) Pattern dimensions from 1 μm to 100 μm .

The photoresist patterns were transferred onto the underlying substrate using the Ti inductively coupled plasma deep etch (TIDE) process (Figure 2.2) [167]. The TIDE process is based on anisotropic dry etching of titanium in Cl_2/Ar plasma and provides, for the first time, the capability for machining titanium with unprecedented resolution in a manner that is intrinsically scalable to low-cost/high-volume manufacturing, owing to its reliance on batch processing techniques derived from the microelectronics industry. Following etching, the photoresist was stripped using ultrasonic agitation, first in acetone, then in isopropanol. The samples were then

rinsed in dH₂O and dried with N₂ gas. They were sterilized by autoclaving and ultra violet (UV) exposure for 30 minutes on both sides.

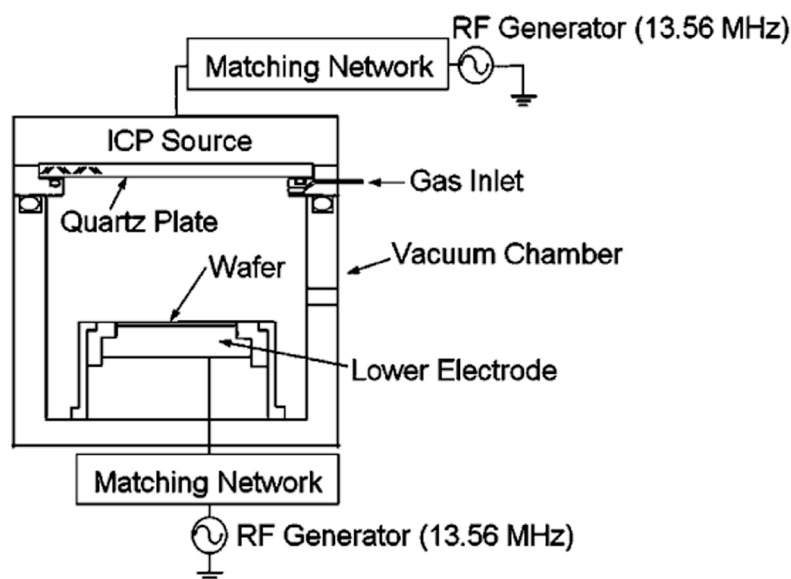


Figure 2.2 Design schematic of the Panasonic E640-ICP dry etching system [167].

2.1.5. Nano and Sub-micron Titanium Surface Roughness on Borosilicate Glass Coverslips

Pure titanium (99.8% of purity: T-2069, Cerac Inc.) was deposited on glass coverslips (12-550-15, Fisher-Scientific) with 50 nm of thickness using an e-beam evaporator (in a vacuum state: 10^{-7} torr, Figure 2.3a) to generate flat titanium surfaces (labeled as F-Ti). The default e-beam deposition rate used in this experiment was 2Å/sec with an e-beam current density of 60-70 mA/cm² (F-Ti in Figure 2.3a). Previous studies have generated rough surfaces by altering the deposition parameters

using an ion-beam deposition process [168, 169]. In this study, to generate nano surface features, a high deposition rate ($20\text{\AA}/\text{sec}$ with an e-beam current density of $130\text{mA}/\text{cm}^2$) was used to generate nano surface topography with a deposition of 50 nm thickness on F-Ti (defined as N-Ti in Figure 2.3b). In addition, to generate sub-micron surface features, the same e-beam current density was used with a deposition of $1\mu\text{m}$ thickness on F-Ti (defined as S-Ti in Figure 2.3b). The e-beam energy was set to 7KeV during the experiments.

2.1.6. Highly Aligned Patterns of Titanium Flat, Nano and Sub-micron Surface Roughness

For generating two different titanium roughness values (i.e., those either flat and nano or flat and sub-micron) on one substrate, an aligned shadow mask or conventional grid (G-400P-Ni, Electron Microscopy Sciences) was used to block titanium deposition from the e-beam which allowed for the deposition of titanium nano particles only in open spaces (Figure 2.3c). Then, nano structured titanium particles (obtained by the high current e-beam) were deposited within the aligned open spaces and this, thus, generated two different surface roughness features (Figure 2.3c). The advantage of this method is that it allows one to generate different patterns (with different sizes of surface features) on one substrate by altering the deposition parameters.

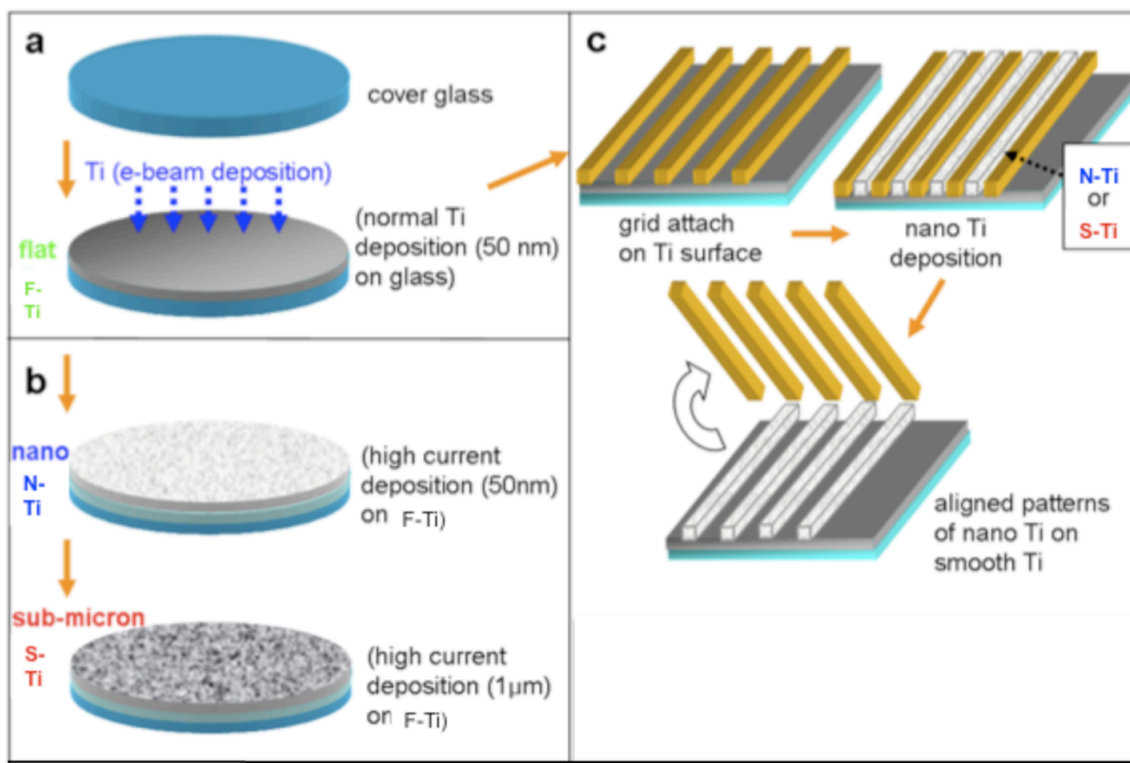


Figure 2.3 Fabrication of flat, nano and sub-micron surface roughness on bulk and aligned titanium surfaces. (a) A conventional e-beam deposition rate ($2\text{\AA}/\text{sec}$) generated flat titanium (F-Ti) surfaces on glass coverslips (50nm of thickness). (b) A high e-beam deposition rate ($20\text{\AA}/\text{sec}$) generated nano surface roughness (N-Ti) after a thickness of 50nm on F-Ti. High deposition rates with a thickness of $1\mu\text{m}$ generated sub-micron surface structures (S-Ti). The inset image shows different transparency for these substrates because of the different deposition thickness on glass coverslips. Sub-micron titanium surfaces were opaque. (c) Schematic of aligned micron patterns of different surface roughness (submicron, nano surface or flat). A titanium thin layer (50nm) on glass coverslips was used as a base substrate. A metal grid (Ni/Au/Cu or shadow mask) with aligned grooves (width of $30\mu\text{m}$) was placed on F-Ti (see (a)). Nano or sub-micron rough titanium was coated through the open grooves (respectively) as described in (a) and (b). Then, the shadow mask was removed from F-Ti to generate aligned patterns of two different surface roughness values on titanium.

2.2. Surface Chemistry and Topography Characterization (FESEM, EDS and AFM)

2.2.1. Scanning Electron Microscopy

The surfaces morphologies of all the titanium samples (with different patterns and different roughness) were investigated by a field emission scanning electron microscope (SEM, LEO 1530 VP) at 5-7kV. SEM was also used to characterize cell morphology on the various samples. For these non-conductive samples, they were sputter-coated with AuPd before imaging using a sputter coater for 3 minutes.

Chemical composition of specific regions of the samples on substrates was examined by energy-dispersive spectroscopy (EDS) using an Oxford INCA 250 detector which was attached to the SEM and associated software (Oxford Instrument America, Inc., Concord, MA).

2.2.2. Atomic Force Microscopy

Surface roughness measurements of the samples were completed using a multimode atomic force microscope (AFM; Dimension 3100, Veeco) to compare flat, sub-micron and nanometer surface features. Scan areas of both $5\mu\text{m}\times 5\mu\text{m}$ (sub-micron resolution) and $500\text{nm}\times 500\text{nm}$ (nano resolution) were used to analyze sub micron and nano scale surface features. Commercially available AFM tips (radius of tip curvature was less than 10 nm, NSC15/ALBS, Micro-Masch) were used in tapping mode

with a scan rate of 0.5 Hz. Full tip cone angles were 30° but less than 10° at the 200nm tip apex (tip height was 25 µm with a 40 N/m of force constant). Note that all of the vertical surface features did not exceed 200nm, so negligible convolution effects (from the tip) were anticipated. Surface area was calculated as a summation of triangles over the surfaces of the analyzed area (5µm×5µm), which was defined by two-by-two pixel kernels (Veeco, Technical Manual).

AFM was also used to investigate different titanium patterns. Scan areas of the patterns whose spacing was less than 5µm were 10µm×10µm and for the patterns whose spacing was 5µm scan areas of 30µm×30µm were used.

2.2.3. Surface Energy and Contact Angles

A drop shape analysis system (Easy drop contact angle system, Kruss, Germany) with analysis software (DSA1) was used to determine the surface contact angles on the titanium samples. Distilled water was used as a contacting solvent. All data were obtained five seconds after placing the droplet on the surfaces under ambient conditions. For the surface energy calculations, $E_s = E_{lv} \times \cos\theta$ was used where $E_{lv} = 72.8 \text{ mJ/m}^2$ at 20°C for pure water and θ is the static contact angle. Here E_s is the surface energy of the contacting surface and E_{lv} is the surface energy between the water and air under ambient conditions. Additionally, the critical surface tensions of the samples were measured by Zisman plot.

2.3. Vascular Cell Responses

2.3.1. Vascular Cell Culture

2.3.1.1. Rat Aortic Endothelial Cell (RAEC) Culture

Rat aortic endothelial cells (RAEC) were used in this study (purchased from VEC Technologies) at the passage numbers between 2 and 10. Frozen cells stored in cryogenic vials were brought to 37 °C and transferred to cell culture flasks when needed. RAEC were then cultured in complete medium (MCDB-131, VEC Technologies) under standard cell culture conditions (37°C, humidified, 5% CO₂/95% air environment). Medium was replenished every other day during cell culturing. When necessary (before cell seeding), cells were lifted from the bottom of cell culture flasks by incubating them in a sterile trypsin solution (containing: 0.05% trypsin, 0.02% EDTA solution (Sigma)) for 5 minutes at 37°C. When the cells rounded up, they were detached loosely by gently striking side of the flasks.

2.3.1.2. Rat Aortic Smooth Muscle Cell (RASMC) Culture

Rat aortic smooth muscle cell (RASMC; VEC Technologies, passage numbers 2-7) culture was similar to that of the RAEC. They were also cultured in MCDB-131 complete medium was used under standard cell culture conditions and the medium was replenished every other day during cell culturing.

2.3.2. RAEC Responses on Highly Controllable Nanostructured Surface

Features: The Role of Pattern Dimension on RAEC Functions

2.3.2.1. RAEC Adhesion on Highly Controllable Nanostructured Surface

Features

For cell adhesion tests, RAEC were seeded at 4500 cells/cm² in MCDB-131 Complete Medium onto nanopatterned titanium surfaces, random nanostructured Ti surfaces, and etched glass coverslips for 4 hours under standard cell culture conditions. Then, the samples were rinsed with phosphate buffered saline (PBS) three times to prepare them for cell viability analysis as described in section 2.3.2.3.

2.3.2.2. RAEC Proliferation on Highly Controllable Nanostructured Surface

Features

For longer term cell density cell proliferation tests, RAEC were seeded at 2500 cells/cm² onto both patterned Ti surfaces, random nanostructured titanium surfaces and etched glass coverslips. Cells were cultured in MCDB-131 Complete Medium for 1, 3 and 5 days. Medium was refreshed on the 2nd and 4th day. At the end of 1st, 3th and 5th day, non-adherent cells on all the substrates were removed by rinsing three times with PBS to prepare them for cell viability analysis as described in section 2.3.2.3.

2.3.2.3. RAEC Viability on Highly Controllable Nanostructured Surface

Features

RAEC viability on highly controllable nanostructured surface features was determined by a LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes). Briefly, the cells were stained with 3.3 μM of calcein AM and 1.7 μM of ethidium homodimer-1 without fixation and incubated for 30 min before visualization using fluorescence microscopy.

Specifically, live and dead cells were quantitatively investigated by using a 530 nm wavelength for calcein AM and a 560 nm wavelength for ethidium homodimer-1 through a Zeiss Axiovert 200 M light microscope with image analysis software (Axiovision).

2.3.3. RAEC Responses on Random Nanostructured NiTi (Nitinol) Surface

Features

2.3.3.1. RAEC Adhesion on Random Nanostructured NiTi (Nitinol) Surface

Features

Before cell experiments, the fine grain NiTi, the coarse grain NiTi as well as commercially purchased flat titanium (Alfa) were immersed in 5 of mL media in 6-well culture plates. For cell adhesion experiments, RAEC were seeded at a density of 4500 cells/cm² onto the substrates of interest to this study using similar procedures to that just described. Cells were allowed

to adhere onto the substrates for 5 hours under standard cell culture conditions. Cell proliferation experiments were conducted similarly for 1 and 5 day durations. The media was changed every other day during the proliferation assay. At the end of each cell experiment, all non-adherent cells were removed from the substrate surfaces by washing with PBS three times. Adherent cells were fixed with a 4% formaldehyde solution. After being rinsed with PBS, the samples were stained using a Hoechst dye (33258, 1:30 in PBS, Sigma) for 5 minutes. Data was collected by counting cells under the Zeiss Axiovert 200 M light microscope with image analysis software (Axiovision).

2.3.4. RAEC and RASMC Responses on Non-patterned and Patterned Titanium Substrates with Different Roughness by E-beam Evaporation: The Role of Nano and Sub-micron Surface Features

2.3.4.1. RAEC Adhesion Bulk and Patterned Titanium Substrates with Different Roughness

For the endothelial cell adhesion test, RAEC were seeded at a density of 3,500 cells/cm² on non-patterned (bulk) titanium surfaces. In addition, for cell adhesion tests on patterned (aligned with 30µm spaces) titanium surfaces, RAEC were seeded at a density of 5000 cells/cm². The cells were incubated for 4 hours in MCDB-131 Complete Medium under standard cell culture conditions.

After 4 hours, non-adherent RAEC cells were removed by rinsing in PBS. Cells were then fixed with 4% formaldehyde (Fisher) at the end of the time period (4 hours). Once fixed, the substrates were washed three times with PBS. The samples were permeabilized in 1% Triton X-100 in PBS for 10 minutes and blocked with 5% FBS/0.1% Triton X-100 in PBS solution for 20 minutes. After being rinsed by PBS twice, the cells were stained with Rhodamine Phalloidin (R415, 1:40 in 5% FBS/0.1 Triton X-100 in PBS, Molecular Probes) for 30 minutes to visualize F-actin filaments as well as a Hoechst dye (33258, 1:30 in PBS, Sigma) for 10 minutes to visualize the cell nucleus. RAEC cell number was counted under a Leica DM5500 fluorescence microscope with Image-Pro software (MediaCybernetics).

2.3.4.2. Co-culture Cell Adhesion on Bulk Titanium Substrates with Different Roughness

Competitive, co-culture, cell adhesion was conducted on all the samples. Both RAEC and RASMC were labeled separately before seeding. To do this, RAEC and RASMC were treated individually with MCDB-131 complete medium supplemented with fluorescent carbocyanine dyes (DiD for RAEC and DiO for RASMC (Molecular Probes)) for 30 minutes before cell seeding. The cell suspensions were then centrifuged twice to remove the dye reagents. Both RAEC and RASMC were seeded at a density of 4,500 cells/cm² in MCDB-131 complete medium onto the substrates. After

4 hours, the non-adherent cells were removed by rinsing in PBS three times. The samples were fixed with 4% formaldehyde (Fisher-Scientific) for 10 min, stained with 4',6-Diamidino-2-phenylindole (DAPI, 1:30 in PBS, Sigma) for 5 min to visualize the cell nucleus, and counted via a Leica DM5500 fluorescence microscope.

2.3.4.3. RAEC and RASMC Proliferation on Bulk Titanium Substrates with Different Roughness

For cell proliferation assays, RAEC and RASMC were seeded at 10,000 cells/cm² in MCDB-131 Complete Medium onto the substrates. The cells were cultured for 1, 3 and 5 days under standard cell culture conditions. Medium was refreshed on the second and fourth days. At the end of the 1st, 3th and 5th day, non-adherent cells on all the substrates were removed by rinsing three times with PBS to prepare them for cell viability analysis. The number of viable cells in the proliferation assays was determined by MTS assays (CellTiter 96 Aqueous Assay, Promega). Briefly, the substrates were incubated with a MTS reagent (contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES)) for 4 hours. The aliquots were then transferred to 96-well plates. The content was detected by a microplate

spectrophotometer (SpectraMax 340PC, Molecular Devices) at an absorbance of 490 nm.

2.3.4.4. Co-culture Cell Proliferation

For co-culture cell proliferation assays, both RAEC and RASMC suspensions were treated the same as described in the co-culture cell adhesion section. The cells were seeded at 2000 cells/cm² in MCDB-131 Complete Medium onto the substrates. Cells were cultured for 1, 3 and 5 days under standard cell culture conditions. Medium was refreshed on the 2nd and 4th days. At the end of the 1st day, 3rd day and 5th day, non-adherent cells on all the substrates were removed by rinsing three times with PBS before fixation with 4% formaldehyde for 10 min. The samples were then stained with 4',6-Diamidino-2-phenylindole (DAPI, 1:30 in PBS, Sigma) for 5 minutes and the number of stained cells was counted via a Leica DM5500 fluorescence microscope.

2.3.4.5. RAEC and RASMC Morphology on Bulk Titanium Substrates with Different Roughness

Similar to the MTS assay, RAEC and RASMC were seeded at 10,000 cells/cm² onto the substrates in MCDB-131 Complete Medium to visualize cell morphology. The cells were cultured for 1, 3 and 5 days under standard cell culture conditions. Medium was refreshed on the 2nd and 4th

days. At the end of each time period, the substrates were fixed with 4% formaldehyde (Fisher-Scientific) for 10 minutes. Once fixed, the substrates were washed three times with PBS. The samples were permeabilized in 1% Triton X-100 in PBS for 10 minutes and blocked with 5% FBS/0.1% Triton X-100 in PBS solution for 20 minutes. After being rinsed by PBS twice, the cells were stained with Rhodamine Phalloidin (R415, 1:40 in 5% FBS/0.1 Triton X-100 in PBS, Molecular Probes) for 30 minutes to visualize F-actin filaments as well as a 4',6-Diamidino-2-phenylindole (DAPI, 1:30 in PBS, Sigma) for 5 minutes to visualize the cell nucleus. To investigate altered endothelial cell morphology on the different titanium features (i.e., flat, nano and sub-micron), aspect ratios (cell length/cell width) for each cell were calculated by Image-Pro software (MediaCybernetics).

2.3.4.6. Immunofluorescence of Vinculin during RAEC Attachment on Bulk Titanium Substrates with Different Roughness

RAEC were seeded at a density of 50,000 cells/cm² in MCDB-131 Complete Medium on the different titanium substrates (i.e., flat, nano and sub-micron) as well as etched glass coverslips. After 24 hours of culture, all the cells were fixed in 4% formaldehyde (Fisher) for 10 minutes at room temperature. Once fixed, the substrates were washed three times with PBS and immersed with 1% Triton X-100 in PBS for 10 minutes to

permeabilize the cells. After being washed in PBS three times, the samples were incubated for 1 hour at room temperature in 1% BSA (in PBS) followed by the treatment with an anti-vinculin antibody (1:50 in 1% BSA (in PBS), monoclonal anti-vinculin FITC conjugated, clone hVIN-1 purified mouse immunoglobulin, Sigma) for another 1 hour. The cells were washed three times and their vinculin was visualized by a Leica DM5500 fluorescence microscope.

2.3.4.7. Protein Adsorption (Fibronectin, Vitronectin and Fibrinogen) on Bulk Titanium Substrates with Different Roughness

Protein adsorption plays an important role in the adhesion of anchorage-dependent cells (such as vascular endothelial cells and vascular smooth muscle cells) and platelet aggregation. To test the effect of different titanium roughness on protein adsorption, three important proteins (fibronectin, vitronectin and fibrinogen) were chosen in this study for ELISA (Enzyme-Linked Immunosorbent Assay) experiments.

For fibronectin and vitronectin adsorption, sub-micron rough (S-Ti), nanorough (N-Ti), flat (F-Ti) titanium substrates were exposed to 500 µl of FBS for 4 hours under standard cell culture conditions. The samples were then rinsed with PBS three times and blocked with 2% BSA (in PBS) for 1 hour. After being rinsed with PBS twice, the samples were incubated with

anti-bovine fibronectin antibody (6 µg/ml in 1% BSA solution; Chemicon) or an anti-bovine vitronectin antibody (6 µg/ml in 1% BSA solution; Accurate Chemical) for 1 hour under standard cell culture conditions. After that, the samples were rinsed with 0.05% Tween 20 (Sigma) in PBS three times and were incubated with horse radish peroxidase conjugated with anti-rabbit secondary antibody (1:100 BSA solution; Biorad) for 1 hour. The samples were rinsed then with 0.05% Tween 20 three times and were transferred to new cell culture plates before being rinsed once with 0.05% Tween 20. Finally, an ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6sulfonic acid) soluble substrate kit (Vector Labs) was used to detect secondary antibodies using a spectrophotometer (SpectoMAX; Molecular Devices) at 405 nm.

Fibrinogen adsorption was tested similar to fibronectin and vitronectin, except that the substrates were exposed to 500 µl of 20 µg/ml human fibrinogen (in PBS, Sigma) for 4 hours under standard cell culture conditions. The samples were then rinsed with PBS three times and blocked with 2% BSA (in PBS) for 1 hours. After being rinsed with PBS twice, the samples were incubated with anti-human fibrinogen antibody (0.5 µg/ml in 1% BSA solution; Abcam) for 1 hour under standard cell culture conditions. The samples were then rinsed with 0.05% Tween 20 three times and were transferred to new cell culture plates before being rinsed once with 0.05% Tween 20. Finally, an ABTS (2, 2'-azino-bis (3-

ethylbenzthiazoline-6sulfonic acid) soluble substrate kit (Vector Labs) was used to detect the secondary antibodies using a spectrophotometer (SpectoMAX; Molecular Devices) at 405 nm.

2.3.4.8. RAEC NO_x and Endothelin-1 Functional Assays on Bulk Titanium Substrates with Different Roughness

NO_x and Endothelin-1 assays were conducted in this study to investigate the function of RAEC on the different substrates. RAEC were seeded at 100,000 cells/cm² in MCDB-131 Complete Medium on sub-micron rough (S-Ti), nanorough (N-Ti), flat (F-Ti) titanium surfaces and etched glass coverslips and were cultured for 24 hours. After 24 hours, DMEM (Invitrogen) supplemented with 1% penicillin/streptomycin (Hyclone) was added to the cells for 4 hours, followed by an incubation with MCDB-131 Complete Medium for another 24 hours. The culture medium was collected after a select period of time and analyzed by NO_x (Nitrate/Nitrite Colorimetric Assay Kit; Cayman Chemical Company) and Endothelin-1 (Endothelin-1 Colorimetric Assay Kit; Cayman Chemical Company) following the manufacturer's instructions (Cayman Chemical Company) and as described below.

2.3.4.8.1. NO_x Assay on Bulk Titanium Substrates with Different Roughness

The NO_x assay was based on the Griess reaction, which includes two steps: the conversion of nitrate to nitrite by means of nitrate reductase and the addition of the Griess Reagents which converts nitrite into a deep purple azo compound. To do this, the culture medium collected was treated by nitrate reductase for 1 hour in the presence of 5mM NADPH, which is an essential cofactor for the function of the NOS enzyme. The sample was then incubated with the Griess Reagents (0.1% N-(1-naphthyl)ethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% H₃PO₄) for 10 minutes at room temperature. The aliquots were transferred to a 96-well plate. The photometric measurement of the final purple compound was determined by a microplate spectrophotometer (SpectraMax 340PC, Molecular Devices) at 540 nm. The final NO_x concentration was expressed as μ M.

2.3.4.8.2. Endothelin-1 Assay on Bulk Titanium Substrates with Different Roughness

The Endothelin-1 assay was based on double-antibody “sandwich” technique (ELISA), in which the first antibody (a monoclonal antibody specific for endothelin) and the secondary antibody (an

acetylcholinesterase conjugate which selectively binds a different epitope of the endothelin molecule) were added to the collected medium. The two antibodies bind to opposite sides of the endothelium molecule. A colored compound was produced when incubating the mixture with Ellman's reagent, which contains a substrate to acetylcholinesterase. Photometric measurements of the absorbance due to the final colored compound is proportional to the concentration of endothelin, which was detected by the microplate spectrophotometer (SpectraMax 340PC, Molecular Devices) at 540 nm. The final endothelin concentration was expressed as pg/ml.

2.3.4.9. Long-term RAEC and RASMC Functional Assays on Bulk Titanium Substrates with Different Roughness

To measure intracellular collagen and elastin concentration, RAEC and RASMC were separately seeded on sub-micron rough (S-Ti), nanorough (N-Ti), flat (F-Ti) titanium surfaces and etched glass coverslips at a density of 10,000 cells/cm² and cultured in MCDB-131 complete medium for 7 and 14 days under standard cell culture conditions. Medium was refreshed every two days. At the conclusion of 7 and 14 days, the medium was removed and the adherent cells were lysed in a solution of Triton X-100 by three freeze-thaw cycles. The cell lysates were collected in microtubes and stored at -80°C.

Amounts of total protein in the cell lysates were determined by a Micro BCA Protein Assay Reagent Kit (Pierce Chemical Co.) according to the manufacturer's instruction. Briefly, 25 μ l of each sample lysate was incubated with 200 μ l of a working reagent (containing cupric sulfate and bicinchoninic acid) at 37 °C for 30 min. Then, the light absorbance of these samples was measured by a spectrophotometer (SpectoMAX; Molecular Devices) at 562 nm. Total intracellular protein synthesized by RAEC and RASMC for 7 and 14 days was determined from a standard curve of absorbance versus known concentrations of albumin run in parallel with experimental samples. The total intracellular protein synthesis was normalized by substrate surface area.

The Amount of intracellular elastin was determined by the FastinTM Elastin Assay kit (Biocolor, UK), which is a quantitative dye-binding method for the analysis of elastin from cells. For this, 100 μ l of each sample lysate was incubated with an equal volume of the Elastin Precipitating Reagent for 10 minutes to precipitate and isolate elastin from the rest of the content. After centrifugation, the precipitated elastin was packed and the liquid content was removed. 1 ml of the Fastin Dye Reagent (containing 5, 10, 15, 20-tetraphenyl-21, 23-porphine tetra-sulfonate, TPPS) was added to each sample which interacted with the elastin for 90 minutes. Gentle mechanical mixing was used during this period to ensure maximum elastin dye binding. The elastin-dye complex is insoluble in the presence

of ammonium sulfate contained in the Fastin Dye Reagent. To recover it, the elastin-dye complex was separated from the unbound dye reagent and treated with a Dye Dissociation Reagent. Then, the light absorbance of the samples was measured by a spectrophotometer (SpectoMAX; Molecular Devices) at 513 nm. The total amount of intracellular elastin synthesized by RAEC and RASMC for 7 and 14 days was determined from an elastin standard curve of absorbance versus known amounts of elastin. The amount of intracellular elastin was normalized by substrate surface area.

Finally, the amount of intracellular collagen in the cell lysates was determined by the Sircol™ Collagen Assay kit (Biocolor, UK). It is based on a quantitative dye-binding method for the analysis of collagen released into cell culture medium by mammalian cells during *in vitro* culture. The dye bounded to soluble collagen is Sirius Red. 50 µL aliquots of each sample were incubated with 1 ml Sircol dye reagent (containing Sirius Red), gently mixed at room temperature for about 30 minutes. After 30 minutes, the mixture was centrifuged to pack the collagen-dye complex pellet. The pellet was then collected and treated with 1 ml of the Alkali reagent for 5 minutes to bring the collagen bound dye back to the solution. Finally, the light absorbance of the samples was measured by the spectrophotometer at 540 nm. The total amount of intracellular elastin synthesized by RAEC and RASMC for 7 and 14 days was determined from a collagen standard curve of absorbance versus known amounts of

collagen. The amount of intracellular collagen was normalized by substrate surface area.

2.3.4.10. RAEC Mobility Assay on Bulk Titanium Substrates with Different Roughness

RAEC mobility on the substrates of interests was studied by time dependent RAEC migration assay. The borderline between RAEC-occupied and non-RAEC-occupied areas on the samples was constructed by covering half area of the sample surfaces with a hemicycle PDMS (Polydimethylsiloxane) piece (diameter: 18 mm).

The PDMS pieces were fabricated by a well established process described in [134]. Briefly, SYLGARD 184 Silicone Elastomer® (Dow Corning) was mixed (10 : 1 wt/wt %) with a silicone resin curing agent (Dow Corning) by gently stirring for 15 minutes. The elastomer was poured into each well of a 12-well plate. The plate was exposed to vacuum pressure (25 in Hg) for 10-15 minutes and left partially uncovered at room temperature in air to cure for 2 days. The PDMS pieces were removed from the plate and cut into hemicycles by a blade, sterilized by 75% ethanol soakings.

The sterile PDMS pieces were attached to sub-micron rough (S-Ti), nanorough (N-Ti), flat (F-Ti) titanium surfaces and etched glass coverslips to cover half of their surface area. The air bubbles trapped between the samples and the PDMS pieces were removed by gently squeezing the bubbles out. Specifically, RAEC were placed on the complexes in MCDB-131 Complete Medium at a density of 500,000 cells/cm² for 36 hours until RAEC grew to confluence. After the cell monolayer was formed, the PDMS pieces were carefully removed and the samples were rinsed with PBS three times. The cells were stained by 3.3 μ M of calcein AM and incubated for 30 min before visualization using fluorescence microscopy at 530 nm. Through a fluorescence microscope, the cell migration was determined by recording migration distance into the open area created by removing the PDMS portion after 0, 12 and 24 hours.

2.3.5. Osteoblast Responses

2.3.5.1. Human Osteoblast (HOB) Culture

For bone cell experiments, human osteoblasts (HOB, CRL-11372, ATCC, population numbers 2-5) were cultured under standard cell culture conditions and were cultured in Dulbecco's modified eagle medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (P/S, Hyclone), under standard cell culture conditions. Medium was replenished every other day during cell culturing.

2.3.5.2. Osteoblast (HOB) Responses on Patterned Titanium Substrates Created with Different Roughness by E-beam Evaporation

For osteoblast adhesion, non-adherent HOB were removed by rinsing in PBS while adherent HOB viability was determined by a LIVE Viability/Cytotoxicity Kit (Molecular Probes). Briefly, the cells were stained with 3.3 μ M of calcium AM and 1.7 μ M of ethidium homodimer-1 and incubated for 30 min before visualization using a fluorescence microscope (Axiovert 200M, Zeiss) which was equipped with a sensitive monochrome camera (Hamamatsu ORCA-ER). The live cells on the aligned patterned titanium surfaces were counted at 530 nm (excitation for calcein AM) and 560 nm (ethidium homodimer-1) wavelengths using the fluorescence microscope.

For the quantification of HOB, Hoechst dye (33258, Sigma) was used to visualize the HOB nucleus. HOB morphology on the aligned substrates (after 4 hours) was also examined using SEM. For SEM investigation, HOB were fixed (after 4 hours) with formalin for 5 min and then, they were dehydrated by soaking serially in 10%, 30%, 50%, 70%, and 90% ethanol for 30 min followed by soaking in 100% ethanol for 15 min (three times). Lastly, the substrates were critically point dried for SEM visualization.

2.3.6. Immune Responses

2.3.6.1. Mouse Macrophage Culture

The mouse IC-21 macrophage cell line was cultured (TIB-186; ATCC; passage number between 2 and 20) in RPMI 1640 medium (ATCC) supplemented with 10% FBS (Hyclone) and 1% P/S (Hyclone) under standard cell culture conditions. Medium was replenished three times per week during cell culturing.

2.3.6.2. Human Monocytes

The human THP-1 monocyte cell line (TIB-202; ATCC; between 2 and 5) was used in this study. The cell culture procedures followed ATCC instructions. The monocytes were cultured in RPMI 1640 medium (ATCC) supplemented with 10% FBS (Hyclone) and 1% P/S (Hyclone) under standard cell culture conditions. Medium was replenished every two-three days during cell culturing. To do this, cell cultures were centrifuged with subsequent resuspension in fresh medium.

2.3.6.3. Macrophage Density Assays

For macrophage adhesion tests, TIB-186 cells were seeded at a density of 4,500 cells/cm² on sub-micron rough (S-Ti), nanorough (N-Ti), flat (F-Ti) titanium surfaces and etched glass coverslips in RPMI 1640 medium supplemented with 10% FBS and 1% P/S. Lipopolysaccharide (LPS, 200

ng/ml Sigma-Aldrich) was added to only one set of experiments. After 12 and 24 hours, non-adherent macrophages were removed by rinsing in phosphate-buffered saline (PBS). Once fixed, the substrates were washed three times with PBS. The samples were permeabilized in 1% Triton X-100 in PBS for 10 minutes and blocked with 5% FBS/0.1% Triton X-100 in PBS solution for 20 minutes. After rinsing with PBS for twice, the cells were stained with Rhodamine Phalloidin (R415, 1:40 in 5% FBS/0.1 Triton X-100 in PBS, Molecular Probes) for 30 minutes to visualize F-actin filaments as well as a 4',6-Diamidino-2-phenylindole (DAPI, 1:30 in PBS, Sigma) for 5 minutes to visualize the cell nucleus. The cell number as well as cell morphology was investigated by a Leica DM5500 fluorescence microscope.

2.3.6.4. Monocyte Adhesion on Titanium Substrates with Different Roughness

For monocyte adhesion tests, TIB-202 cells were seeded at a density of 1,000,000 cells/cm² on sub-micron rough (S-Ti), nanorough (N-Ti), flat (F-Ti) titanium surfaces and etched glass coverslips in RPMI 1640 medium supplemented with 10% FBS and 1% P/S under activation with 1 µg/ml of a tumor-promoting phorbol ester (12-O-Tetradecanoylphorbol 13-acetate, PMA, TPA, 4β,9α,12β,13α,20-Pentahydroxytiglic-1,6-dien-3-one 12-

tetradecanoate 13-acetate, P1585, Sigma). The cells were incubated for 4 hours under standard cell culture conditions.

After 4 hours, non-adherent TIB-202 cells were removed by rinsing in PBS. The number of adherent viable cells in the proliferation assays was determined by MTS assays. Briefly, the substrates were incubated with the MTS reagent (contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES)) for 4 hours. Aliquots were then transferred to 96-well plates. The content was detected by a microplate spectrophotometer (SpectraMax 340PC, Molecular Devices) at an absorbance of 490 nm.

2.3.6.5. Monocyte Adhesion on RAEC Monolayers on Titanium Substrates with Different Roughness

RAEC were seeded on the samples at 500,000 cells/cm² and cultured in MCDB-131 Complete Medium for 36 hours to reach confluency. After reaching confluency, TIB-202 cells, pre-stained with 3.3 µM of calcein AM, were seeded at a density of 3,500 cells/cm² on the RAEC monolayers in RPMI 1640 medium (supplemented with 10% FBS and 1% P/S) containing 1 µg/ml of TPA. The cells were incubated for 4 hours under

standard cell culture conditions. After 4 hours, all the samples were rinsed three times with PBS. The monocytes were quantified through a Leica DM5500 fluorescence microscope by using a 530 nm wavelength for calcein AM.

2.3.6.6. Monocyte Differentiation on Titanium Substrates with Different Roughness

TIB-202 cells were separately seeded on sub-micron rough (S-Ti), nanorough (N-Ti), flat (F-Ti) and etched glass coverslips at a density of 100,000 cells/cm² and were cultured in RPMI 1640 medium (supplemented with 10% FBS and 1% P/S) containing 1 µg/ml of TPA for 5 days under standard cell culture conditions. Medium was refreshed every two days. Non-adherent TIB-202 cells were removed by rinsing in PBS. Cells were then fixed with 4% formaldehyde (Fisher) at the end of the time period (5 days). Once fixed, the substrates were washed three times with PBS. The samples were permeabilized in 1% Triton X-100 in PBS for 10 minutes and blocked with 5% FBS/0.1% Triton X-100 in PBS for 20 minutes. After being rinsed with PBS twice, the cells were stained with Rhodamine Phalloidin (R415, 1:40 in 5% FBS/0.1 Triton X-100 in PBS, Molecular Probes) for 30 minutes to visualize F-actin filaments as well as a 4',6-Diamidino-2-phenylindole (DAPI, 1:30 in PBS, Sigma) for 5 min to visualize the cell nucleus. Monocyte cell number as well as cell

morphology was investigated by a Leica DM5500 fluorescence microscope.

2.3.6.7. Cytokine Release from Macrophages Attached on Titanium Substrates with Different Roughness

TIB-186 cells were placed on the samples at a seeding density of 1,000,000 cells/cm² and were cultured in RPMI 1640 medium (supplemented with 10% FBS and 1% P/S). LPS (200 ng/ml, Sigma) was added to one set of the experiments. After 4, 12, 24 and 36 hours, the culture medium was collected and centrifuged to remove any suspended cells. The presence of cytokines (TNF- α , IL-1 β and IL-6) in the cell culture supernatant solution produced by the activated macrophages adherent on all the titanium surfaces were investigated by commercially available ELISA kits (R&D Systems, Minneapolis).

For the analysis of all three cytokines, the process was similar. Briefly, 50 μ l of the assay diluent (a buffered protein solution with preservatives) was added to each well of the 96 well microplates precoated with an antibody specific for mouse TNF- α (polyclonal), IL-1 β (polyclonal) or IL-6 (monoclonal). 50 μ l of each sample was then added and mixed well by gently tapping the plate frame for 1 minute. The plates were incubated for 2 hours at room temperature. After the incubation, the aliquots in each

well were aspirated and the wells were rinsed five times with wash buffer (a buffered surfactant) to remove any remaining liquid. Each well was treated with 100 μ l of polyclonal antibodies against mouse TNF- α , IL-1 β or IL-6 conjugated to horseradish peroxidase and was incubated for another 2 hours at room temperature. Each well was aspirated and washed 5 times after 2 hours. The samples were finally incubated with 100 μ l of substrate solution (mixture of stabilized hydrogen peroxide and chromogen tetramethylbenzidine) for 30 minutes at room temperature. The optical density of each well was determined within 30 minutes after 100 μ l of a stop solution (diluted hydrochloric acid solution) and was added to the wells. The microplate reader was set to 450 nm. The readings were subtracted by the readings at 540 nm. Readings made directly at 450 nm without correction might be higher and less accurate. The concentration of each released cytokine was determined from the standard curves of absorbance versus known concentrations of TNF- α , IL-1 β or IL-6.

2.4. Laminar Flow in the Vessel Model

2.4.1. The Vessel Flow Model in a Home-made Flow System

Parts which were included in the home-made flow system are listed as follows:

- ✓ Glycotech rectangular parallel plate flow chamber kit (Figure 2.4, Glycotech)
 - – flow deck
 - – Silastic[®] laboratory tubing (1/16" inner diameter)
 - - flow chamber rubber gasket (0.010" thick)
 - – straight and L-shape polypropylene connectors
- ✓ Digital peristaltic pump (3200, Welch Scientific)
- ✓ Vacuum pump (25468-01, Welch Scientific)
- ✓ Hot plate (33993-908, VWR)
- ✓ Media reservoir with a 3-hole cap (Figure 2.5, Kontes)
- ✓ 95% air/5% CO₂ gas tank

To assemble the custom rectangular parallel flow chamber, the fittings were first installed by wrapping one or two layers of Teflon tape on the threads of each fitting and fitting them into the four threaded holes in the flow deck. The flow deck was then aligned on the gasket and pressed on top of it. Finally, a microscope slide with a titanium coating of the various roughness was placed on the deck-gasket complex. Air bubbles were removed in the flow area by squeezing them out.

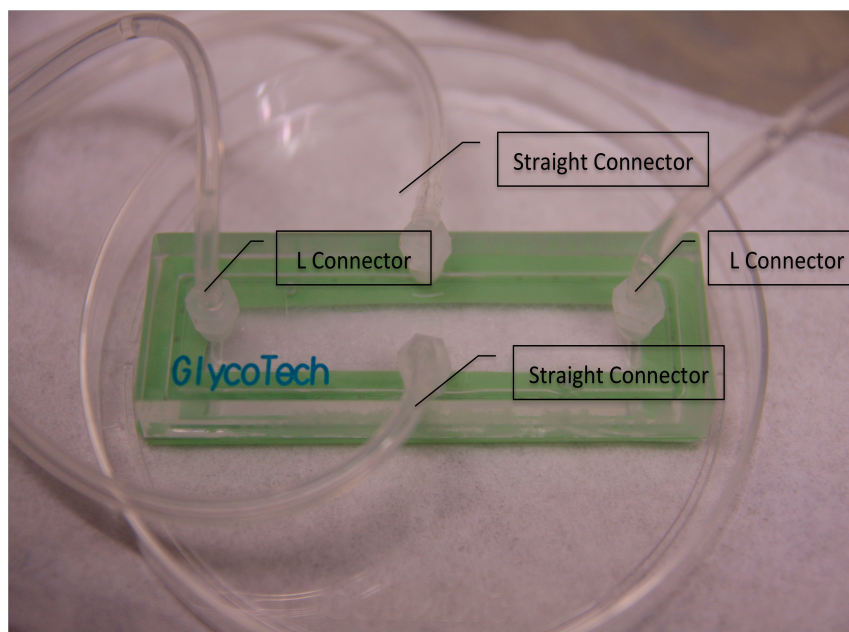
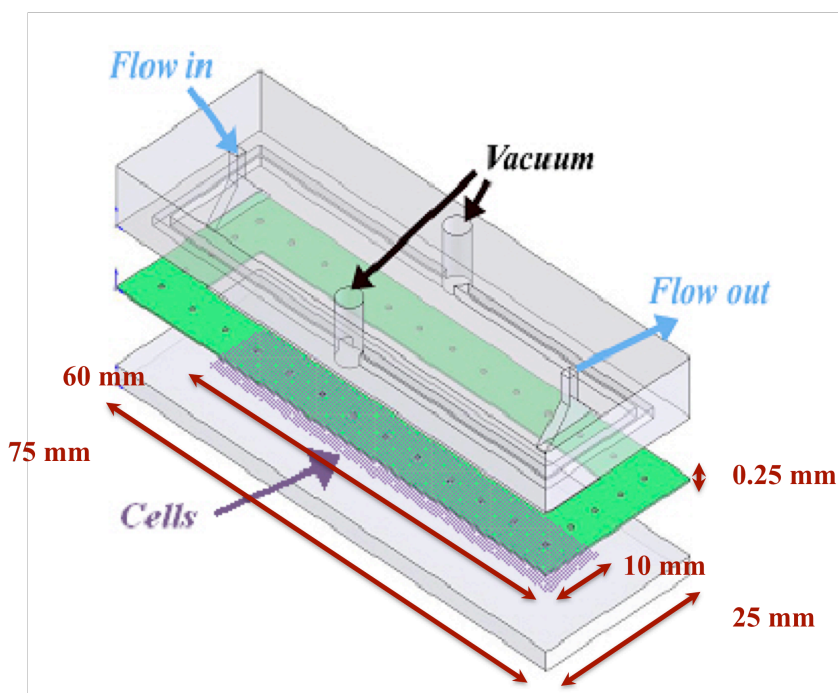
**A****B**

Figure 2.4 Parallel flow chamber showing (A) images of the installed flow chamber with the fittings. And (B) schematic diagram of the flow chamber structure.

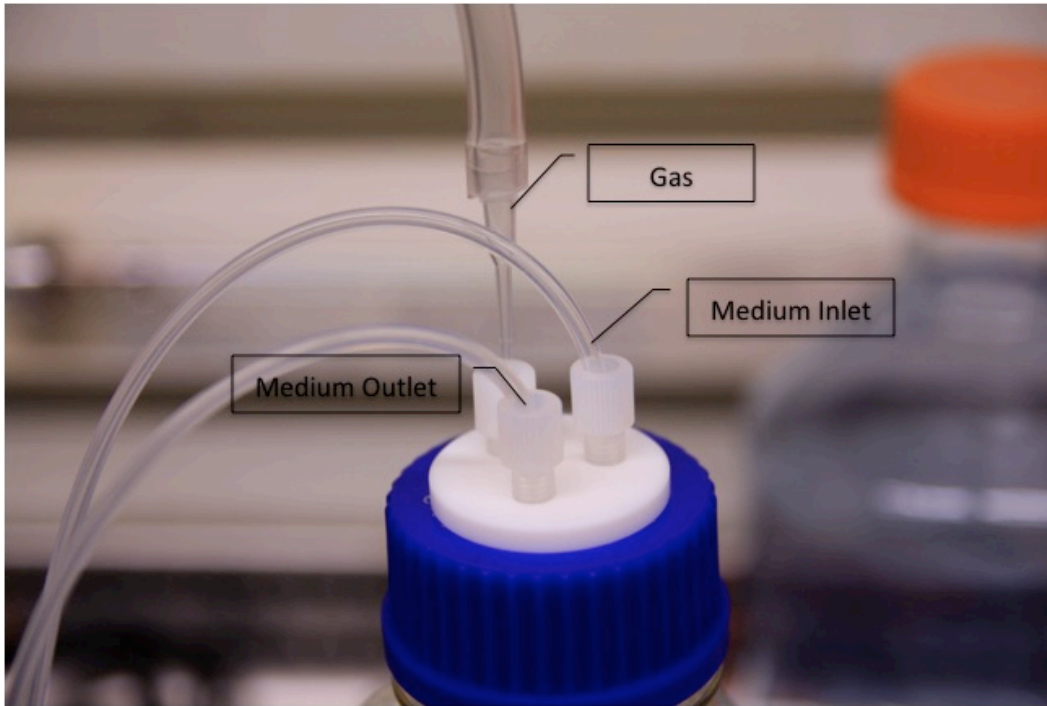


Figure 2.5. Image of the medium reservoir with the 3-hole cap.

The calibration curve for the peristaltic pump described the relationship between RPM (revolutions per minute) of the pump and the volumetric flow rate (ml/sec), and the wall shear stress of the stimulated flow. The normal shear stress in blood flow varies from 15 to 50 dynes/cm², which is in the range of the custom flow system.

Prior to each use, the flow system was cleaned and sterilized by passing 70% of ethanol through the system for 1 hour and rinsing with sterile PBS twice.

2.4.2. Coating Stability Test

A 1"× 3" standard microscope slide coated with sub-micron rough (S-Ti), nanorough (N-Ti) and flat (F-Ti) was placed on the deck-gasket complex before the experiments. The wall shear stress applied was 20 dynes/cm². The samples were exposed to the laminar flow for 24 hours using PBS. At the end of the time period, the samples were removed from the flow system. The surface topography of the samples was observed by SEM and compared with the topography before exposure to the PBS flow.

2.4.3. Dynamic RAEC Adhesion Test on Titanium Substrates with Different Roughness

RAEC were seeded on standard microscope slides coated with sub-micron rough (S-Ti), nanorough (N-Ti) and flat (F-Ti) titanium at 500,000 cells/cm² in MCDB-131 Complete Medium for 36 hours until confluent. After growing to confluency, the samples were rinsed with PBS three times and placed on the flow deck. The wall shear stress applied was set at 10 dynes/cm². The samples were exposed to the laminar flow for 24 hours using MCDB-131 Complete Medium. At the end of 24 hours, the substrates were removed from the flow system and rinsed with PBS three times. They were then fixed with 4% formaldehyde (Fisher-Scientific) for 10 minutes. Once fixed, the substrates were washed three times with PBS. The samples were permeabilized in 1% Triton X-100 in PBS for 10 minutes and blocked with 5% FBS/0.1% Triton X-100 in PBS solution for

20 minutes. After rinsing with PBS twice, the cells were stained with Rhodamine Phalloidin (R415, 1:40 in 5% FBS/0.1 Triton X-100 in PBS, Molecular Probes) for 30 minutes to visualize F-actin filaments as well as a 4',6-Diamidino-2-phenylindole (DAPI, 1:30 in PBS, Sigma) for 5 minutes to visualize the cell nucleus. The cell morphology on the samples after exposure to the stimulated vessel model was observed with a Leica DM5500 fluorescence microscope.

2.4.4. Dynamic Monocyte Adhesion Test on Titanium Substrates with Different Roughness

RAEC were seeded on standard microscope slides coated with sub-micron rough (S-Ti), nanorough (N-Ti) and flat (F-Ti) titanium at 500,000 cells/cm² in MCDB-131 Complete Medium for 36 hours until confluent. After growing to confluency, the samples were rinsed with PBS three times and placed on the flow deck. TIB-202 cells, pre-stained with 3.3 μ M of calcein AM, were placed in 50 ml of RPMI 1640 medium (supplemented with 10% FBS and 1% P/S) containing 1 μ g/ml of TPA at a density of 50,000 cells/ml in the medium reservoir. The wall shear stress applied was 10 dynes/cm². The samples were exposed to laminar flow for 4 hours using PBS. A magnetic stir bar was used in the medium reservoir during this time period. At the end of 4 hours, the samples were removed from the flow system and rinsed with PBS three times. The adherent cells on the samples after exposure to the stimulated vessel model was quantified with a Leica DM5500 fluorescence microscope.

2.5. Statistical Analysis

All experiments were conducted in triplicate and repeated at three different times. Experimental data were analyzed statistically using ANOVA with single factor and commercially available programs: Excel (Microsoft, WA) and Origin 7.5 (Origin Lab, MA).

CHAPTER 3. RESULTS

3.1. Characterization of Nanostructured Titanium Surfaces

3.1.1. Random Nanostructured Titanium

Choudhary *et al.* for the first time created random nanostructured titanium surface features to enhance vascular cell (specifically, vascular endothelial cells and vascular smooth muscle cells) function. As a result, these surfaces were used as controls in this study. As shown in Figure 3.1, random nanoparticulate topographies for the random nanostructured titanium. The titanium nanoparticles, whose dimensions ranged from 50 nm to 200 nm with an average of 77.5 nm, and interparticle voids were distributed heterogeneously on the surface. Compared to conventional titanium surface features, Figure 3.1 (b) provided evidence of increased nanometer roughness for the random nanostructured titanium.

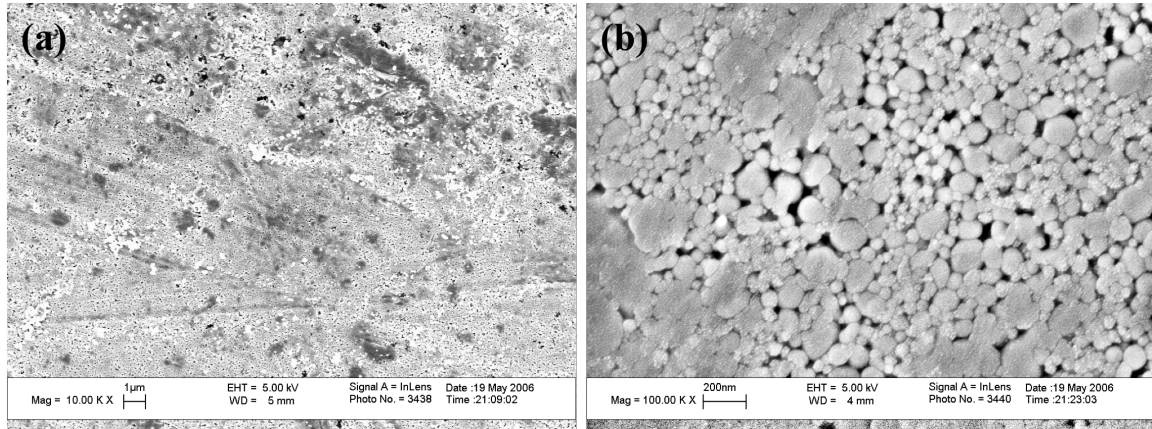


Figure 3.1 SEM image of random nanostructured titanium surface features at (a) low magnification, bar = 1 μm and at (b) high magnification bar = 200 nm.

3.1.2. Random Nanostructured Nitinol (NiTi)

SEM images in Figure 3.2 showed a more rough surface topography for the fine grained NiTi as compared with the course grained NiTi. The fine grained surface was also visibly more compacted with a greater percentage of small particles. Though the largest particle size on the fine grain surface was approximately 50 μm while the smallest particle sizes were less than 1 μm , the average particle size on the fine grained surface was around 19.1 μm and that on the course grained surface was 30.4 μm . The EDS result confirmed similar surface chemistry between the two substrates. The fine grained NiTi had an average surface composition of 55.1 wt.% Ni and the course grained had 54.5 wt.% Ni.

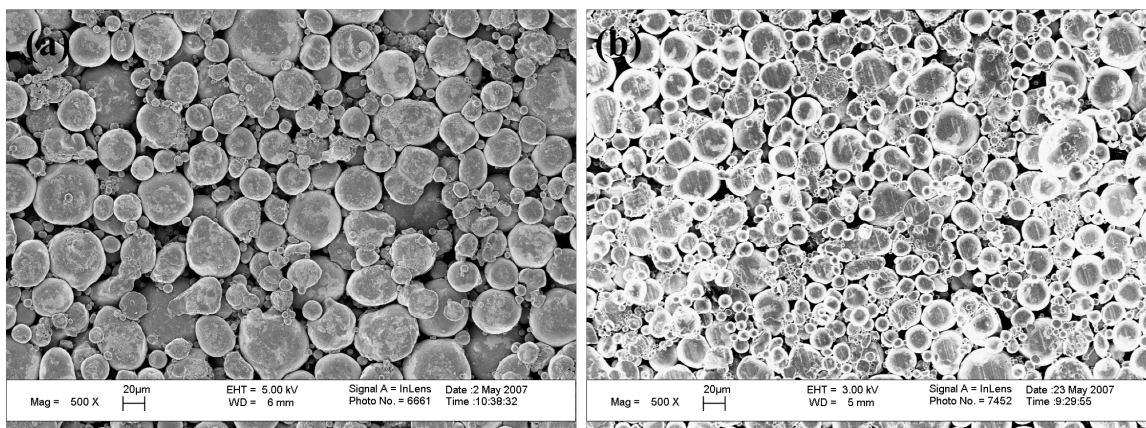


Figure 3.2 SEM images of micro and sub-microstructured NiTi surfaces. (a) Microstructured NiTi, bar = 20 μm. And (b) sub-microstructured, bar = 20 μm.

3.1.3. Highly Controllable Nanostructured (Nano/Micropatterned) Titanium Surface Features Created by TIDE

SEM (Figure 3.3) and AFM images (Figure 3.4) demonstrated the high resolution patterning capability of the TIDE process. Periodic arrays of uniform grooves with nearly vertical sidewalls were created with width and spacing ranging from the nanometer (750 nm) regime to a hundred micrometers. The sporadic thin bright lines and spots observed on the etched grooves (darker stripes) in the SEM micrographs for the patterned samples were artifacts due to the dry etching process. They were caused by the presence of impurities in the titanium substrate material that were not etched by the Cl_2/Ar plasma. Their incidence decreased considerably with decreased feature size and they were virtually non-existent in the nanometer patterned samples.

AFM 3D and line profiles again showed that TIDE had the capability to produce ordered line patterns. These linear patterns possessed smooth top surface with an average rms roughness of around 0.

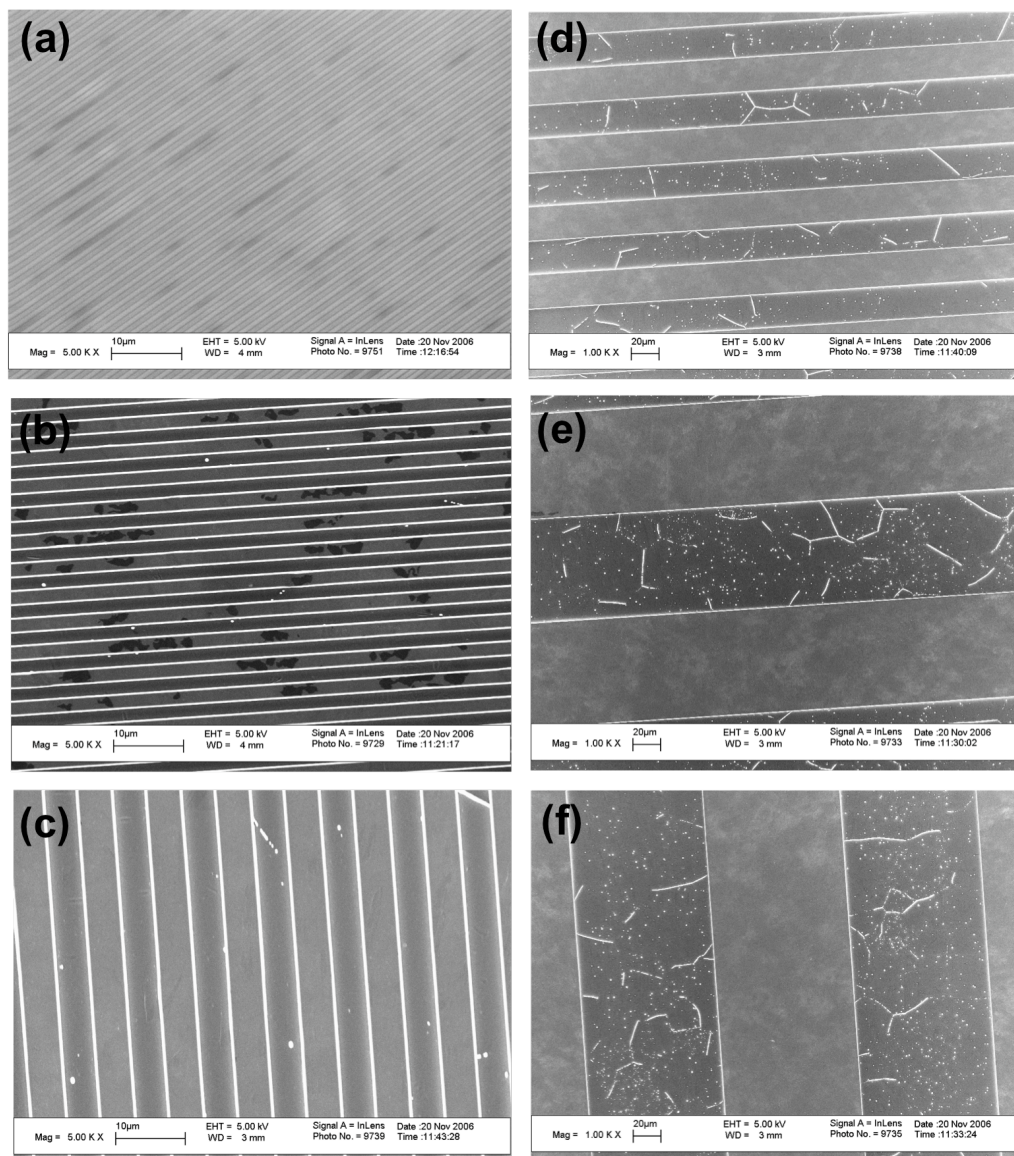


Figure 3.3 SEM images of the titanium TIDE patterned surface with groove widths of (a) 750 nm, bar = 1 μm; (b) 2 μm, bar = 1 μm; (c) 5 μm, bar = 1 μm; (d) 25 μm, bar = 20 μm; (e) 75 μm, bar = 20 μm; and (f) 100 μm, bar = 20 μm. The thin, non-uniform bright lines and spots observed in the etched grooves (darker stripes) were artifacts of the dry etching process.

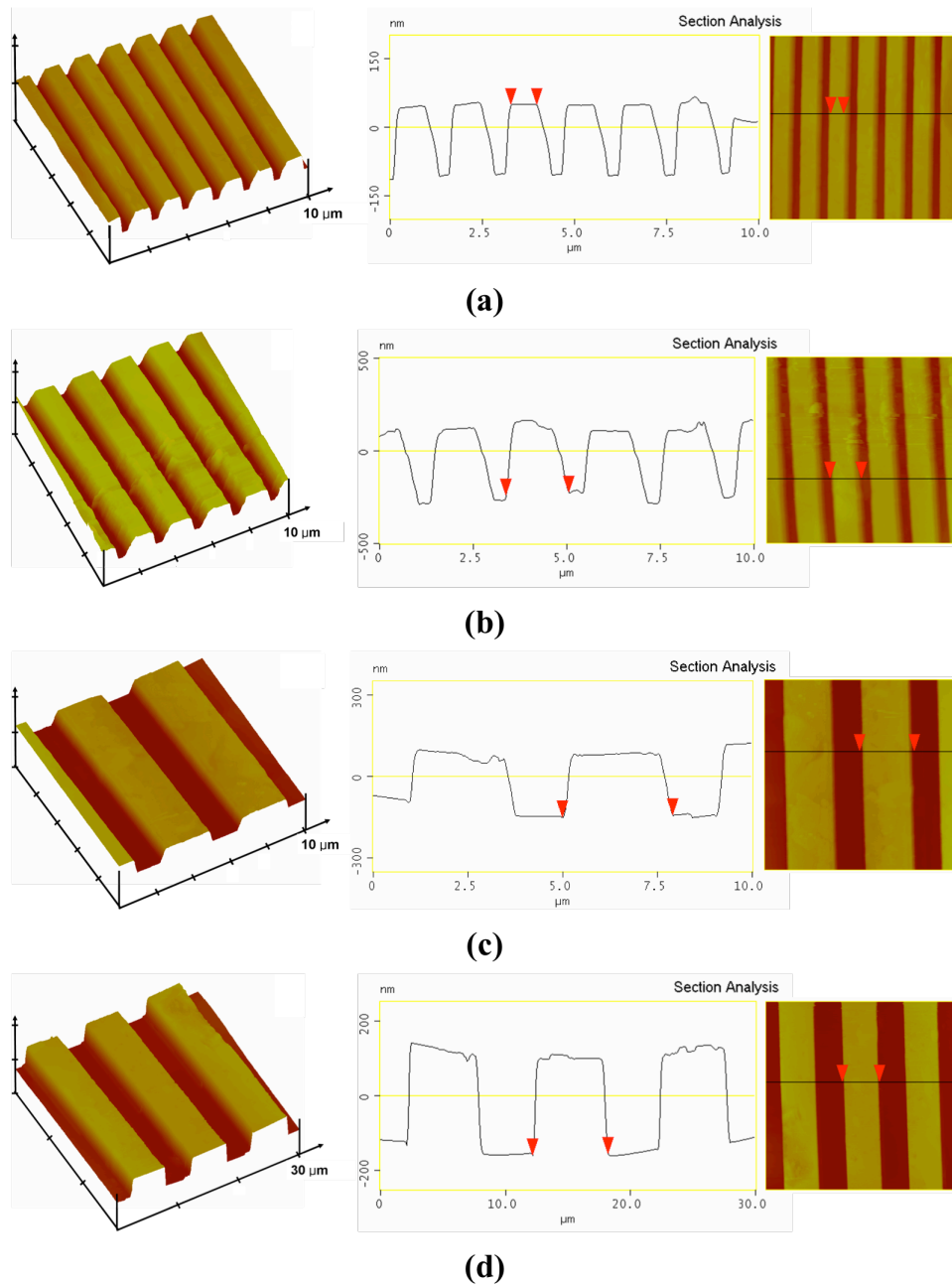


Figure 3.4 AFM images of the titanium TIDE patterned surface with groove widths of: (a) $0.75\ \mu\text{m}$, scan scale = $10\ \mu\text{m}$; (b) $1\ \mu\text{m}$, scan scale = $10\ \mu\text{m}$; (c) $2\ \mu\text{m}$, scan scale = $10\ \mu\text{m}$ and (d) $5\ \mu\text{m}$, scan scale = $30\ \mu\text{m}$.

3.1.4. Nano and Sub-micron Titanium Surface Roughness on Borosilicate Glass Coverslips Created by E-beam Deposition

Figure 3.5 (SEM) and 3.6 (AFM) demonstrated the surface features of sub-micron rough (S-Ti), nanorough (N-Ti) and flat titanium (F-Ti) created by the E-beam deposition technique used in this study. Since cover glass is very smooth, it provided an underlying substrates with minimal (close to zero) roughness. When titanium thin films were deposited onto cover glass using slower deposition rate (such as $2\text{\AA}/\text{sec}$), the structure of deposited titanium was flat (in both micron and nano scales: Figure 3.6 a-b). Specifically, sectional analysis indicated that the surface was flat (in both nano and sub-micron scale: defined as F-Ti). Inset image (depth analysis) also showed negligible depth (vertical) hits on flat surfaces (no meaningful variation on both vertical and lateral scales). However, high e-beam current densities ($130\text{ mA}/\text{cm}^2$) and, thus, fast deposition rates ($20\text{\AA}/\text{sec}$) with a deposition thickness of 50 nm generated some surface roughness at the sub-micron scale (Figures 3.6c) and sectional analyses showed that nanostructures were clearly visible at the nanoscale (defined as N-Ti: structures were $30\text{-}40\text{ nm}$ in the lateral and $2\text{-}6\text{ nm}$ in vertical scales). Since both vertical and lateral structures were less than 100 nm , all the surface roughness came from these features should be considered as nano surface roughness. Vertical depth (inset images of N-Ti) hits also showed that most of the depth hits were obtained from $2\text{ nm}\text{-}6\text{ nm}$ and a majority of hits were about 4 nm in the vertical scale). Figures 3.6 e-f

showed very drastic differences in surface structures at the sub micron and nano-scale. In contrast to N-Ti with increased surface roughness at the nanoscale than at the submicron scale (Figure 3.6 c-d), high current density (130 mA/cm^2 with increased deposition rate of 20 Å/sec) and e-beam deposition thickness of 1 μm generated the best rough structures at the sub-micron scale (defined as S-Ti: Figure 3.6 e). However, at the nanoscale (Figure 3.6 f), those structures were relatively smooth (in height variation) compared to N-Ti (Figure 3.6 d). This clearly demonstrated that sub-micron scale surface roughness (larger than 100nm in lateral width) was dominant on S-Ti surfaces (but vertical structures were still less than 100 nm). The depth of S-Ti (inset image) also shows variation of vertical structures were $0\text{-}70 \text{ nm}$ with maximum hits within $30\text{-}40 \text{ nm}$ (in vertical scale).

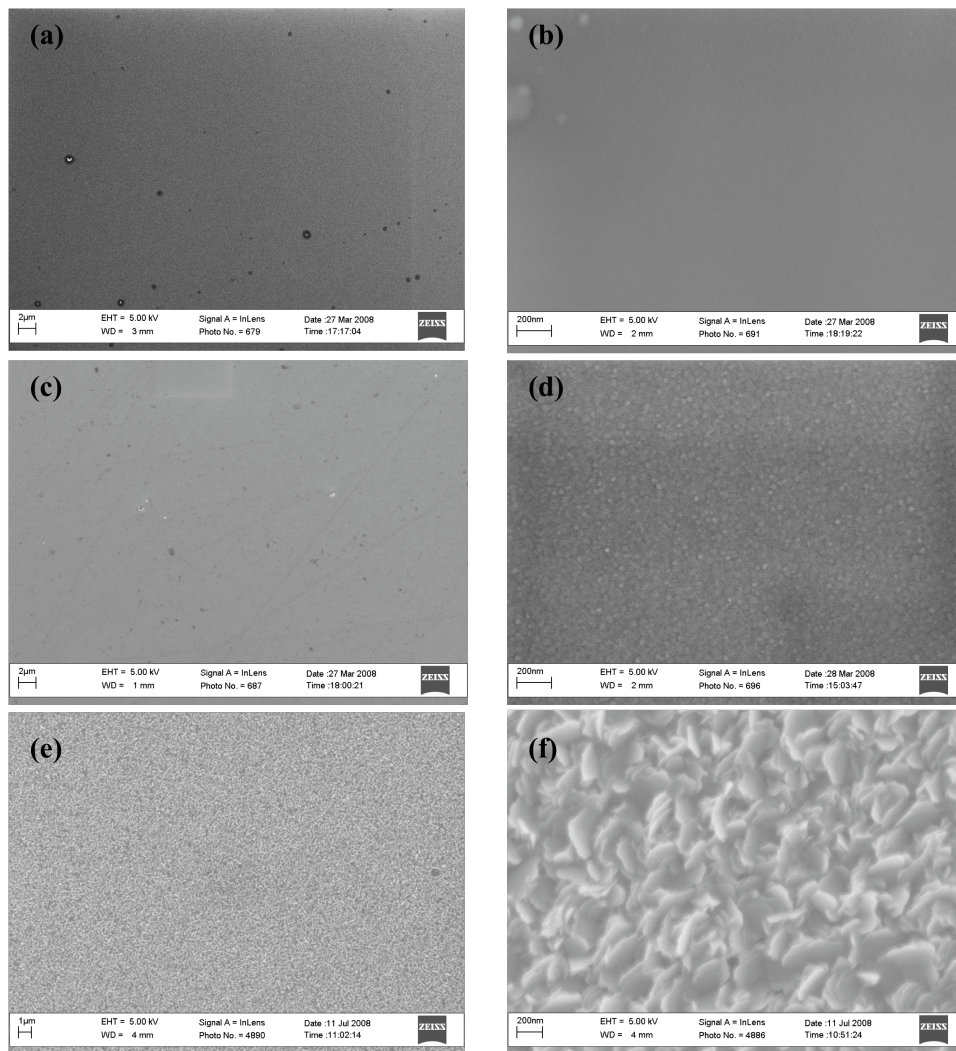


Figure 3.5 SEM images of titanium surface roughness. (a) Flat surface (F-Ti), bar=2 μm ; (b) Flat surface (F-Ti), bar=200 nm; (c) Nanorough surfaces (N-Ti), bar=2 μm and (d) Nanorough surfaces (N-Ti), bar=200 nm; (e) Sub-micron rough surfaces, bar=1 μm and (f) Sub-micron rough surfaces (S-Ti), bar=200 nm.

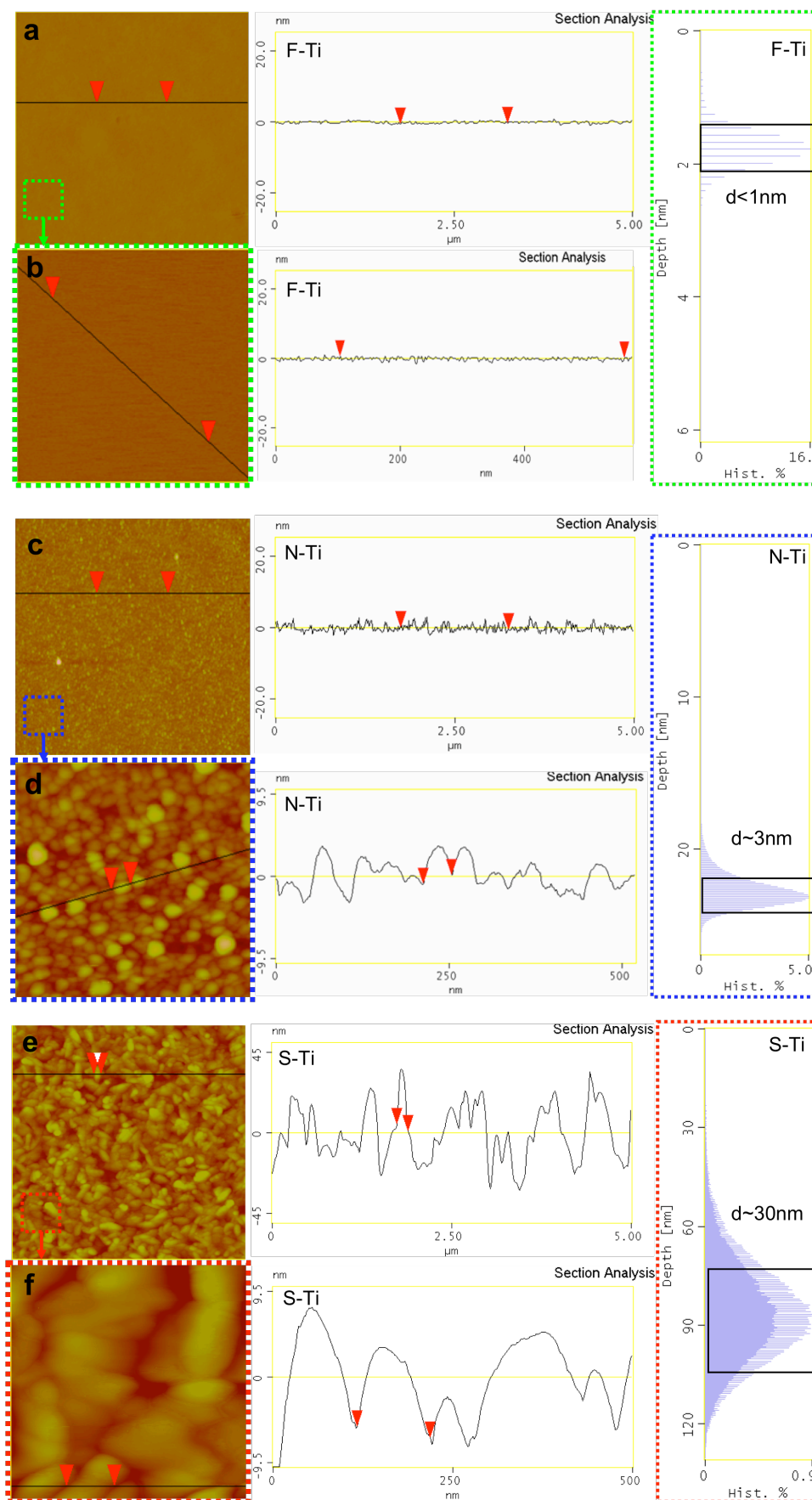


Figure 3.6 AFM characterization of titanium surface roughness. (a)-(b) AFM analysis of F-Ti showed no surface features at the sub-micron and nano scale. A sectional analysis showed that F-Ti had flat (or smooth) structures in both the nano and sub micron scales. Depth analysis (inset image) showed negligible hits of depth features (less than 1 nm). (c)-(d)

AFM analysis showed clear differences in surface features at the submicron and nano scales. AFM sectional analysis showed that N-Ti was very rough at the nano scale (structure widths were 30-40 nm) whereas surface roughness was less on S-Ti. Depth analysis (inset image) showed that a majority of hits were distributed within 2-6 nm. (e) AFM analysis showed that S-Ti had very rough surfaces at the sub-micron scale but it had rather smooth surface structures at the nano-scale: less lateral scale variation compared to N-Ti in the nano scale in (f). The widths of lateral structures were above 100 nm. Depth analysis (inset image) showed that the depth of titanium surface sizes were around 20-40 nm. All depth profiles were measured from $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ AFM scans.

Interestingly, the above developed titanium samples had altered transparency (Figure 3.7). This was due to the different deposition thickness generating not only different sizes of surface features on titanium, but also different transparencies for each sample. The glass, flat titanium (50nm deposition of titanium) and nano titanium structures (100nm deposition in total) were still transparent (although the transparency was very low compared to flat structures) under standard illumination. However, sub micron structured surfaces were completely opaque because of the high deposition thickness ($1\text{ }\mu\text{m}$) of titanium.

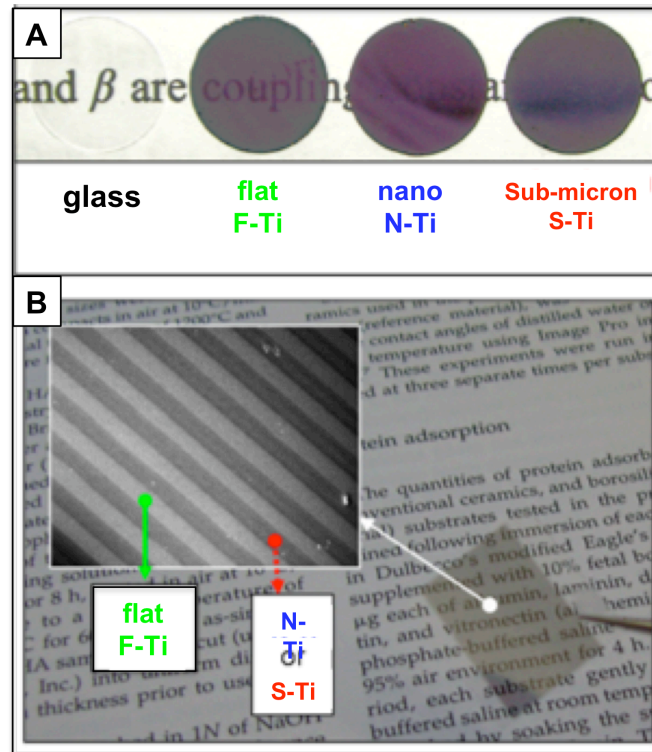


Figure 3.7 Transparency of e-beam deposited titanium on glass. (a) Shows different transparency for these substrates because of the different deposition thickness on glass coverslips. Sub-micron titanium surfaces were opaque. (b) Shows that the aligned titanium patterned surfaces were still transparent (SEM image shows aligned patterns with 30µm spaces: aligned dark regions are nano or sub-micron layers and bright regions correspond to flat surfaces (F-Ti)).

3.1.5. Highly Aligned Patterns of Flat, Nano and Sub-micron Surface

Roughness Created by E-beam Deposition

SEM images showed that highly aligned patterns were successfully generated with different surface roughness in 30 µm patterns created by e-beam deposition (Figure 3.8 a-b). Figure 3.8a showed aligned patterns of nano-titanium (N-Ti) on flat titanium (F-Ti). Because of a small difference of height (50 nm) and small nano surface roughness (N-Ti in

Figure 3.6a), SEM images did not show clear differences of the two structures as observed in AFM (Figure 3.6 d). The inset image (N-Ti) in Figure 3.8a showed very tiny spots that were already observed in Figure 3.6 c. Nanoscale resolution demonstrated again no nano or sub-micron surface structures on F-Ti (inset image in Figure 3.8 a). In comparison, after high thickness deposition by e-beam deposition (1 μm), SEM images demonstrated clear differences in surface structures even at low magnification (Figure 3.8 b) and the inset image in S-Ti demonstrated drastic increments in surface roughness at the sub-micron scale (as already verified in Figure 3.6 e). AFM depth hits showed clear height differences in the top and bottom layers of about 1 μm (Figures 3.8 c-d). AFM sectional analysis also showed very rough surface structures on the sub micron region compared to the flat region (Figures 3.8 d-e).

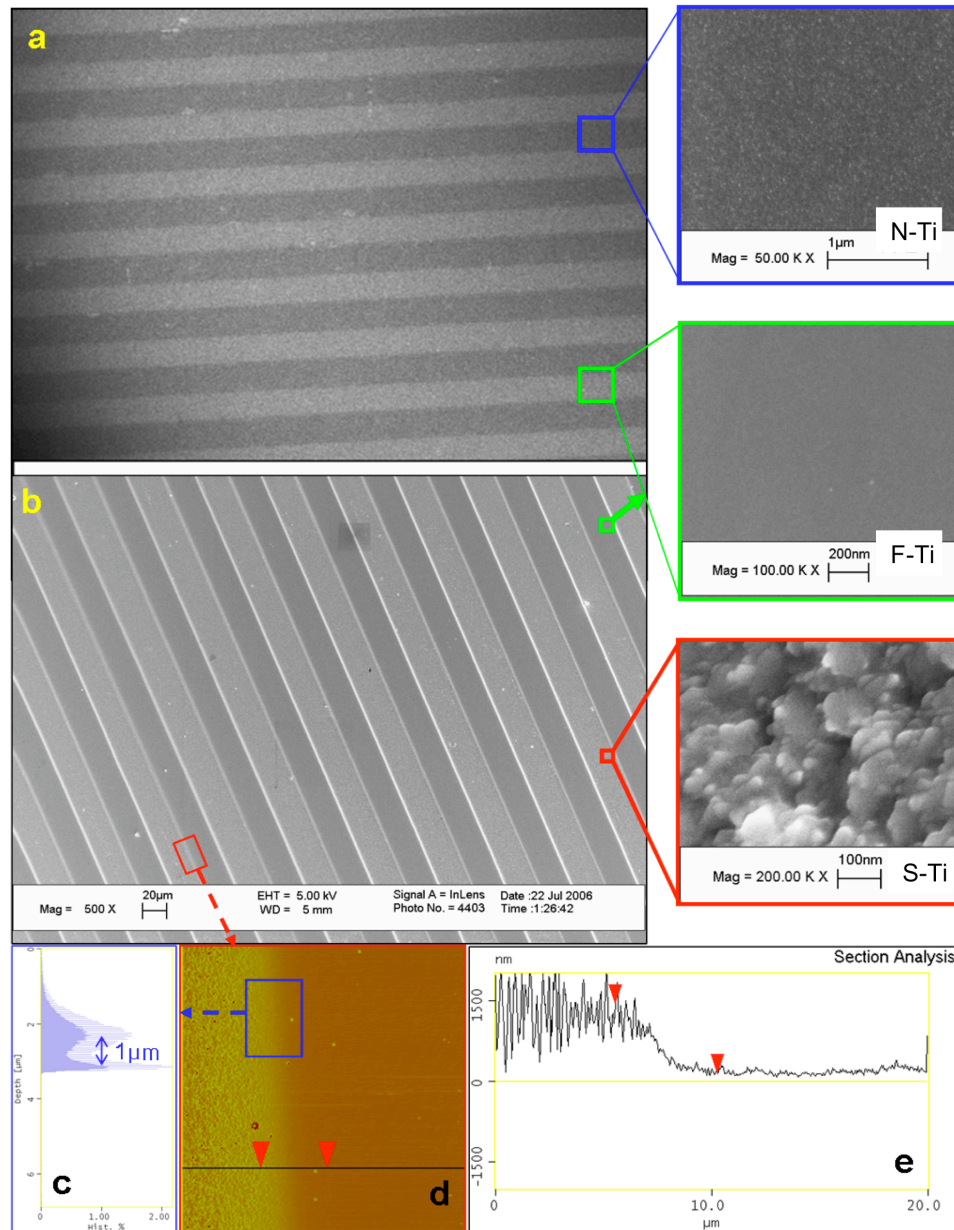


Figure 3.8 SEM characterization of titanium surface roughness. (a) Highly aligned micron patterns of nano structures (N-Ti) on flat titanium (F-Ti). Inset image (upper) showed surface spots due to nano scale surface structures (N-Ti). The flat region (below inset) did not show any surface structures at the nano scale. (b) Highly aligned micron patterns of S-Ti (sub-micron-titanium) on flat titanium (F-Ti). Inset magnified images showed a very rough morphology of S-Ti in the submicron scale. (c)-(d) Depth analysis showed the height difference between S-Ti and F-Ti and the difference was about 1µm (confirmed deposition thickness). (d)-(e) AFM analysis at the aligned intersection between S-Ti and F-Ti. (e) AFM sectional analysis of (d) showed two clear differences in surface roughness (flat and sub-micron).

3.1.6. Surface Chemistry and Surface Energy

As expected, surface chemistry results showed no difference in chemistry between each surface (i.e., flat, nano and sub-micron). This was anticipated since the source of titanium was pure (99.9%) and evaporated in a high vacuum state (10^{-7} torr). The presence of some Si and K signals (less than 4 % by weight: Table 3.1) was due to the glass cover slip since EDS measures at a depth greater than 1 μm . Thus, all surfaces, regardless of surface structures and roughness, possessed identical chemistry.

Table 3-1 EDS analysis for flat, nano and sub-micron titanium surfaces. There were no significant changes in surface chemistry for flat (F-Ti), nano (N-Ti) and sub-micron (S-Ti) structured titanium surfaces (errors were $\pm 0.031\%$). Some Si and K signals were noticed due to the presence of the glass substrate beneath the titanium deposition.

Element	Weight %	Atomic %
Si K	1.72 (± 0.024)	2.91 (± 0.036)
K K	0.68 (± 0.012)	0.82 (± 0.012)
Ti K	97.22(± 0.331)	96 (± 0.2)
Totals	100.00	

The size of the surface structures on both the nano and sub-micron scale affected water contact angles (Figure 3.8). Flat titanium surfaces (F-Ti) were hydrophilic (less than 45° in water contact angle). In comparison, the

nano roughness titanium surfaces (N-Ti) were more hydrophilic than the flat surface (Figure 3.8b), at less than 35° water contact angles. Sub-micron titanium surfaces (S-Ti) were the most hydrophilic of all. The average contact angle of S-Ti was less than 20° .

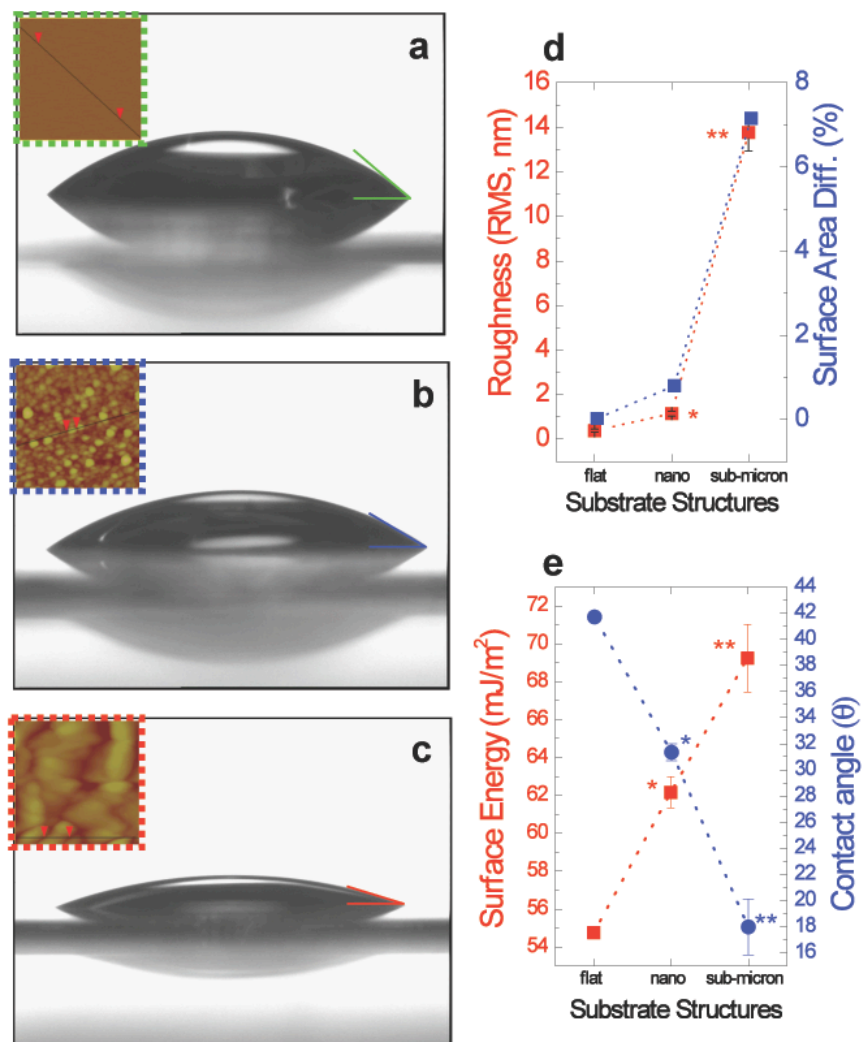


Figure 3.9 Surface roughness, area and energy characterization showing contact angle measurements on (a) flat (F-Ti), (b) nano (N-Ti) and (c) sub-micron (S-Ti) titanium surface features. (d) Surface roughness (RMS) versus surface area (%) variance on the substrates. (e) Surface energy versus contact angles. All error bars are mean $\pm$ SEM; $n=3$; * $p < 0.01$ (compared to flat) and ** $p < 0.01$ (compared to nano).

3.2. Vascular Cell Responses on Various Titanium Surfaces Created in the Present Study

3.2.1. RAEC Responses on Highly Controllable Nanostructured Surface Features: The Role of Pattern Dimension on RAEC Functions

Results from rat aorta endothelial cell (RAEC) density assays on patterned titanium surfaces with different spacings are shown in Figure 3.10 (4 hours) and Figure 3.11 (1 day). Results of cell adhesion tests (4 hours) on the TIDE created groove-patterned samples indicated that the RAEC density significantly increased as the dimension of the pattern decreased to the nano-scale with the exception of the 25 μm patterns. The highest RAEC adhesion density was detected on the 750 nm patterns, more than three times as many as RAEC adhered to the 750 nm patterns than to the 100 μm patterns. When compared with the cell density on the random nanostructured and smooth Ti surface features, that on the nanopatterned Ti was also greater (more than 100 %) (Figure 3.10).

The cells began to spread on the nanopatterned Ti surfaces where the dimensions were less than 10 μm (Figure 3.12), which could not be observed on the micropatterned Ti and random nanostructured Ti surfaces (Figures 3.13 and 3.14). Moreover, although the density of dead cells were similar on these substrates, since the density of live cells greatly increased on nanopatterns, cell viability on the nanopatterned substrates

greatly improved compared to the smooth and random nanostructured titanium surfaces.

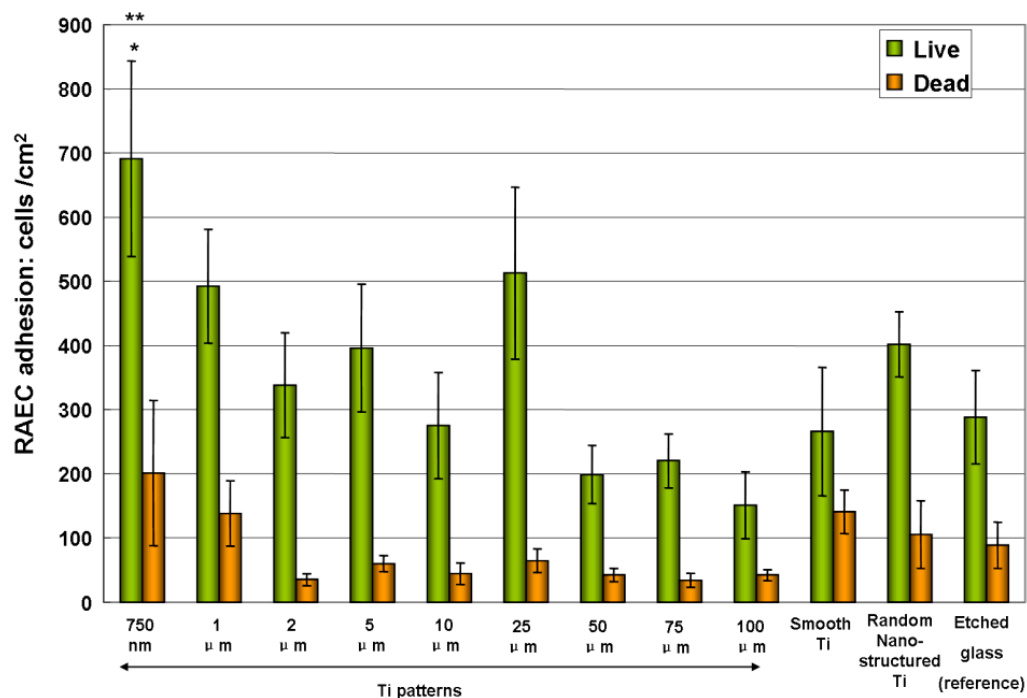


Figure 3.10 Increased rat aortic endothelial cell (RAEC) adhesion after 4 hours culture on nano to sub-micrometer patterns fabricated by the TIDE process compared to micrometer-scale patterns and random nanostructured titanium features. Data = mean $\pm$ SEM; N = 6; * $p < 0.01$ and ** $p < 0.01$ (compared to titanium patterns of 100 μm and random nanostructured titanium, respectively).

From cell adhesion experiments, it can be concluded that RAEC preferred attaching to the nanopatterned Ti surfaces after 4 hours. From the results in Figure 3.12 (ii), it was clear that RAEC grew along all the patterns created in this study after 1 day, more so on the nanopatterns.

Furthermore, on the micro patterned titanium, RAEC were more round than on the nanopatterns.

The quantitative analysis (Figure 3.11) was consistent with the observation of the fluorescence images. Specifically, the endothelial cell density on the 750 nm patterned titanium surface was twice as great as that on the 100 μm patterned surface after 1 day. Among the small dimension patterns (750 nm, 1 μm and 2 μm), the RAEC density on 750 nm patterned titanium increased by 25% and 10% compared to that on 1 μm and 2 μm , respectively. For the dead RAEC, similar cell densities were observed on all the substrates without statistical significance, which indicated that RAEC viability was still the highest on the 750nm patterned titanium surfaces.

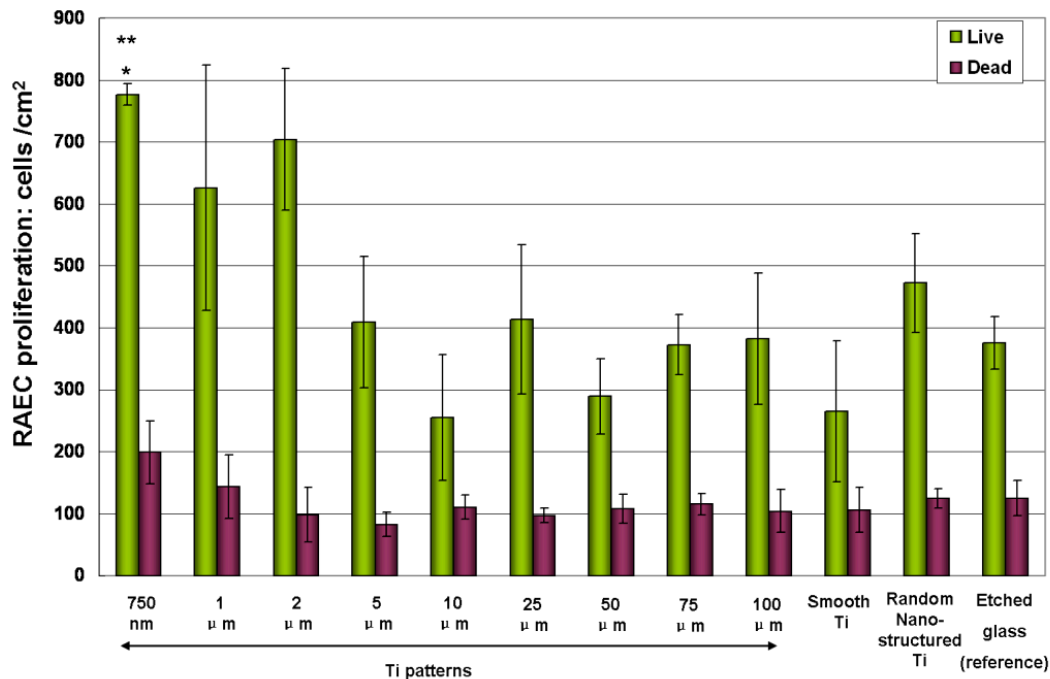
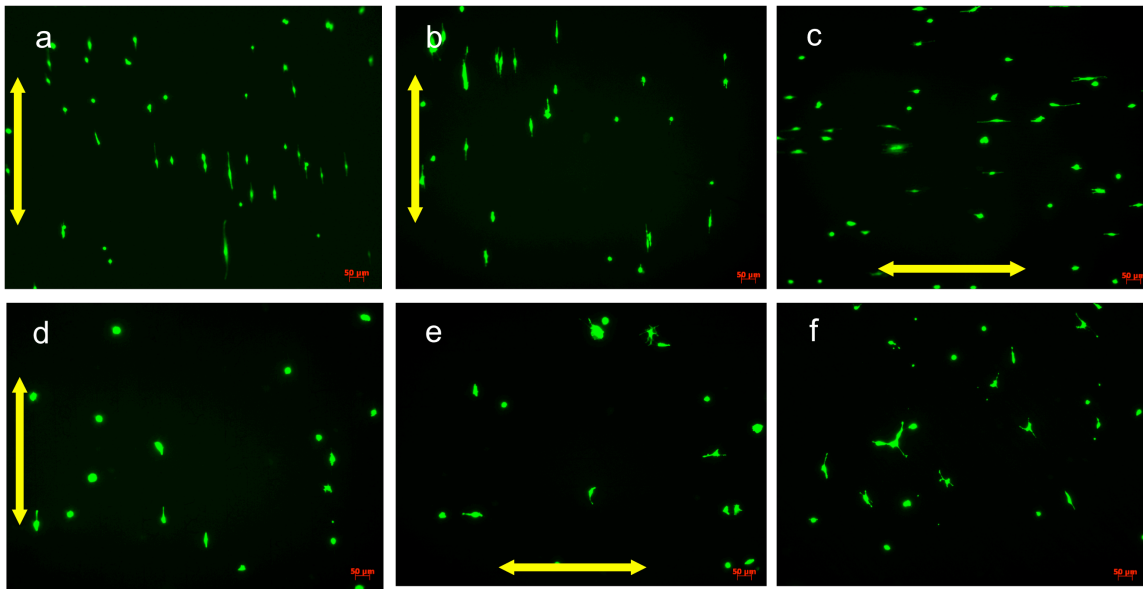


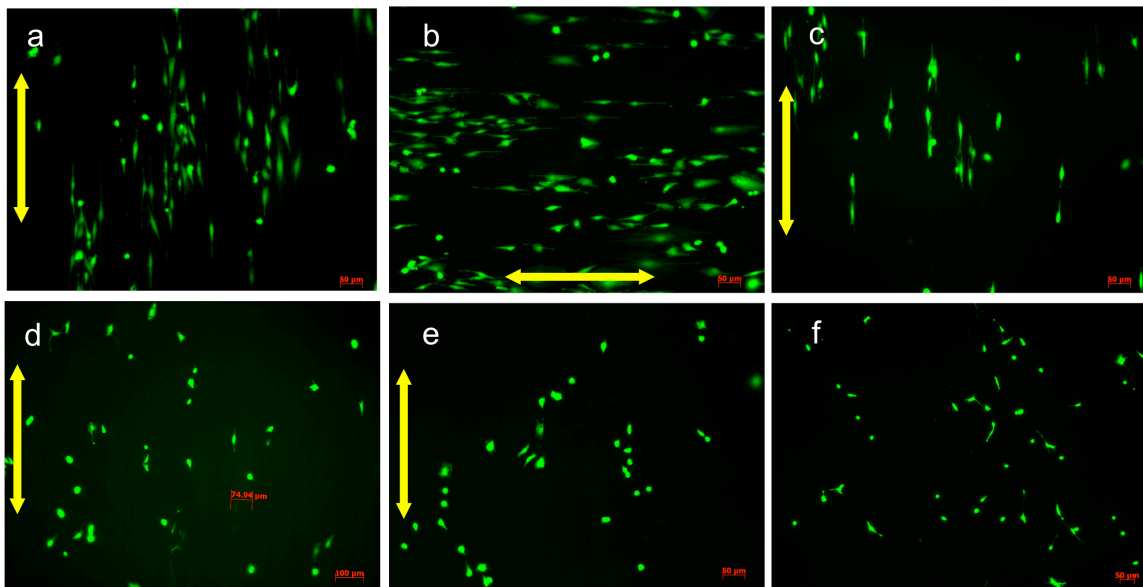
Figure 3.11 Increased rat aortic endothelial cell (RAEC) cell density after the first day of culture on the TIDE-made patterned titanium surface features. Data = mean $\pm$ SEM; N = 3; * $p < 0.05$ (compared to the patterns of 100 and 75 μm) and ** $p < 0.05$ (compared to random nanostructured surface features).

After three days, from both qualitative and quantitative analysis (Figure 3.12iii), the endothelial cell density was still higher on the patterns less than 10 μm than those larger than 10 μm . For the patterns greater than 10 μm , the cells were less uniform and less aligned. Importantly, a monolayer of endothelial cells on all of the substrates was formed after 5 days (Figure 3.12 (iv)). But according to RAEC adhesion and growth, the speed of endothelialization was the greatest on the patterns less than 10 μm with the 750 nm pattern being the best. Despite full coverage of RAEC on all

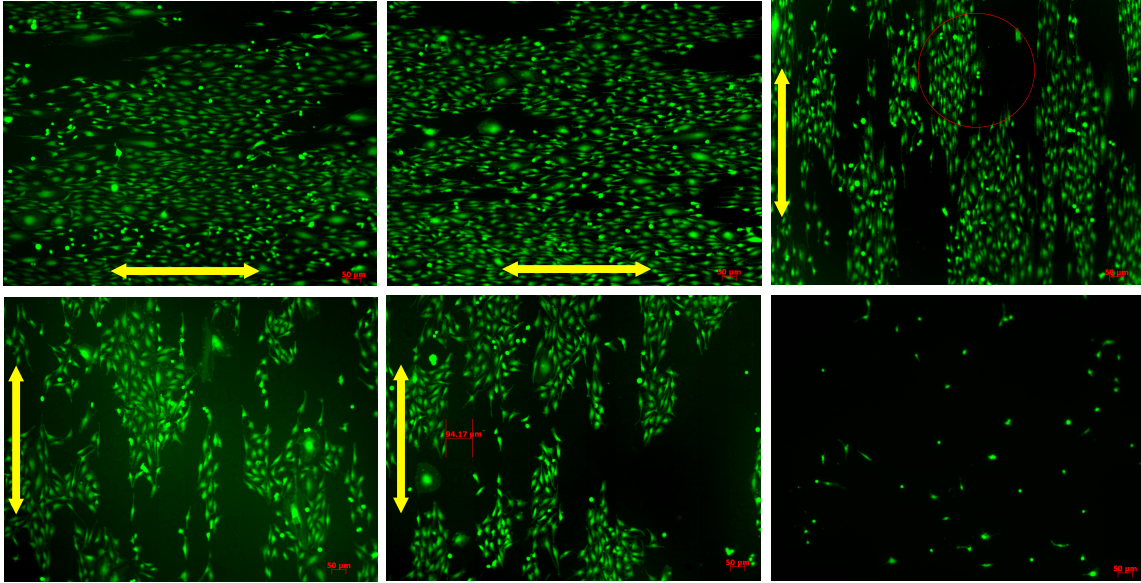
the substrates, the effect of pattern dimension on cell shape on the 5th day was still very obvious (Figure 3.12 (iv)): the length/width ratio of the cells on the 1 μm patterns substantially increased when compared with those on the 100 μm patterns, in which RAEC formed round shapes.



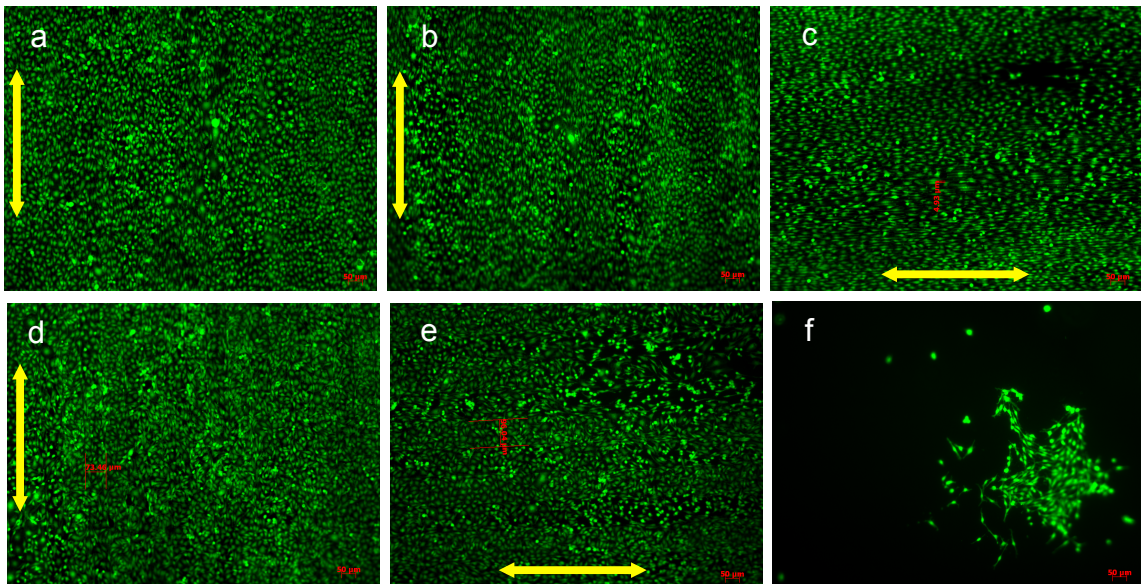
(i)



(ii)



(iii)



(iv)

Figure 3.12 Rat aortic endothelial cell (RAEC) cell density after (i) Four hours; (ii) One (iii) Three and (iv) Five days of culture on titanium patterns of (a) 750 nm; (b) 2 μm ; (c) 5 μm ; (d) 75 μm ; (e) 100 μm and (f) random nanostructured titanium surfaces. (a) to (f) bars = 50 μm . Arrows indicate groove alignment direction on patterned samples.

The SEM images (Figure 3.13 and 3.14) of RAEC spreading on nano and micron patterned titanium surfaces confirmed the observations from the fluorescence images (Figure 3.12). The filopodia of RAEC extended because of the existence of nanopatterns after 4 hours (Figure 3.13). As a result, RAEC morphology on 750 nm patterned titanium was much different from that on 100 μm patterns. RAEC on the nanopatterned titanium (750 nm) was elongated but on the micron patterned titanium surface (100 μm) they still maintained a round shape.

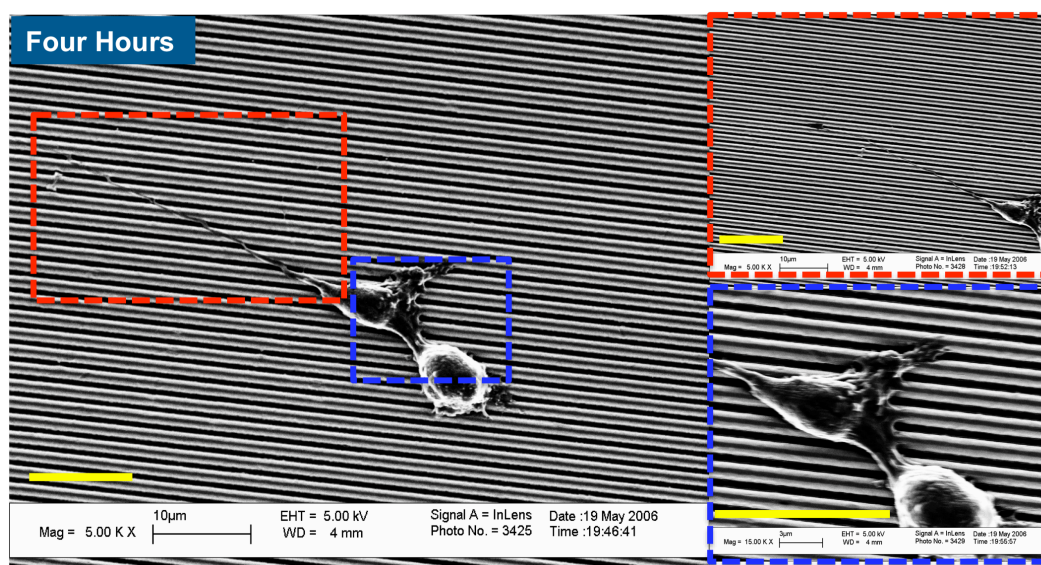


Figure 3.13 SEM images of RAEC morphology on the 750 nm nanopatterns after 4 hours of culture: The filopodia extended along the nanolines. Bars = 10 μm

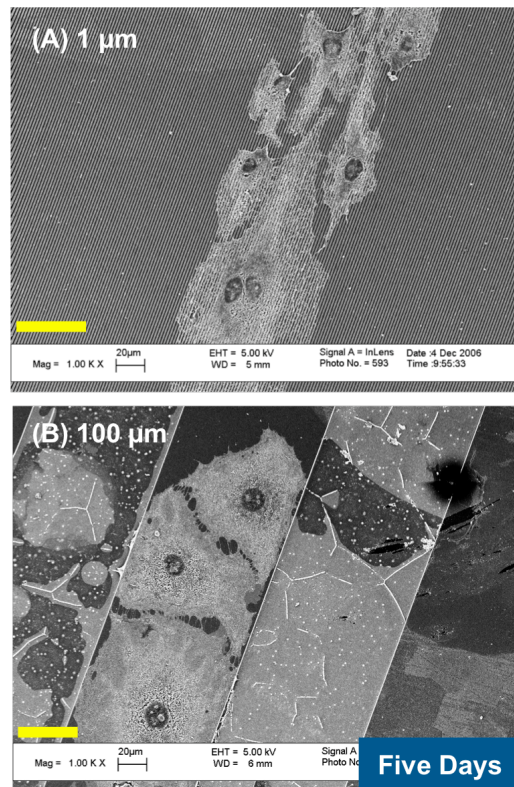


Figure 3.14 SEM images of RAEC morphology after the fifth day of culture on titanium patterns of (A) 1 μm and (B) 100 μm . Bar = 20 μm .

3.2.2. RAEC Responses on Random Nanostructured NiTi (Nitinol)

Surface Features

Cell experiment results after 5 hours showed that endothelial cells adhered much better on fine grained ($p < 0.01$) compared to coarse grain NiTi and conventional (c.p.) titanium substrates (Figure 3.15). Coarse grained NiTi compacts and conventional titanium promoted similar levels of endothelial cell adhesion. It is observed on the fluorescence microscope images that cells adhered at the boundaries between particles; cell adhesion was greater on the fine grained sample with more grain boundaries. The results after 1 day showed an almost linear increase in

endothelial cell density with a decrease in NiTi surface feature dimensions into the sub-micron and nanoscale (Figure 3.16 and Figure 3.17). The difference between fine grained and all other sized NiTi compacts were again significant ($p < 0.01$). However, the difference in cell density between the fine grained NiTi and conventional titanium was not significant.

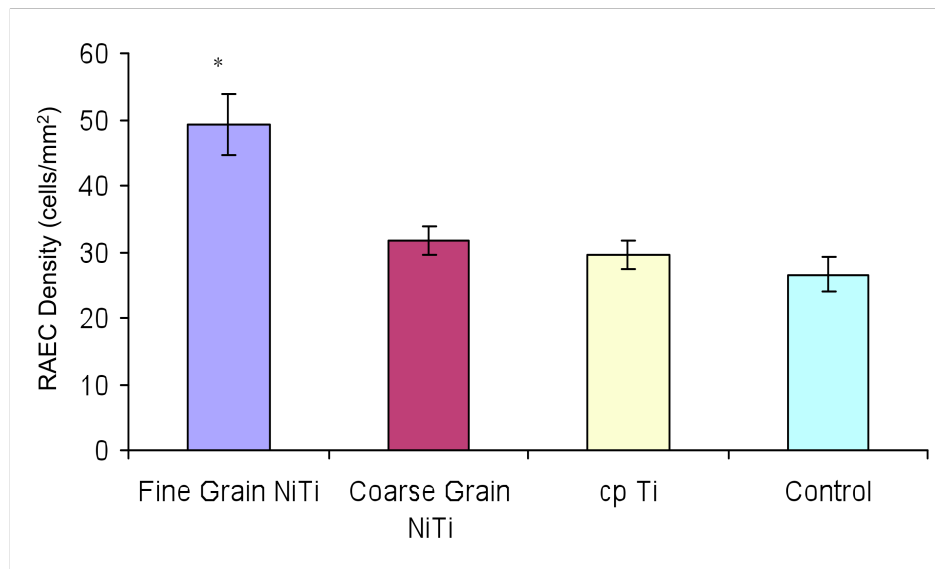


Figure 3.15 RAEC density increased on fine grained NiTi substrates with greater submicron to nanometer surface features after 5 hours, * $p < 0.01$ compared to all other substrates. The difference in endothelial cell adhesion between coarse grain NiTi, c.p. Ti, and the control was not significant. Data = mean $\pm$ SEM; N = 3. Control = tissue culture polystyrene.

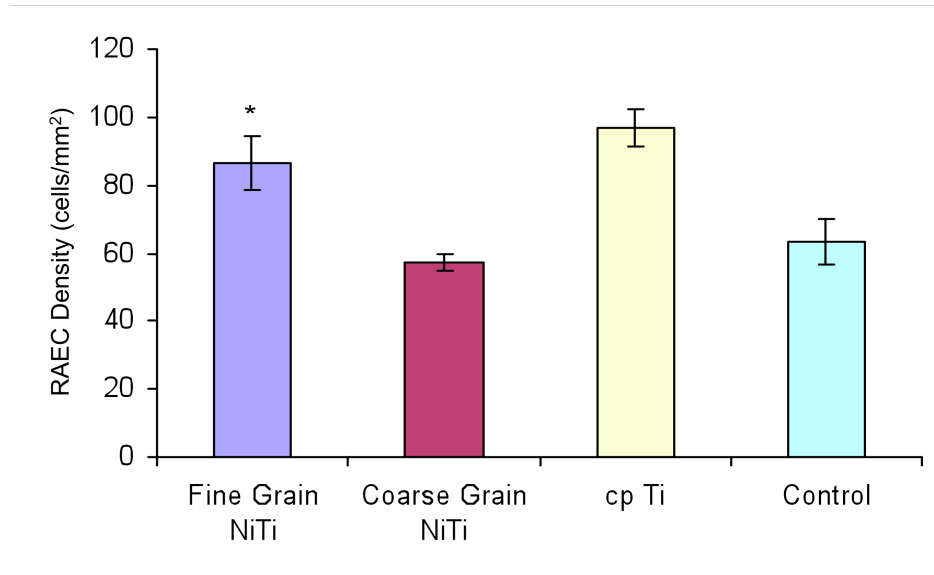


Figure 3.16 RAEC density increased on fine grained NiTi substrates with greater sub-micron to nanometer surface features after 1 day. The difference in the number of endothelial cells between fine and coarse grain NiTi substrates was significant, $*p < 0.01$. However, there was no significant difference in endothelial cell density between fine grain NiTi and c.p. Ti. Data = mean $\pm$ SEM; N = 3. Control = tissue culture polystyrene.

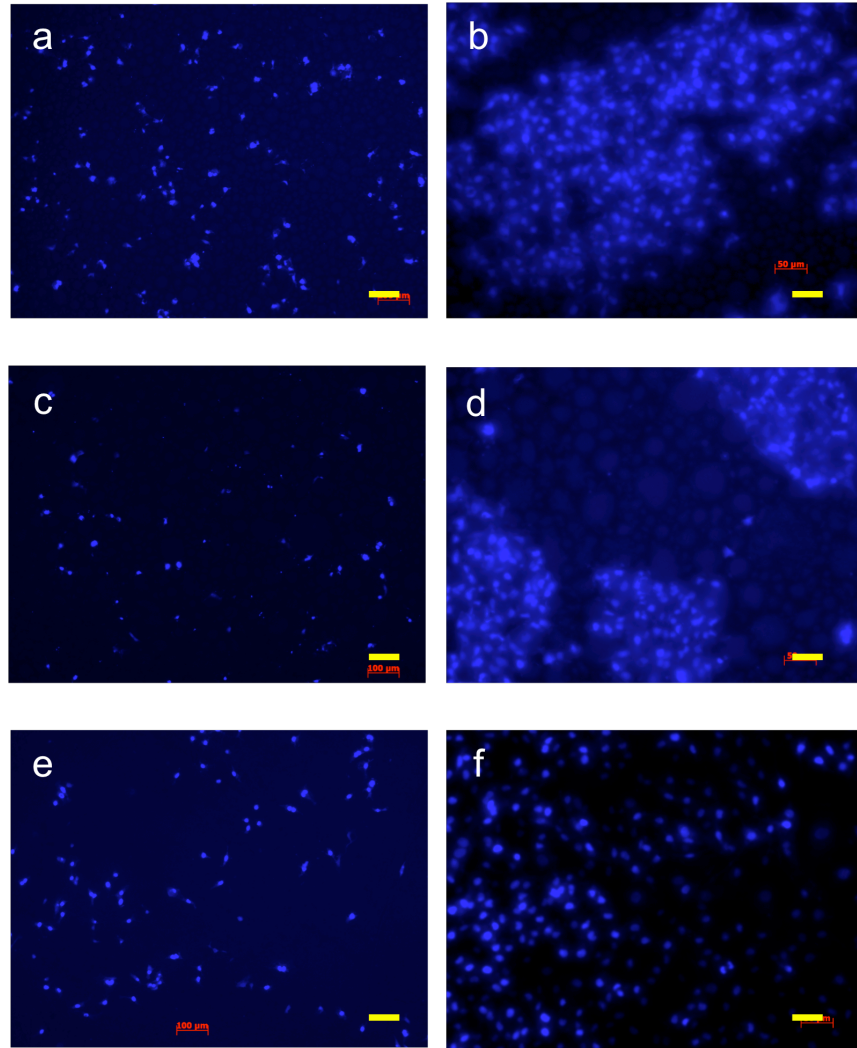


Figure 3.17 Fluorescence microscope images of endothelial cells after (a, c, e) 1 day (Bars = 100 μm) and (b, d, f) 5 days (Bars = 50 μm) on fine and coarse grain NiTi and c.p. Ti substrates. Endothelial cells were more confluent on fine grain NiTi after 5 days compared to coarse grain NiTi.

3.2.3. RAEC and RASMC Responses on Non-patterned and Patterned Titanium Substrates with Different Roughness by E-beam Evaporation: The Role of Nano and Sub-micron Surface Features

3.2.3.1. RAEC Adhesion on Titanium Substrates with Different Roughness Created by E-beam Deposition

Figure 3.18 showed greater adhesion of RAEC on sub-micron rough titanium surfaces (S-Ti) compared to flat surfaces (F-Ti) after 4 h of culture. Importantly, RAEC densities increased on sub-micron (S-Ti) and nano surface features (N-Ti) about 216% and 58%, respectively, compared to cell density on flat titanium surfaces. Magnified images of cell body staining (F-actin) clearly showed different aspect ratios of RAEC on S-Ti (submicron structures) compared to smooth titanium surfaces (Figure 3.19). The quantitative analysis showed that the aspect ratios of RAEC on S-Ti improved by 100% than those on N-Ti and F-Ti (Figure 3.18). Since most of the endothelial cells in the natural inner blood vessels are aligned with blood flow, as determined by cell morphology aspect ratios, sub-micron scale titanium surface features showed more favorable endothelial cell attachment than flat titanium surfaces.

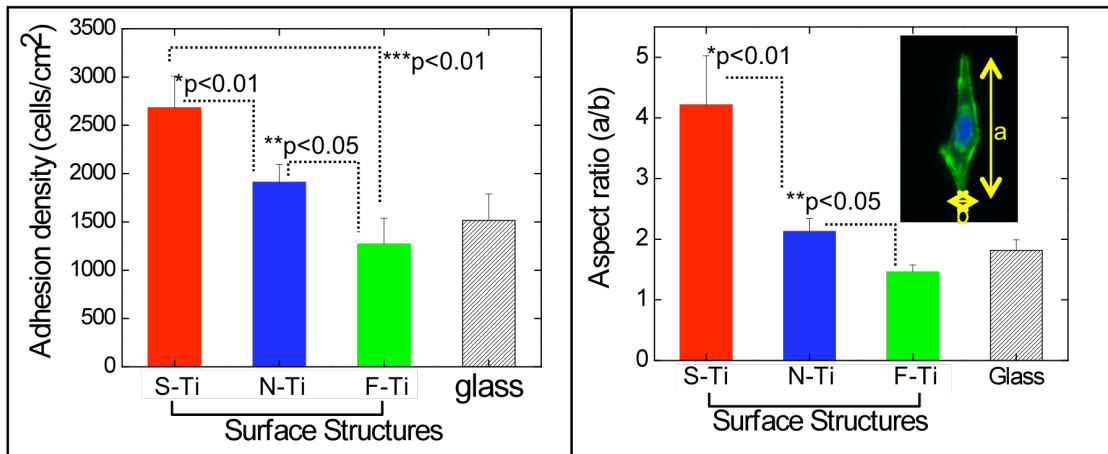


Figure 3.18 RAEC adhesion on titanium substrates with different roughness. Adhesion density showed that sub micron structures led to the best RAEC adhesion density (seeding density was 3500cells/cm²) and highest aspect ratio. All error bars are mean $\pm$ SEM; N=3; * $p<0.01$ and ** $p<0.05$

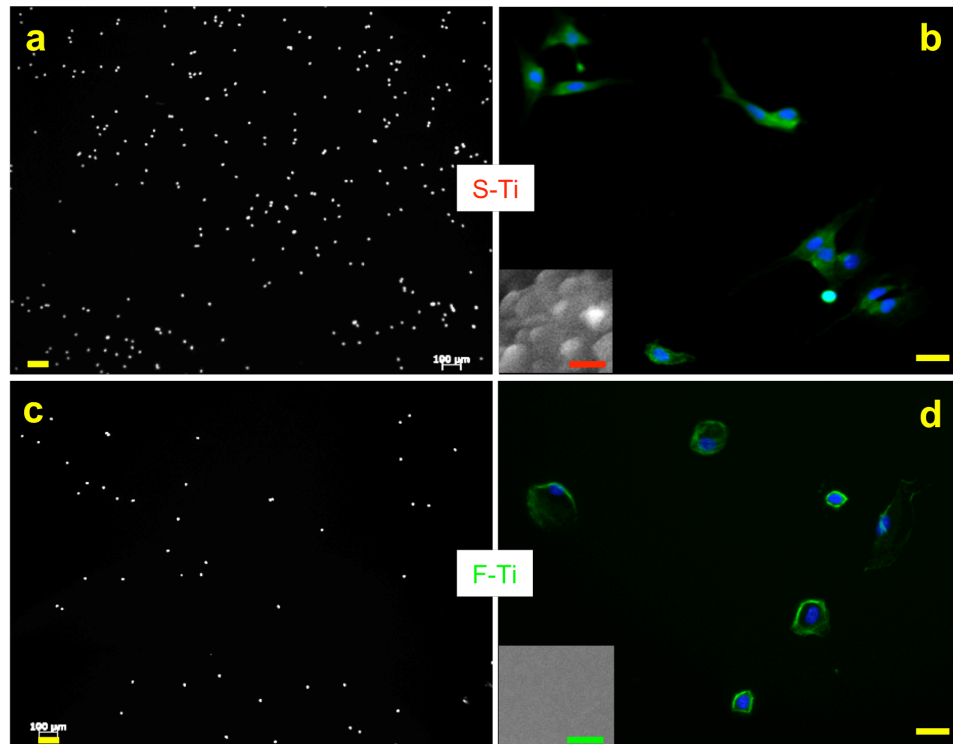


Figure 3.19 RAEC morphology on titanium substrates with different roughness. (a) Fluorescence RAEC density on submicron structured surfaces (S-Ti); (b) Magnified fluorescence RAEC image of submicron surfaces shows high cell aspect ratios. Inset image shows SEM surface structures (bar is 100nm); (c) Fluorescence RAEC density on flat surfaces (F-Ti) and (d) Magnified fluorescence cell images of RAEC on flat surfaces show small cell aspect ratios (more round cellular shapes than on sub micron surfaces). Bar = 100 nm. All yellow bars in (a) and (b) are 100 µm and yellow bars in (b) and (d) are 20 µm.

3.2.3.2. RAEC Adhesion on Patterned Titanium Substrates with Different Roughness

RAEC adhesion on aligned patterns showed that more than 80% of the RAEC selectively adhered onto sub-micron titanium patterns (Figure 3.20; dark regions compared to bright patterns in Figure 3.21 a-b). Results

clearly showed RAEC f-actin filaments tended to orient on the sub-micron features compared to flat regions (Figure 3.21 b).

In addition, 65% of RAEC selectively adhered onto nano surface featured titanium patterns (N-Ti) compared to flat surfaces (Figure 3.21 c). To determine if the selective adhesion of RAEC came from altered surface properties (surface roughness and surface energy), similar height layers (50nm and 1 μ m in height difference) with the same surface properties (both layers were flat) were tested for RAEC adhesion but showed no preferential adhesion on the top and bottom layers (Figure 3.20). More than 80% of endothelial cells adhered on sub-micron structures than flat titanium surfaces. Nano surfaces also led to greater cell adhesion (more than 60%) compared to flat surfaces. The height variation (1 μ m and 50nm) did not affect preferential cell adhesion on the top and bottom layers.

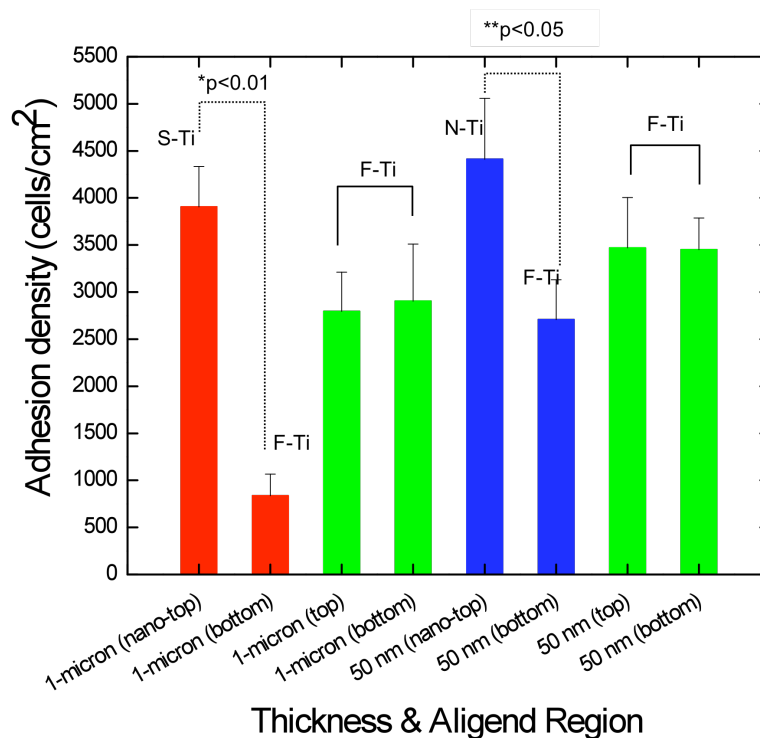


Figure 3.20 Endothelial cell (RAEC) adhesion on aligned patterns. ("top" indicates upper coated layers and "bottom" indicates lower layers). All error bars are mean $\pm$ SEM; N=3; * $p<0.01$ (compared to the respective bottom layer) and ** $p<0.05$ (compared to the respective bottom layer). No significant p values were observed for the flat top and flat bottom (both 50nm and 1 μ m height difference). S-Ti is submicron featured surface, N-Ti is nanofeatured surface, and F-Ti is flat titanium surface.

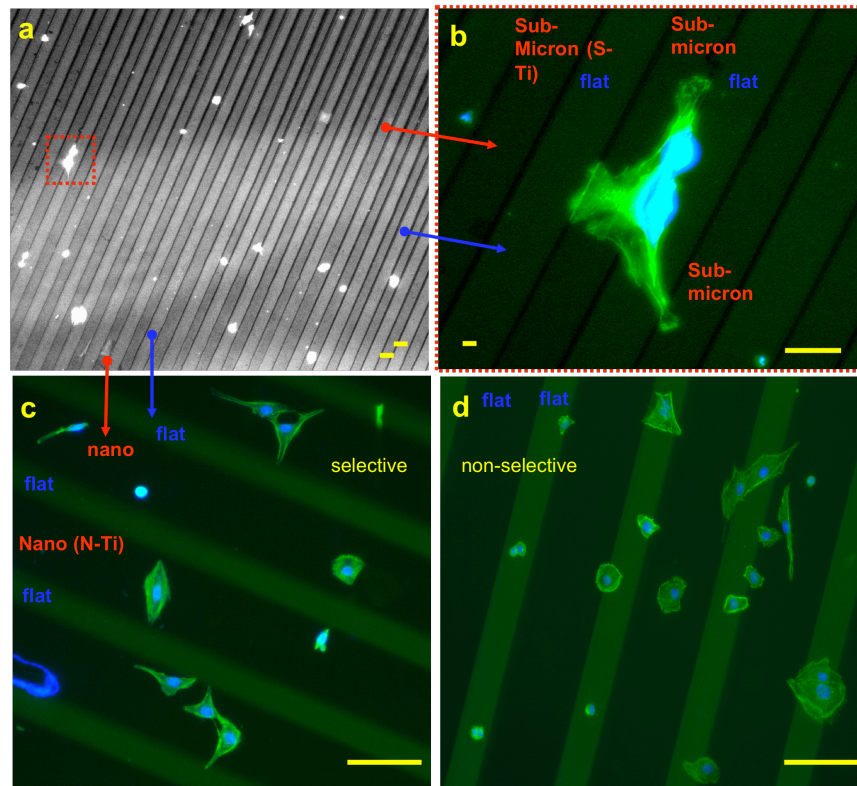


Figure 3.21 RAEC adhesion on aligned patterns. (a) Fluorescence cell image showed RAEC adherent more on aligned submicron spaces (S-Ti) compared to aligned flat titanium (F-Ti). Bar is 50 μ m. (b) Selected cell images (magnified) from (a) showed F-actin filaments oriented more on aligned sub-micron structured patterns than aligned flat surfaces (bar is 50 μ m). (c) Selective adhesion of endothelial cells showed more cells adherent on nanorough surfaces (N-Ti) than F-Ti (height was 50nm). (d) Non-selective cell adhesion on the top layer (F-Ti) and bottom layer (F-Ti) showed no preferential attachment of RAEC cells. All scale bars in (c) and (d) are 60nm.

3.2.3.3. RAEC and RASMC Proliferation on Titanium Substrates with Different Roughness Created by E-beam Deposition

Results of this study showed that the cell density on the sub-micron rough titanium surfaces (S-Ti) was the greatest after 1 day compared to the nanometer rough titanium surfaces (N-Ti; 20% more) and flat titanium

control surfaces (F-Ti; 50 % more), which is consistent with previously reported RAEC adhesion results that indicated enhanced RAEC adhesion on S-Ti compared with N-Ti and F-Ti (Figure 3.22). After 5 days, RAEC density on N-Ti was 11% greater than that on S-Ti; however, both S-Ti and N-Ti significantly increased RAEC proliferation when compared with F-Ti. Specifically, at the same time period, 60% and 75% more RAEC were found on S-Ti and N-Ti than F-Ti, respectively. Since all the Ti substrates in this study had the same chemistry but differed only in surface topography, these results indicated a strong correlation between topography and RAEC density. While N-Ti had lower surface energy and surface roughness than S-Ti (but both higher than F-Ti), both promoted RAEC density compared to F-Ti.

In contrast, results concerning RASMC density were different from that just described for RAEC (Figure 3.23). Specifically, no significant difference in RASMC density after 1 day was observed between S-Ti and N-Ti. But RASMC density on F-Ti increased by 12% and 4% compared to S-Ti and N-Ti, respectively, after 3 days. The average RASMC proliferation rates did not change on S-Ti and F-Ti, but they decreased on N-Ti from 1 to 5 days. After 5 days, RASMC density increased 12% and 14% comparing F-Ti to S-Ti and N-Ti, respectively. In other words, enhanced Ti surface energy and roughness slightly decreased RASMC proliferation from 1 to 5 days.

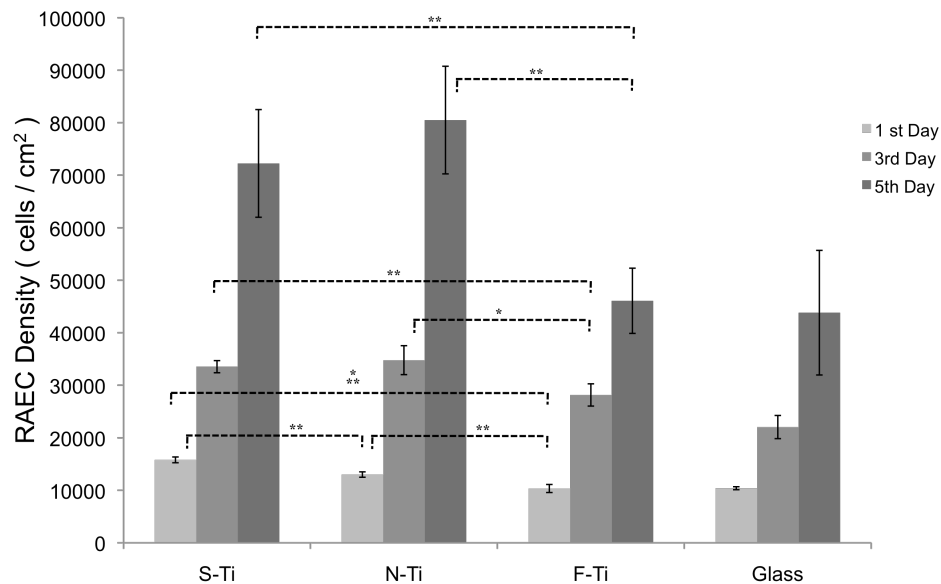


Figure 3.22 RAEC proliferation on submicron (S-Ti), nanometer (N-Ti), flat (F-Ti) titanium surface features and borosilicate glass (Glass) after 1, 3 and 5 days of culture. RAEC seeding density was 10,000 cells/cm². All error bars are mean ± SEM; N=9; **p*<0.1, ***p*<0.05 and ****p*<0.01.

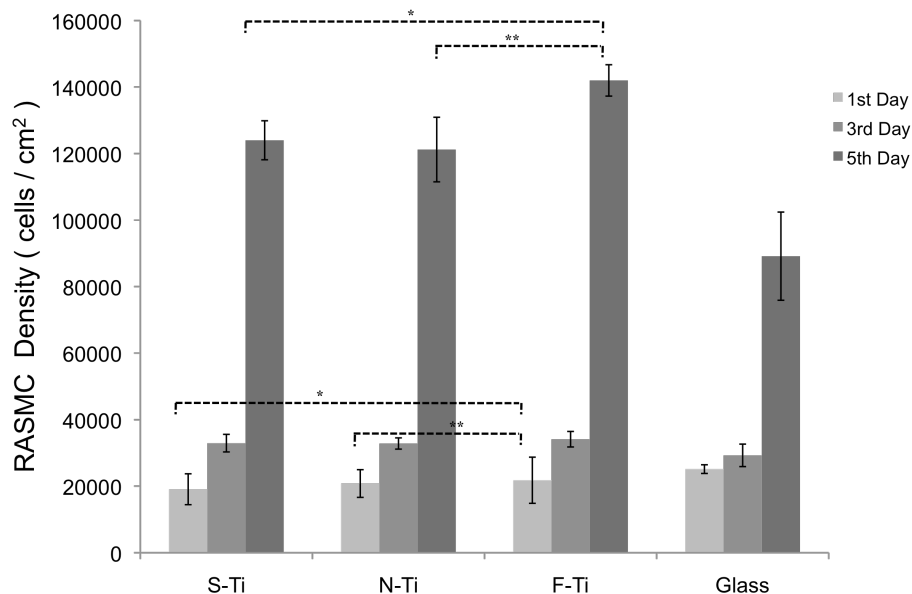


Figure 3.23 RASMC proliferation on submicron (S-Ti), nanometer (N-Ti), flat (F-Ti) titanium surface features and borosilicate glass (Glass) after 1, 3 and 5 days of culture. RASMC seeding density was 10,000 cells/cm². All error bars are mean ± SEM; N=9; **p*<0.1 and ***p*<0.05.

3.2.3.4. RAEC and RASMC Morphology on Titanium Substrates with Different Roughness Created by E-beam Deposition

RAEC (Figure 3.24) and RASMC (Figure 3.25) morphology on sub-micron rough surfaces (S-Ti), nanorough surfaces (N-Ti) and flat surfaces (F-Ti) after 1, 3 and 5 days were investigated by fluorescence microscopy. After 1 day, RAEC on S-Ti were more spread with increased coverage compared to F-Ti (Figure 3.24 and Figure 2.26). More than 45% of the S-Ti surfaces was covered by RAEC, however, on F-Ti, the coverage was less than 15%. Since on the inner side of blood vessels, endothelial cells are parallel with each other with respect to blood flow, which corresponds to larger cell aspect ratios, the present results that S-Ti had greater endothelial cell spreading than F-Ti are promising for vascular stent applications. In addition, because of a faster proliferation rate, S-Ti and N-Ti were covered by a monolayer of RAEC more quickly than F-Ti after 5 days, this is most promising considering the crucial role of endothelialization of vascular stent surfaces towards inhibiting in-stent restenosis.

In this study, it was not easy to distinguish differences between RASMC morphology on the three substrates (Figure 3.25). The cell spreading areas were similar, but larger than those of RAEC. These results

demonstrated that RASMC might not be as sensitive as RAEC to surface nanometer and sub-micron Ti features.

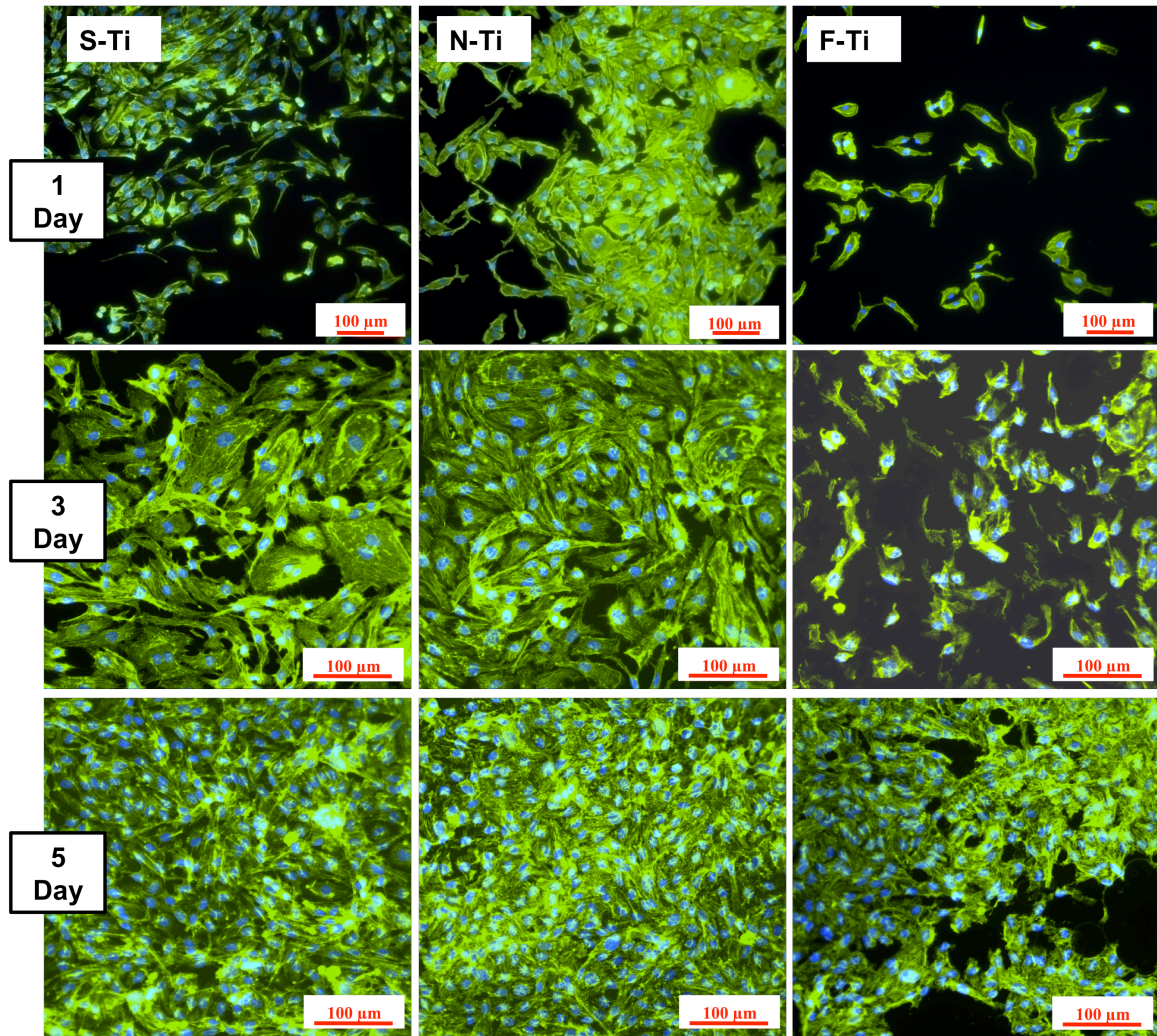


Figure 3.24 Fluorescence cell images showing RAEC morphology on submicron (S-Ti), nanometer (N-Ti) and flat (F-Ti) titanium surface features after 1, 3 and 5 days of culture. All scale bars are 100 μm.

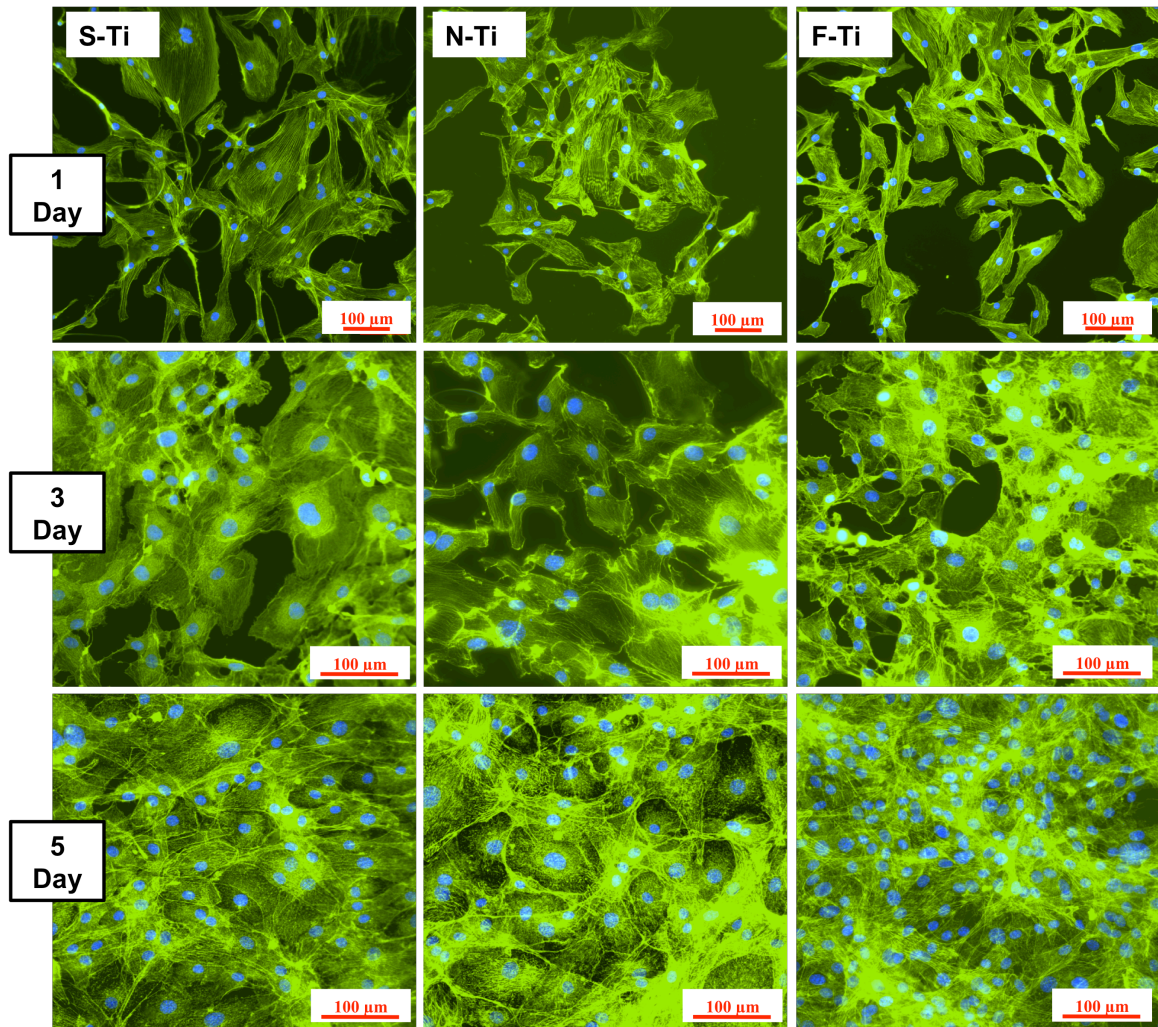


Figure 3.25 Fluorescence cell images showing RASMC morphology on submicron (S-Ti), nanometer (N-Ti) and flat (F-Ti) titanium surface features after 1, 3 and 5 days of culture. All scale bars are 100 μm.

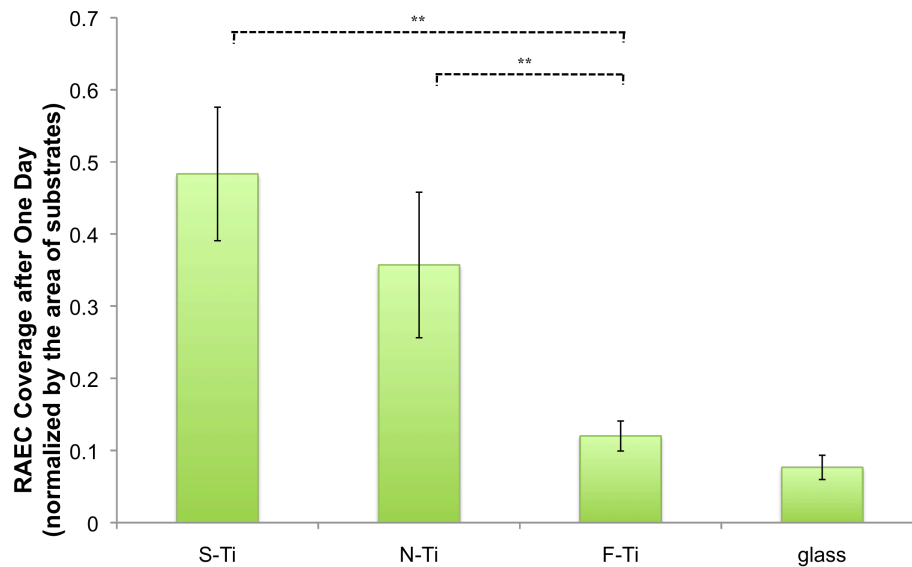


Figure 3.26 RAEC coverage on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) surfaces after one day, showing that the area RAEC covered on S-Ti was more than three times as large as on F-Ti. The spreading area was normalized by the area of the substrates. All error bars are mean $\pm$ SEM; N=3; ** $p < 0.05$.

3.2.3.5. Co-culture of HAEC and RSMC Adhesion on Titanium Substrates with Different Roughness Created by E-beam Deposition

During competitive co-culture experiments, after four hours, 37% more RAEC adhered on sub-micron surface featured titanium (S-Ti) than RASMC (Figure 3.27). However, on N-Ti, the densities of the two cells were around the same after four hours. In contrast, on control flat titanium surfaces (F-Ti), the adhesion of RASMC was greater than RAEC (22% more RASMC than RAEC) after four hours. Coupled with prior results, these results suggest that S-Ti might be a more favorable environment for

RAEC than RASMC compared with the other substrates of interest to this study.

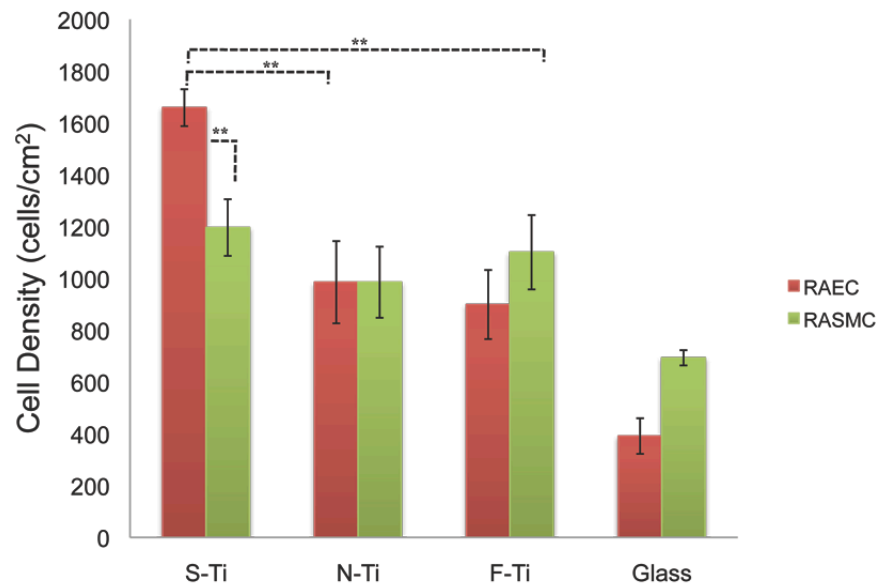


Figure 3.27 Competitive RAEC and RASMC co-culture adhesion on submicron (S-Ti), nanometer (N-Ti), flat (F-Ti) titanium surface features and borosilicate glass (Glass) after 4 hours. The cell seeding densities were 4,500 cells/cm². All error bars are mean $\pm$ SEM; N=9; ** p <0.05.

3.2.3.6. Co-culture Cell Proliferation on Titanium Substrates with Different Roughness

The 5-day proliferation co-culture assays further confirmed this finding (Figure 3.28). Specifically, compared with RASMC densities on S-Ti, RAEC densities increased by 45%, 75% and 134% on S-Ti, after 1, 3 and 5 days, respectively. This indicated that not only did more RAEC adhere on S-Ti at the beginning of the experiments, but also the RAEC

proliferation rate was also greater than RASMC. RAEC density was less competitive on N-Ti than on S-Ti, but was still much greater on these substrates than F-Ti as about 70% more RAEC were observed than RASMC after 5 days on N-Ti. In contrast, on F-Ti, the RAEC densities were 24% and 18% less than RASMC after 1 and 3 days. All of these results suggest that S-Ti or N-Ti might accelerate endothelialization while inhibiting smooth muscle cell proliferation necessary for improving vascular stent efficacy.

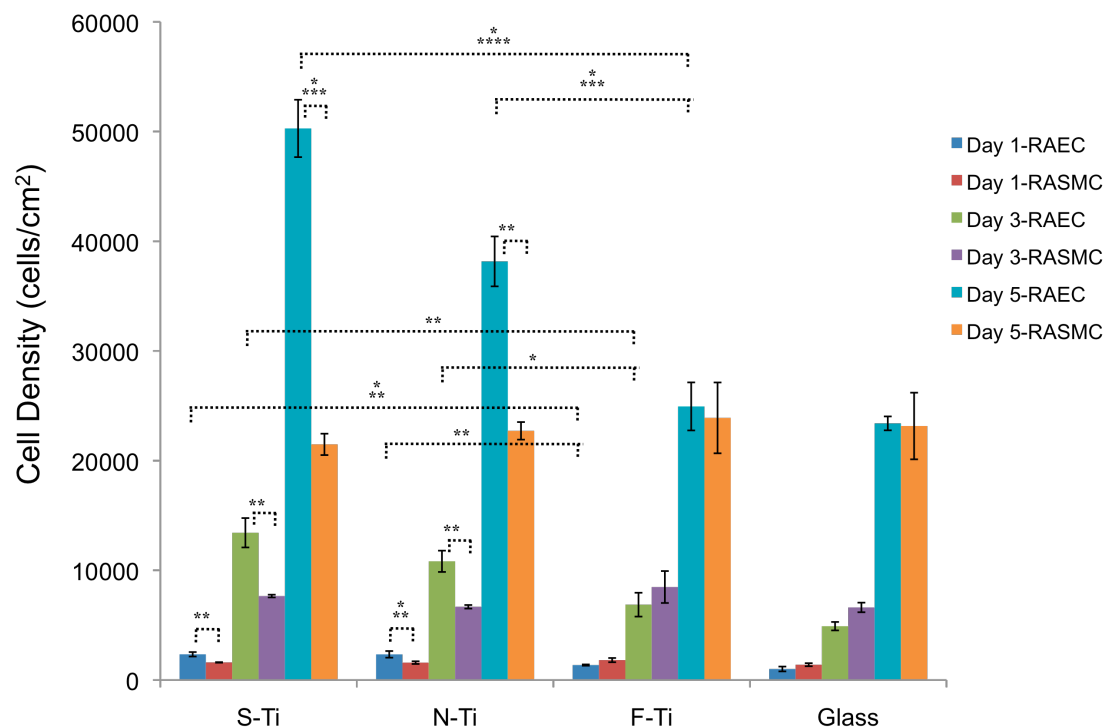


Figure 3.28 Competitive RAEC and RASMC co-culture proliferation on submicron (S-Ti), nanometer (N-Ti), flat (F-Ti) titanium surface features and borosilicate glass (Glass) after 1, 3 and 5 days of culture. The cell seeding densities were 2,000 cells/cm². All error bars are mean ± SEM; n=9; *p<0.1, **p<0.05, ***p<0.01, ****p<0.005 and *****p<0.001.

3.2.3.7. Protein Adsorption (Vitronectin, Fibronectin and Fibrinogen) on Titanium Substrates with Different Roughness Created by E-beam Deposition

Cells attach to material surfaces via pre-adsorbed proteins. In other words, the specific kinds of proteins, their quantity and confirmation, will determine cell responses. Thus, three main proteins involved in endothelial cell adhesion and migration (vitronectin, fibronectin, and fibrinogen) were studied here.

Figure 3.29 showed that both S-Ti and N-Ti had higher vitronectin adsorption after 4 hours than F-Ti (with 10% more adsorption on S-Ti compared to F-Ti). S-Ti and N-Ti also exhibited significantly increased fibronectin adsorption compared to F-Ti after 4 hours, about 60% more adsorption on N-Ti compared to F-Ti (Figure 3.30). Fibronectin adsorption on N-Ti was significantly higher than on S-Ti. Finally, the S-Ti substrate demonstrated much improved fibrinogen adsorption by 15% and 21%, respectively, compared to N-Ti and F-Ti after 4 hours (Figure 3.31).

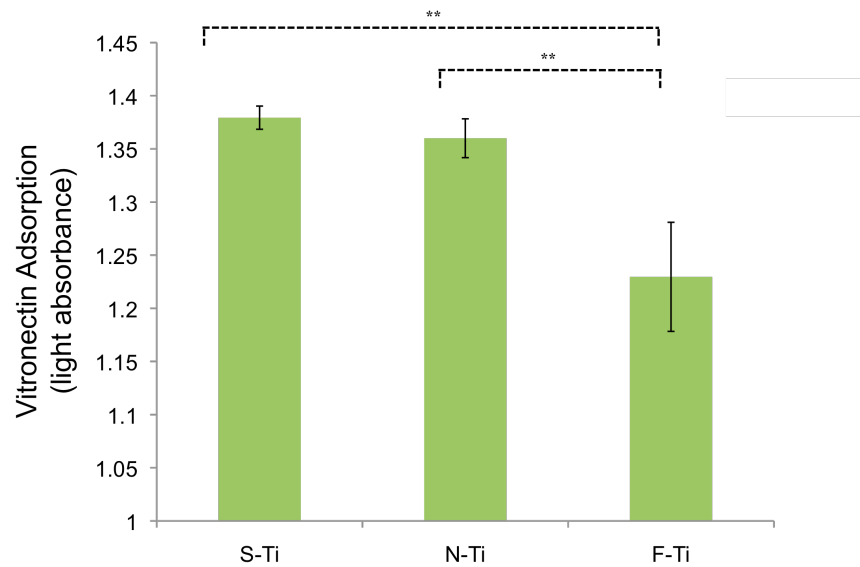


Figure 3.29 Vitronectin adsorption on S-Ti, N-Ti, and F-Ti substrates after 4 hours of exposure. Data=mean $\pm$ SEM, N=3. $**p<0.01$. Y axis is optical density readings from a spectrophotometer which are related to protein adsorption amount.

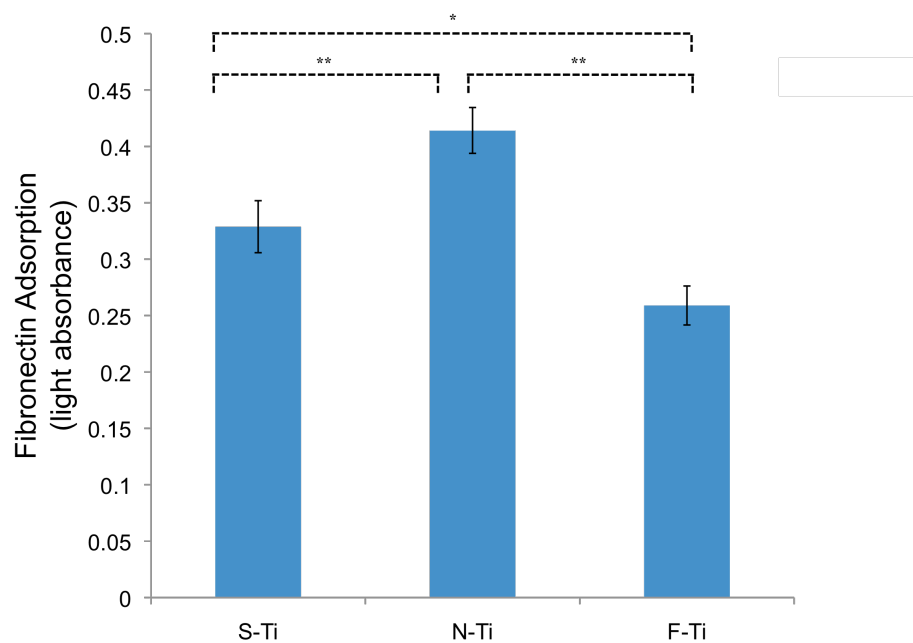


Figure 3.30 Fibronectin adsorption on S-Ti, N-Ti, and F-Ti substrates after 4 hours of exposure. Data=mean $\pm$ SEM, N=3. $*p<0.05$ and $**p<0.01$.

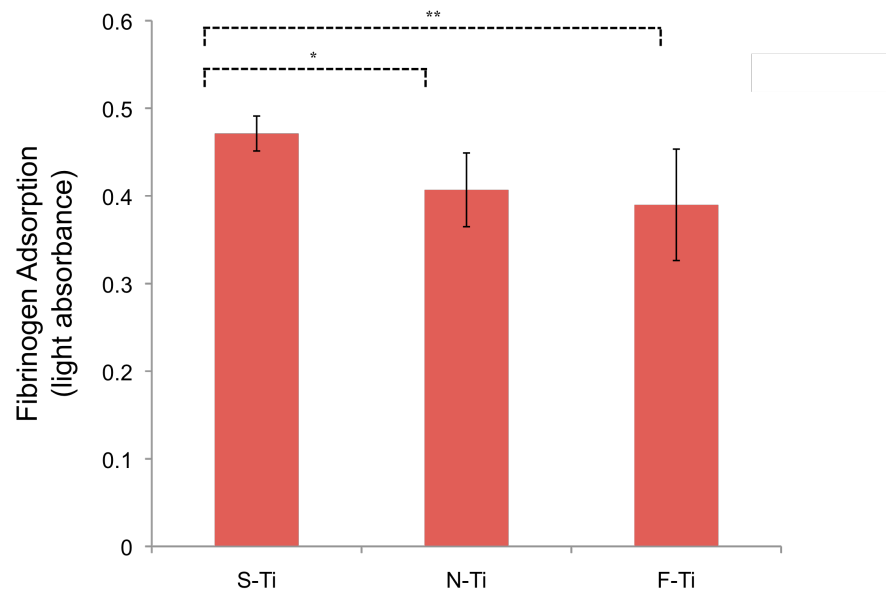


Figure 3.31 Fibrinogen adsorption on S-Ti, N-Ti, and F-Ti substrates after 4 hours of exposure. Data=mean+/-SEM, N=3. * $p<0.05$ and ** $p<0.01$.

3.2.3.8. RAEC NOx and Endothelin-1 Functional Assays on Titanium

Substrates with Different Roughness Created by E-beam Deposition

3.2.3.8.1. NOx assay

In healthy endothelium, nitric oxide (NOx) is continuously secreted by endothelial cells to maintain vascular homeostasis. NOx is a vasodilator and it can also inhibit platelet aggregation. Therefore, NOx synthesis by endothelial cells is important for the proper functioning of vascular stent. The amount of NOx synthesis by endothelial cells cultured on different titanium surfaces was investigated and results reported in Figure 3.32. Sub-micron rough titanium surfaces led to the highest level of NOx synthesis by RAEC, which increased by 70% compared to that on flat

titanium surfaces (F-Ti). There was no significant difference of NOx secretion between nanorough (N-Ti) and flat (F-Ti) titanium surfaces.

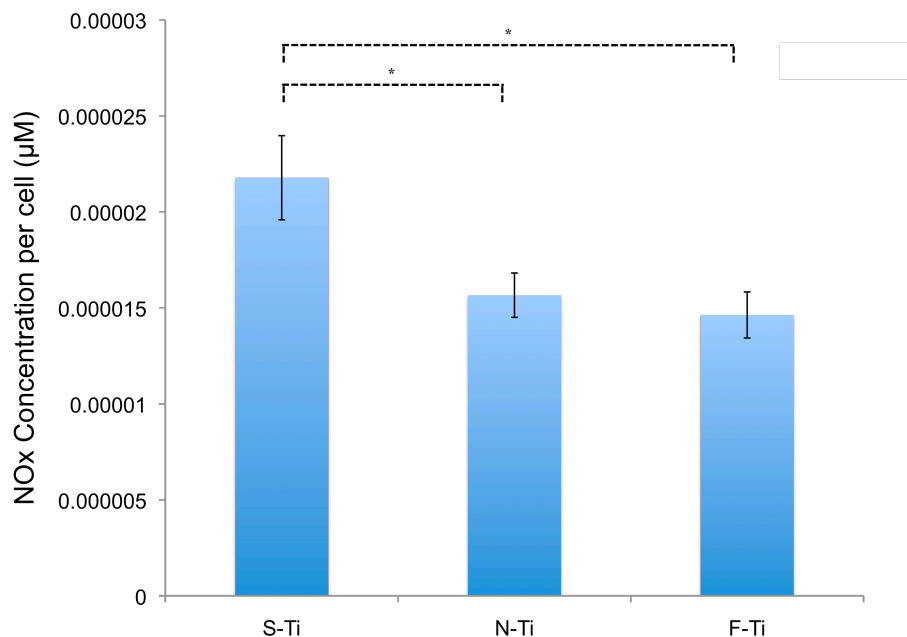


Figure 3.32 NOx concentration synthesized by RAEC on sub-micron rough (S-Ti), nanorough (N-Ti), flat (F-Ti) titanium substrates
Data=mean $\pm$ SEM, N=3. * p <0.05.

3.2.3.8.2. Endothelin-1 Assay

However, endothelin-1, which is also synthesized and secreted by endothelial cells, is a vasoconstrictor and it promotes platelet aggregation.

The amounts of endothelin-1 secreted by endothelial cells on different titanium surfaces were investigated and results reported in Figure 3.33.

There was no significant difference of endothelin-1 levels by endothelial cells on any of the titanium surfaces.

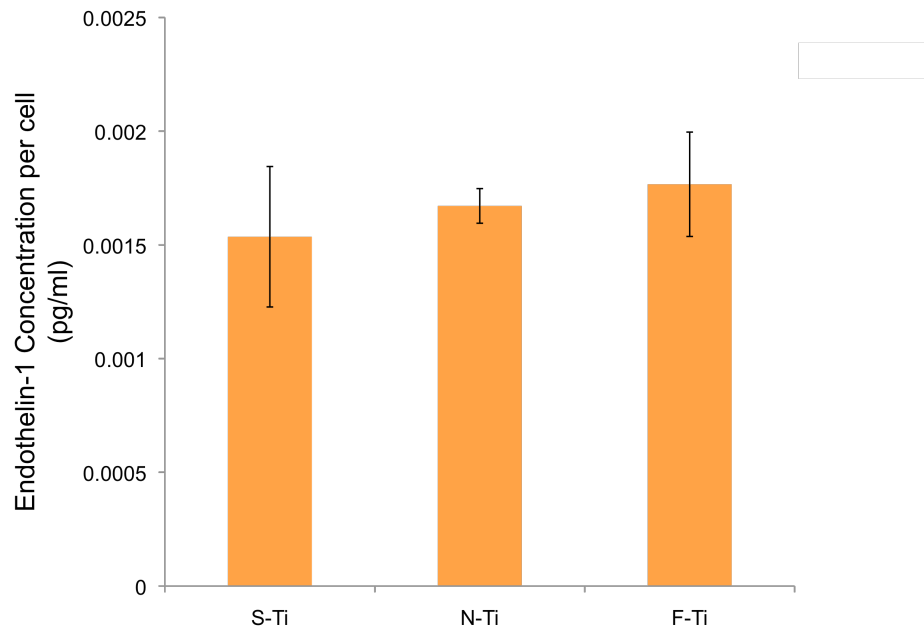


Figure 3.33 Endothelin-1 concentrations synthesized by RAEC on sub-micron rough (S-Ti), nanorough (N-Ti) and flat (F-Ti) titanium substrates
Data=mean $\pm$ SEM, N=3.

3.2.3.9. Long-term Cell Function Assays of RAEC and RASMC on Titanium Substrates with Different Roughness Created by E-beam Deposition

Collagen and elastin are main components of the vascular extracellular matrix and were used to determine endothelial and smooth muscle cell functions after longer time periods.

Both sub-micron rough (S-Ti) and nanorough (N-Ti) substrates showed increased collagen synthesized by endothelial cells compared to flat surfaces (F-Ti) after 1 week (Figure 3.34) but there was no significant

difference between them. At the end of the second week, S-Ti showed the highest amount of collagen (more than twice as much as F-Ti) while N-Ti was the second highest (67% more than F-Ti).

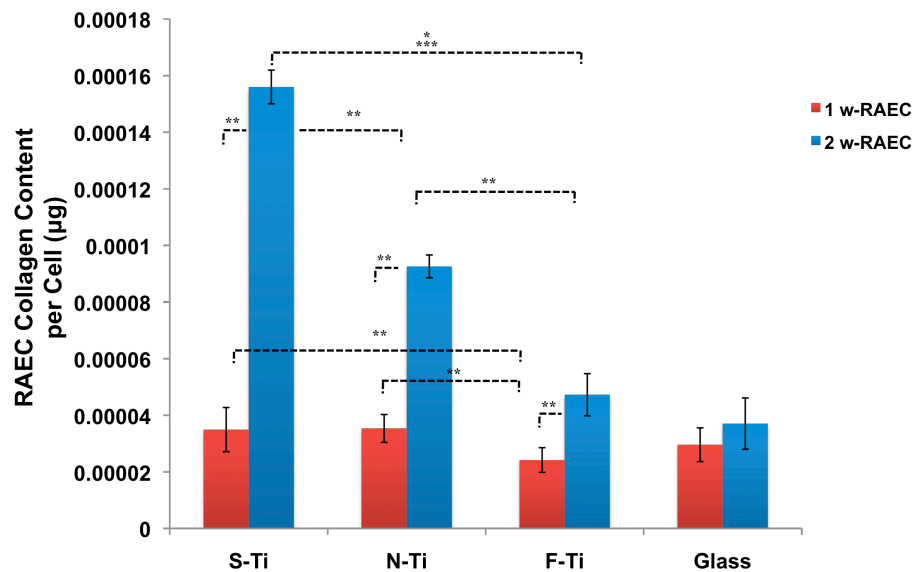


Figure 3.34 Collagen content secreted by endothelial cells after 1 and 2 weeks of culture on titanium substrates with different roughness. Results were normalized with cell numbers. Data=mean $\pm$ SEM, N=3. For each substrate, collagen content at second week was significantly higher than the first week. ** $p<0.01$ and *** $p<0.001$.

Elastin secreted by endothelial cells on various titanium substrates exhibited the same trend as collagen. Specifically, at 1 week, both S-Ti and N-Ti substrates showed increased elastin synthesized by endothelial cells by around 30% compared to F-Ti but there was no significant difference between them. At week 2, S-Ti outperformed N-Ti in elastin content which were all significantly higher than F-Ti (Figure 3.35).

Specifically, S-Ti increased elastin content by 39% and N-Ti increased by 13% both compared to F-Ti.

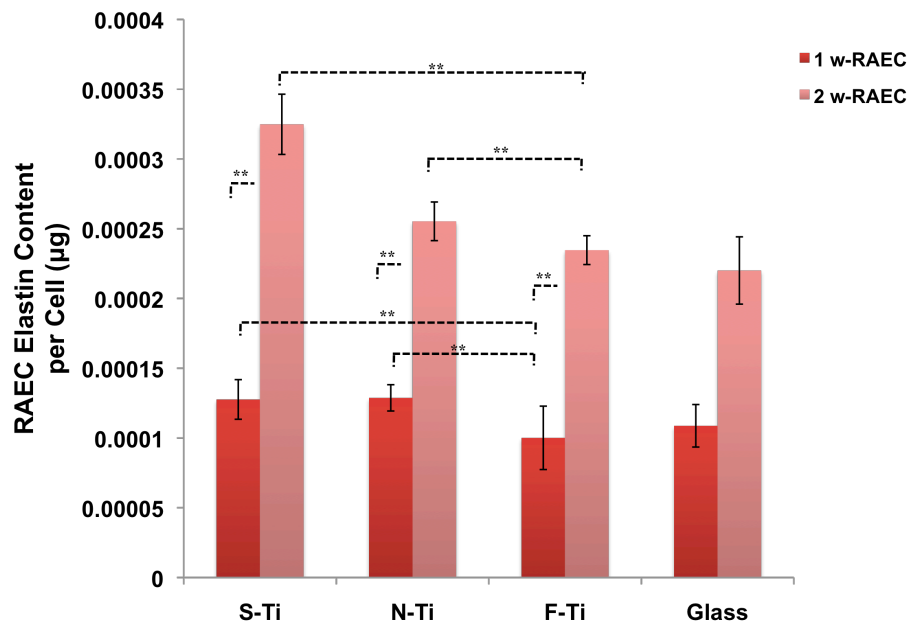


Figure 3.35 Elastin content secreted by endothelial cells after 1 and 2 weeks of culture on titanium substrates with different roughness. Results were normalized with cell numbers. Data=mean $\pm$ SEM, N=3. For each substrate, elastin content at second week was significantly higher than the first week. $**p<0.01$.

Smooth muscle cells showed different behavior from endothelial cells in terms of collagen and elastin production (Figure 3.36 and 3.37). Specifically, S-Ti, N-Ti, and F-Ti promoted similar collagen synthesized by smooth muscle cells after both 1 and 2 weeks (Figure 3.36). N-Ti substrates showed similar elastin content after 1 and 2 weeks while S-Ti substrates showed enhanced elastin content compared to F-Ti by 17% after 1 week and 67% after 2 weeks (Figure 3.37). These results indicated

that smooth muscle cells are not as sensitive to underlying titanium substrate topography as endothelial cells.

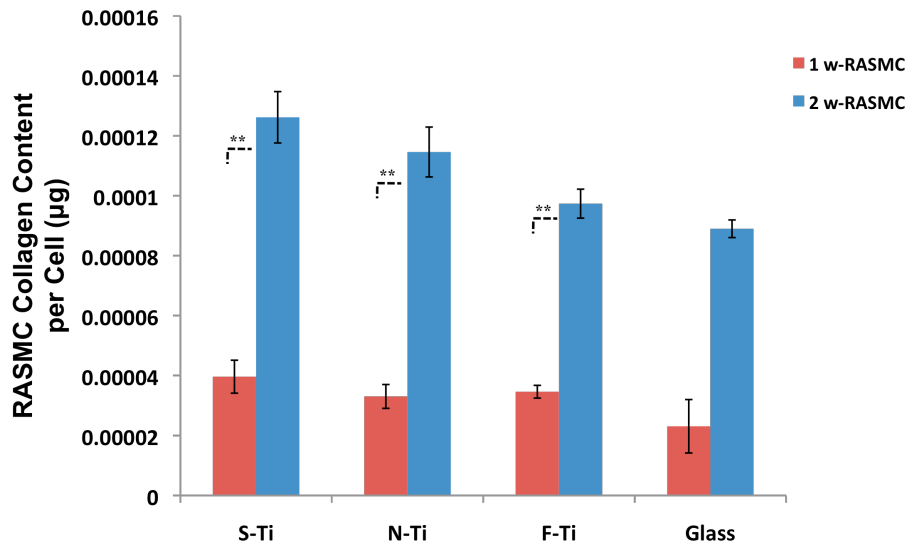


Figure 3.36 Collagen content secreted by smooth muscle cells after 1 and 2 weeks of culture on titanium substrates with different roughness. Results were normalized with cell numbers. Data=mean $\pm$ SEM, N=3. $**p<0.01$.

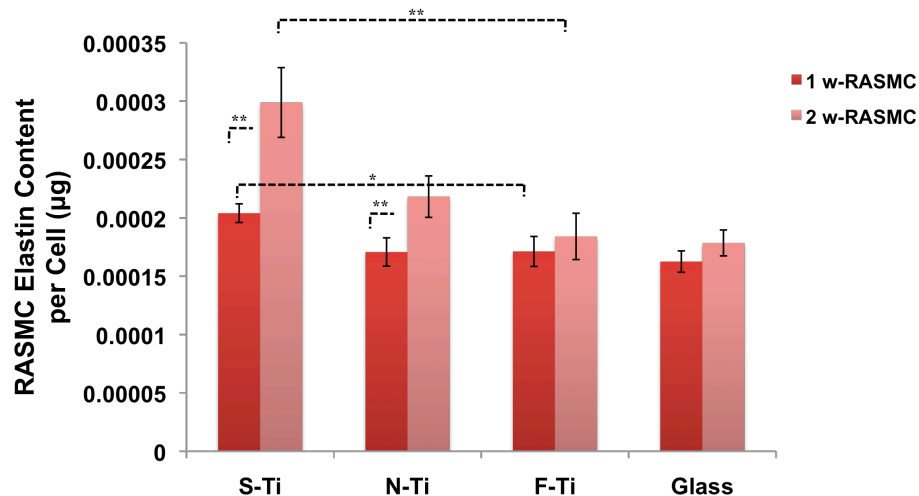


Figure 3.37 Elastin content secreted by smooth muscle cells after 1 and 2 weeks of culture on titanium substrates with different roughness. Results were normalized with cell numbers. Data=mean $\pm$ SEM, N=3. For each substrate, elastin content at second week was significantly higher than the first week. * $p<0.05$ and ** $p<0.01$.

3.2.3.10. RAEC Mobility Assay on Titanium Substrates with Different Roughness

It was hypothesized that the underlying titanium substrate roughness will influence the shape of adherent cells and in turn influence cell migration, which is a critical process for endothelialization. To support this hypothesis, a time-dependent study of endothelial migration was conducted (Figure 3.38). Result showed a clear faster cell movement on S-Ti (about 500 μ m after 96 hours) and N-Ti (about 400 μ m after 96 hours) compared to F-Ti (about 300 μ m after 96 hours; Figure 3.39). Moreover, endothelial cells migrated more directionally on S-Ti than on the other two. This is useful in

vascular repair considering that cells are able to extrude lamellipodia, spread, elongate, and migrate over the stent surface to cover the stent.

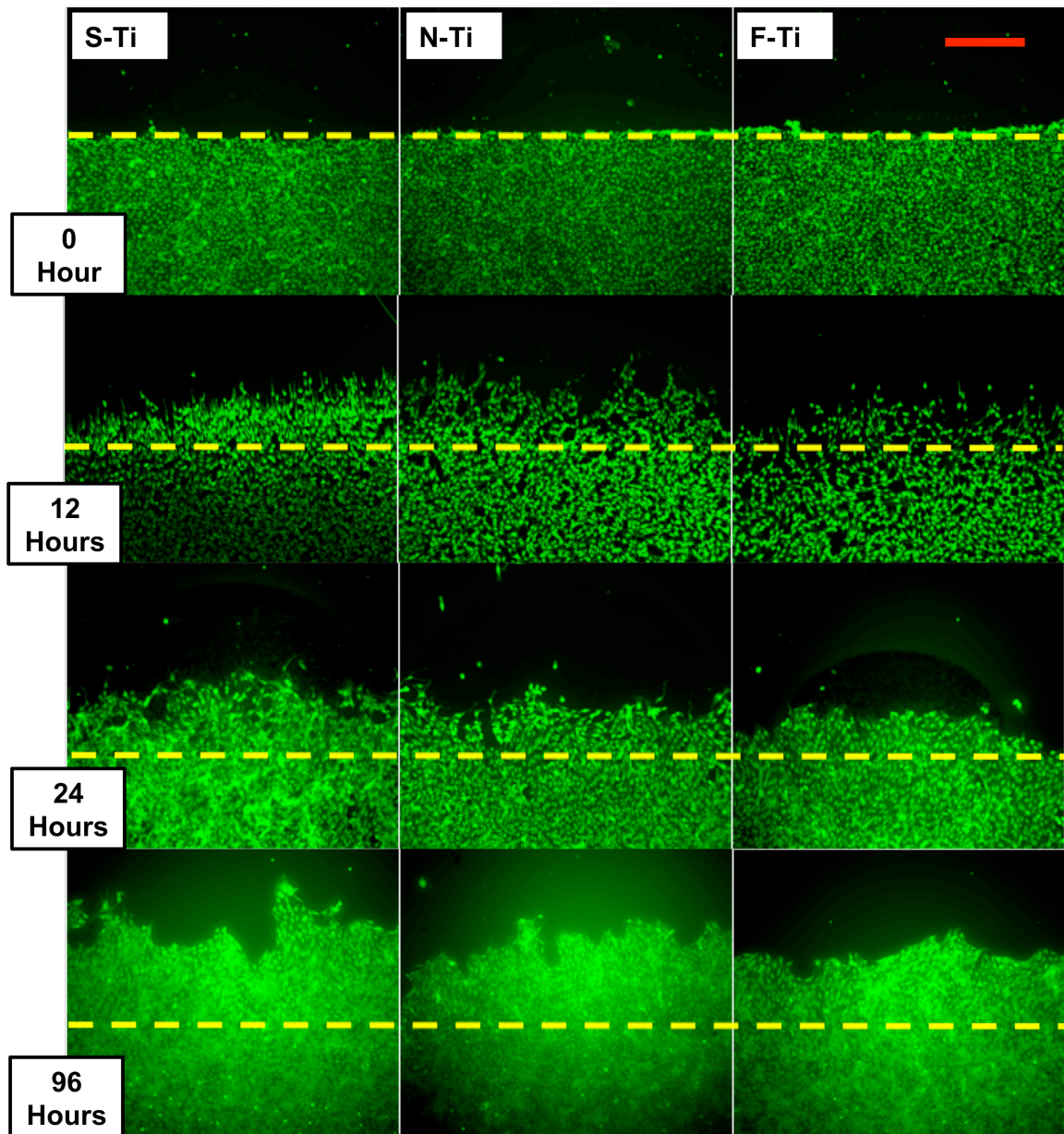


Figure 3.38 RAEC migration on S-Ti, N-Ti, and F-Ti substrates over 12, 24 and 96 hours. Cells were stained with Calcein AM. Scale bars = 200 μm . Dash lines indicate starting points at time zero.

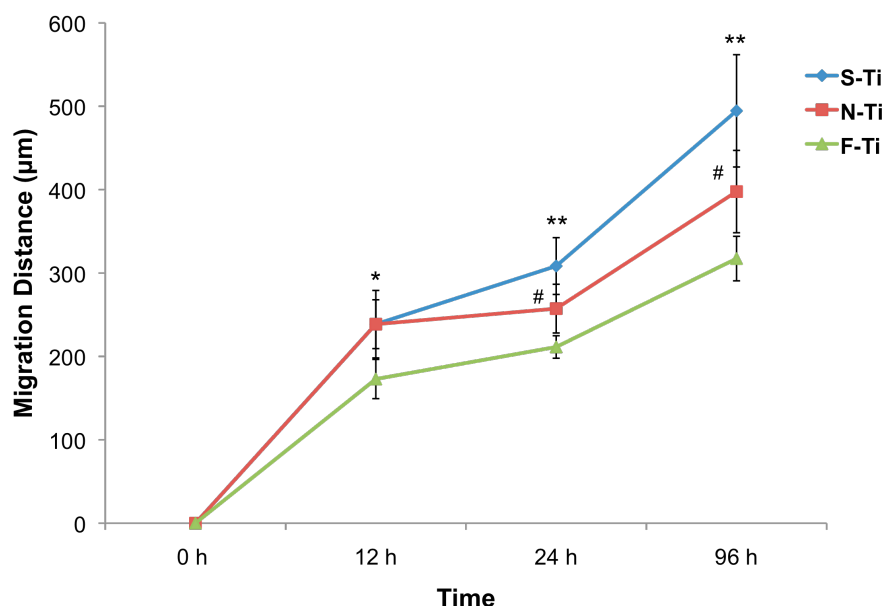


Figure 3.39 RAEC migration on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) titanium surfaces after 12, 24 and 96 hours, showing that RAEC motility greatly increased on S-Ti compared with that on N-Ti and F-T. All error bars are mean $\pm$ SEM; N=3; * $p < 0.05$ (compared to F-Ti after 12 hours), ** $p < 0.05$ (compared to N-Ti and F-Ti after 24 and 96 hours), # $p < 0.05$ (compared to F-Ti after 12, 24 and 96 hours).

3.2.3.10.1. Immunofluorescence of Vinculin of RAEC on Titanium

Substrates with Different Roughness Created by E-beam Deposition

Vinculin, a focal adhesion protein, plays an important role in binding cell surface integrin receptors to extracellular matrix adhesion molecules and controlling cell migration. Therefore, to further study the effects of titanium surface roughness on endothelial cell migration, the endothelial cell focal adhesion protein vinculin was investigated on different titanium roughness here. The fluorescence images of vinculin in Figure 3.34 showed that on sub-micron rough titanium surfaces (S-Ti, Figure 3.34a), the focal

adhesion protein formed small clusters on the binding sites on the end of the cytoskeleton. On the other hand, on flat titanium surfaces (F-Ti, Figure 3.34c), large adhesions were observed. The morphology of focal contacts on nanorough titanium surfaces (N-Ti, Figure 3.34b) was in between that on S-Ti and F-Ti, where in some places they were well distributed while in the other places small clusters were found.

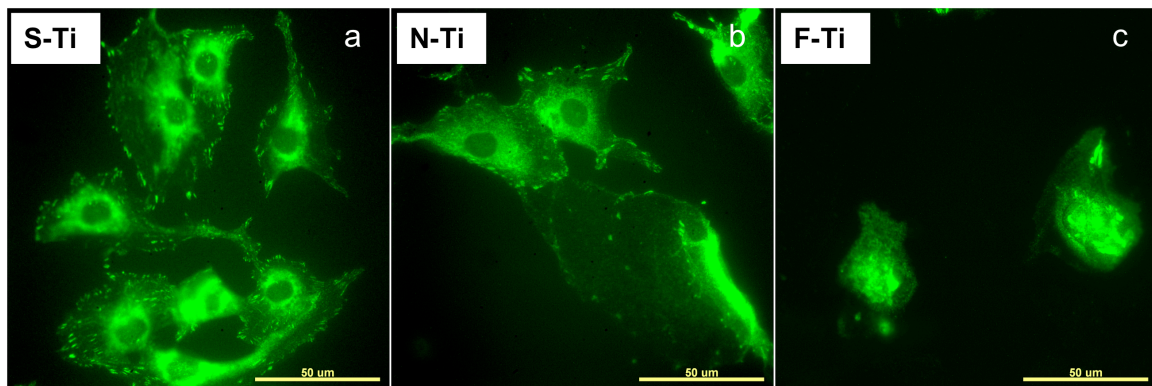


Figure 3.40 Immunofluorescent images of vinculin, which indicated focal adhesion of RAEC, on titanium surfaces of different roughness. (a) Sub-micron rough titanium (S-Ti); (b) Nanorough titanium (N-Ti) and (c) Flat titanium (F-Ti). All yellow scale bars = 100 μm

3.2.4. Immune Cell Responses

3.2.4.1. Macrophage Density Assays Titanium Substrates with Different Roughness

Results showed that nanorough titanium surfaces (N-Ti) had the least macrophage attachment after both 12 and 24 hours (Figure 3.41). In comparison, sub-micron rough titanium surfaces (S-Ti) had about 25%

and 50% more macrophages attached after 12 and 24 hours, respectively. Flat surfaces (F-Ti) showed the most macrophage attachment after 12 and 24 hours, about 40% and 75% more than N-Ti.

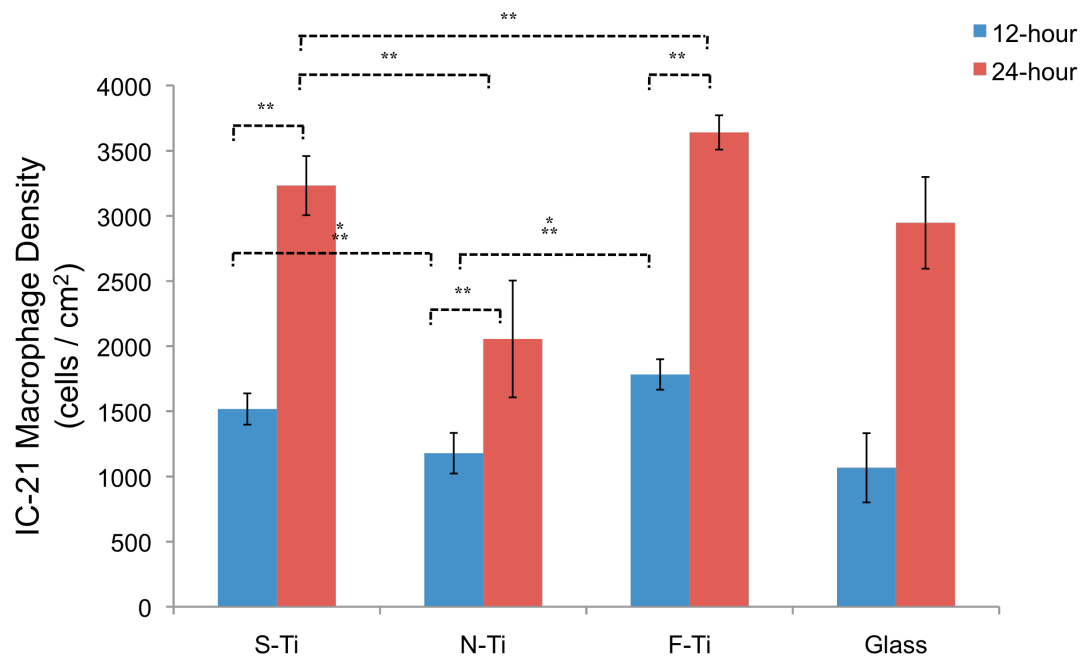


Figure 3.41 IC-21 macrophage attachment on various e-beam titanium coatings after 12 and 24 hours without LPS. Data=mean+/-SEM, N=3. * $p < 0.05$ and ** $p < 0.01$.

When lipopolysaccharide (LPS) was added to activate macrophage function, it was observed that each substrate showed more macrophage attachment compared to without LPS after 12 hours. N-Ti still exhibited least macrophage adhesion after 12 hours. S-Ti had about 30% more and F-Ti had about 50% more, which is consistent with prior results without adding LPS. After 24 hours, this trend remained with N-Ti the least and F-Ti the most. However, it was noticed that the number of adhered

macrophages on each substrate was less than that when LPS was not added after 24 hours (Figure 3.42).

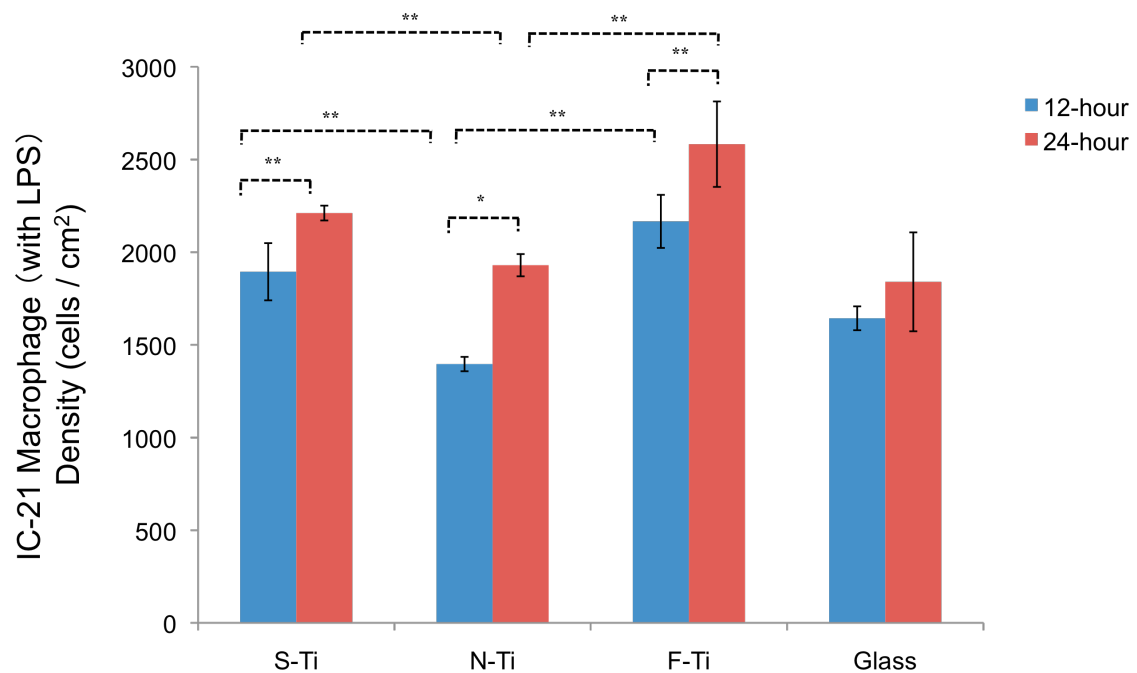


Figure 3.42 IC-21 macrophage attachment on various e-beam titanium coatings after 12 and 24 hours. Data=MEAN $\pm$ SEM, N=3. * $p < 0.05$ and ** $p < 0.01$. 200ng/ml LPS was added in the culture medium to activate macrophage function.

Figure 3.43 shows representative macrophage morphologies attached onto various coatings after 12 hours without LPS. It can be seen that all macrophages attached were well spread. Figure 3.44 shows representative macrophage morphologies to the various coatings after 12 hours with LPS. The quantitative analysis showed that F-Ti not only had the highest amount of macrophages but also had more-spread

macrophage morphologies (compared to S-Ti), while N-Ti had similar cell areas to F-Ti.

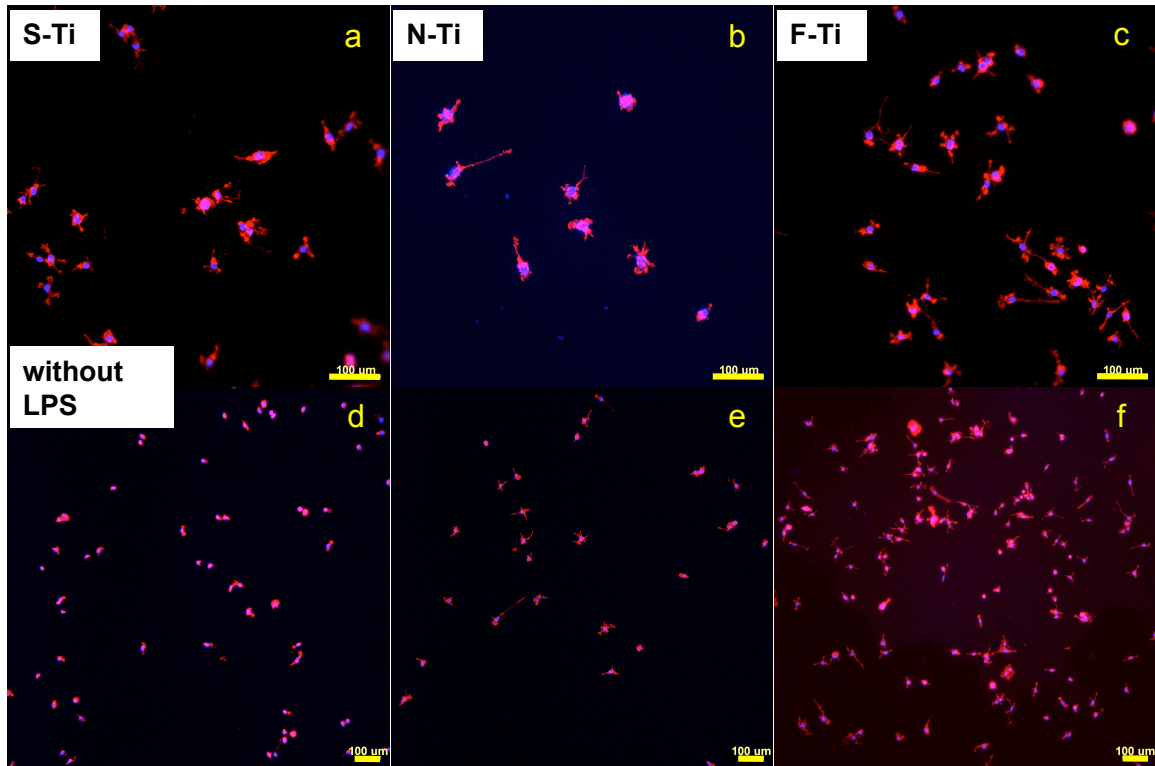


Figure 3.43 Fluorescence images showing macrophages adherent onto (a, c) Sub-micron rough (S-Ti), (b, e) Nanorough (N-Ti), and (c, f) Flat (F-Ti) titanium surfaces after 12 hours. First line is high magnification (x20) and second line is respective low magnification image (x10). Cells were stained with DAPI (blue) and rhodamine-phalloidin (red). Scale bars = 100 μm .

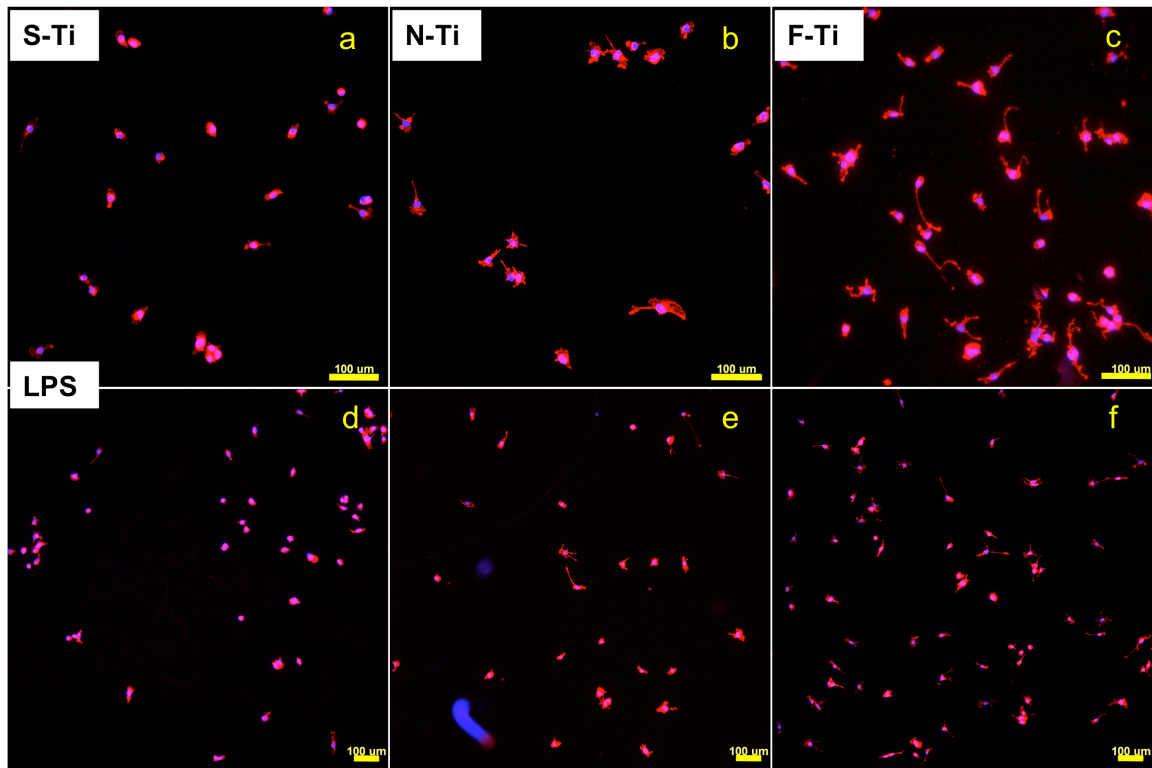


Figure 3.44 Fluorescence images showing macrophages adherent onto (a, d) Sub-micron rough (S-Ti), (b, e) Nanorough (N-Ti), and (c, f) Flat (F-Ti) after 12 hours with LPS activation. First line is high magnification (x20) and second line is respective low magnification image (x10). Cells were stained with DAPI (blue) and rhodamine-phalloidin (red). Scale bars = 100 μm .

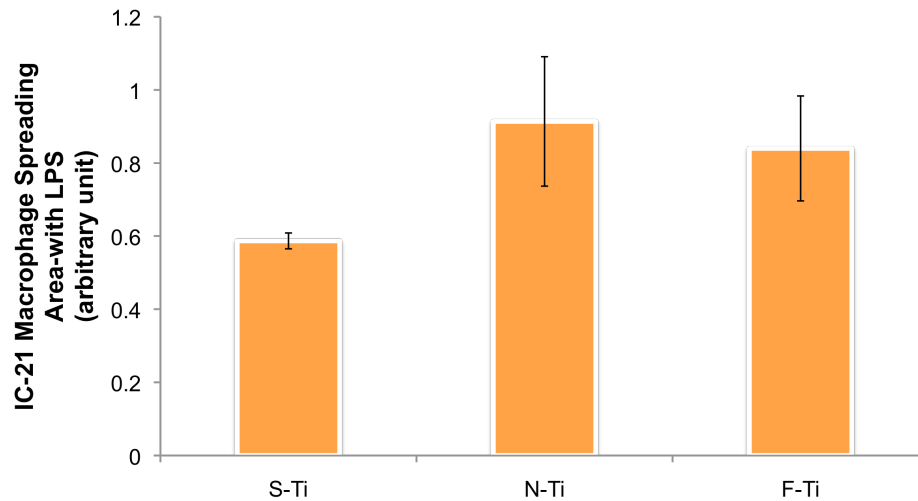


Figure 3.45 The single cell area of IC-21 macrophages on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) surfaces under flow after 12 hours, showing that the cell area on S-Ti was the smallest of all the substrates. All error bars are mean $\pm$ SEM; N=3; $**p<0.05$.

3.2.4.2. Monocyte Adhesion

In this study, monocyte responses to various titanium coatings was also determined considering that macrophages are differentiated monocytes. In human body, monocytes exist in blood and are recruited onto the endothelium during injury. Thus, in this study, both monocyte adhesion onto bare titanium coatings and onto endothelial-covered titanium coatings were performed. It was hypothesized that the underlying substrate would influence monocyte adhesion by changing the state of the intermediate endothelial cell layers.

3.2.4.3. Monocyte Adhesion on Titanium Substrates with Different Roughness Created by E-beam Deposition

The number of monocytes attached onto each substrate was determined by a MTS assay (Figure 3.46). TPA was added to activate monocyte function. It is shown that after 4 hours, N-Ti had the least number of monocytes. In comparison, S-Ti had 40% more and F-Ti had 70% more compared to N-Ti.

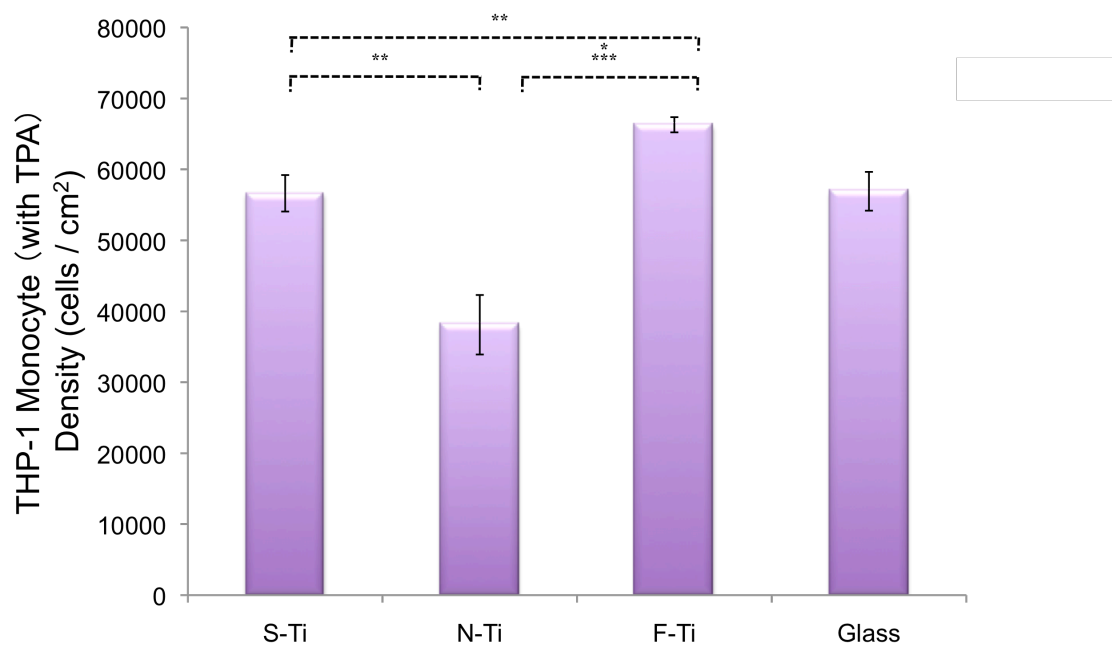


Figure 3.46 THP-1 monocyte adhesion on S-Ti, N-Ti, and F-Ti after 4 hours with TPA activation. Seeding density was 100,000 cells/cm². TPA was added at a concentration of 1 µg/ml. Data=mean±SEM, N=3.

**** $p < 0.01$ and **** $p < 0.001$.**

3.2.4.4. Monocyte Adhesion on RAEC Monolayers on Titanium Substrates with Different Roughness Created by E-beam Deposition

When titanium substrates were coated with an endothelial cell monolayer, monocytes demonstrated a different adhesion trend than that just described. Endothelial cells coated S-Ti had the least amount of monocytes, about one third, attached onto the surface. In comparison, endothelial coated N-Ti had around half of the monocytes attached onto the surface while F-Ti had about two thirds of the monocytes attached. These results indicated that monocyte adhesion was significantly influenced by the underlying endothelial monolayer whose functions were in turn influenced by the underlying titanium substrates of different roughness (Figure 3.47). This will be discussed further in the next chapter.

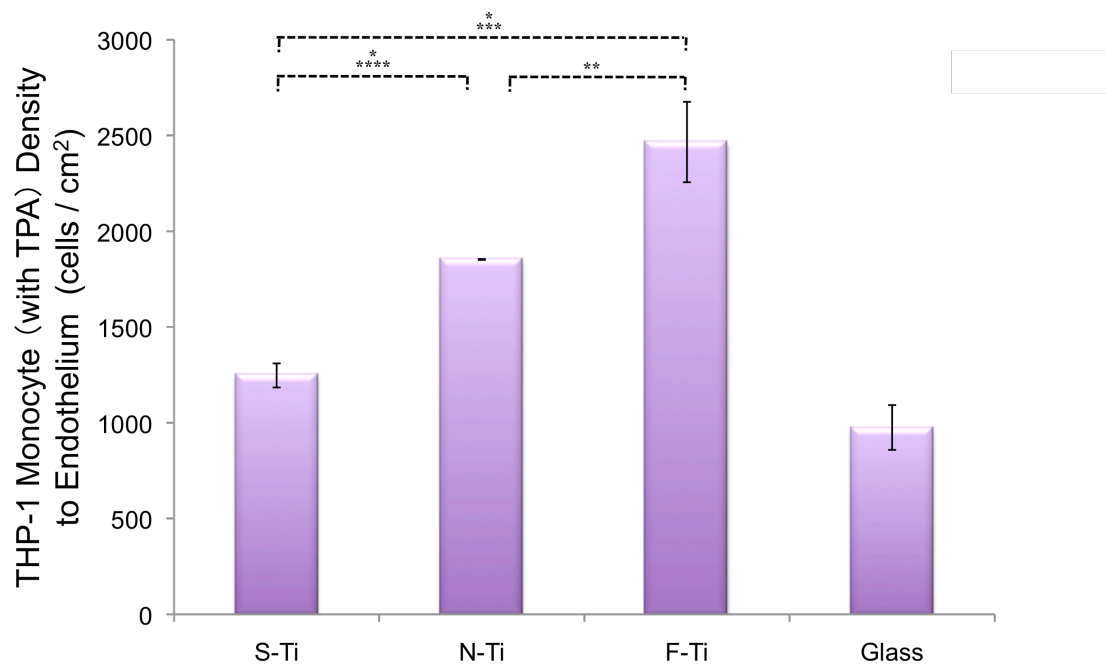


Figure 3.47 THP-1 monocyte adhesion on endothelial monolayers on S-Ti, N-Ti, and F-Ti after 4 hours with TPA activation. Seeding density was 3,500 cells/cm². TPA was added at a concentration of 1µg/ml. Data=mean+/-SEM, N=3. ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0005$.

3.2.4.5. Monocyte Differentiation on Titanium Substrates with Different Roughness Created by E-beam Deposition

Results of this study also showed that monocytes had round shapes when adherent to the substrates (Figure 3.48 a-c). After 5 days, their shape became elongated and more well spread (Figure 3.48 d-f). This is a direct indicator of monocyte differentiation [170]. In this figure, it can also be seen that F-Ti was completely covered by multilayers of monocytes. In comparison, the N-Ti substrates were mostly covered by a layer of monocytes while S-Ti was only partially covered by monocytes.

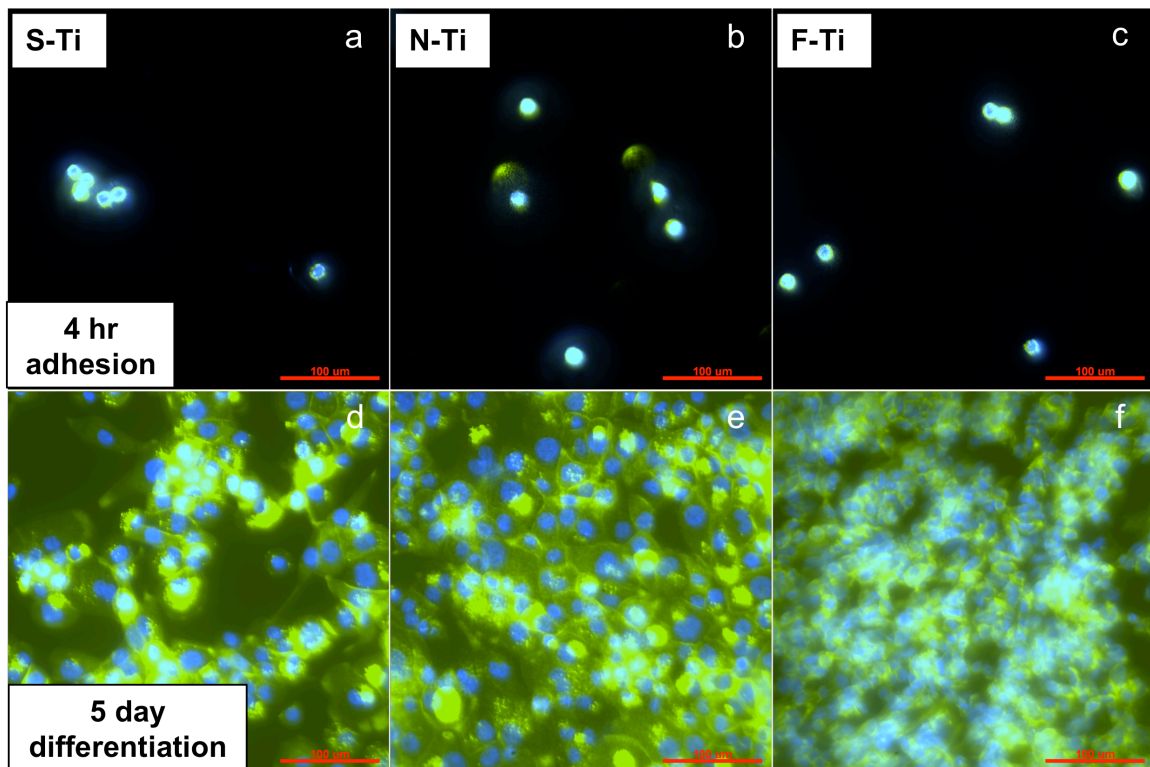


Figure 3.48 Fluorescent images of monocytes on S-Ti, N-Ti, and F-Ti after (a-c) 4 hours and (d-f) 5 days. Cells were stained with DAPI (blue) and rhodamine-phalloidin (green). Scale bars = 100 μm .

3.2.4.6. Cytokine Release from Macrophages on Titanium Substrates with Different Roughness Created by E-beam Deposition

IL-1 β , IL-6, and TNF- α are three main pro-inflammatory cytokines that are released by macrophages and indicate macrophage activity, which regulate the immune response to biomaterials. In this study, the concentrations of these three cytokines were measured after 4, 12, 24, 36 hours.

The amounts of IL-1 β secreted by macrophages were similar on S-Ti and N-Ti substrates over all time points (Figure 3.49). After 4 hours, S-Ti showed significantly lower IL-1 β than F-Ti. Both S-Ti and N-Ti showed significantly lower IL-1 β than F-Ti after 12 hours. N-Ti showed significantly lower IL-1 β than F-Ti after 24 and 36 hours.

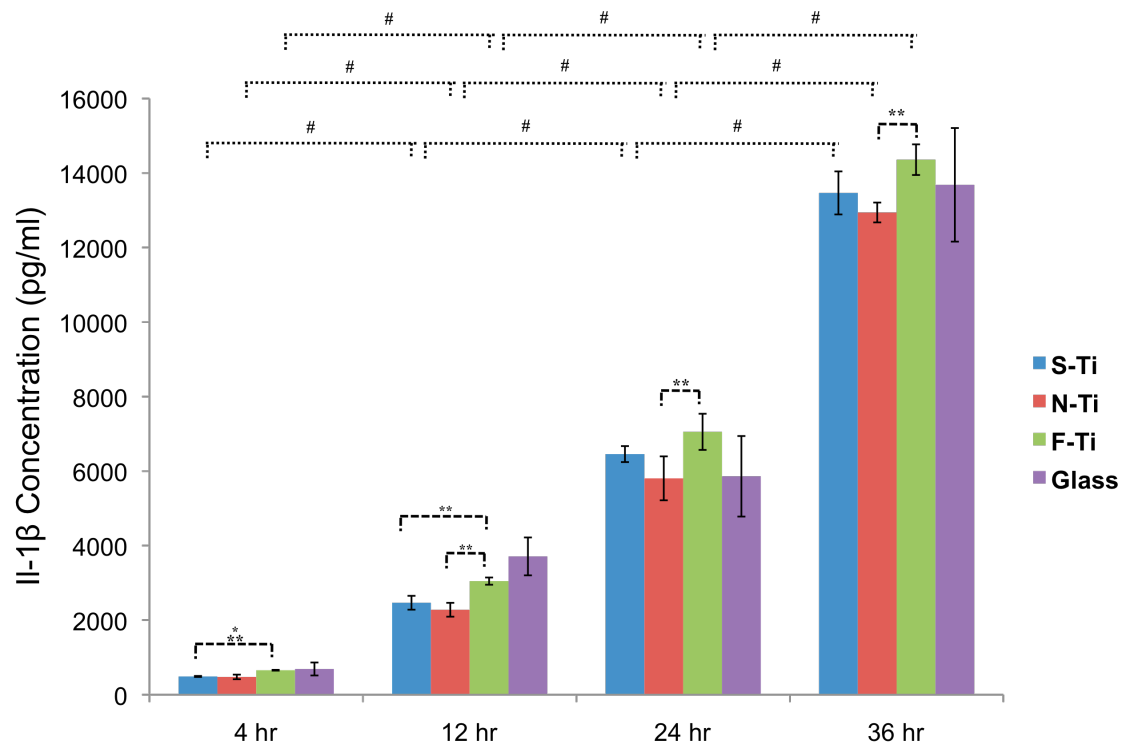


Figure 3.49 Concentration of IL-1 β secreted by macrophages after 4, 12, 24, and 36 hours of culture on titanium substrates with different roughness. Seeding density was 1,000,000 cells/cm². Data=mean $\pm$ SEM, N=3. ** p <0.01, *** p <0.005 and # p <0.05.

In regards to IL-6 release, results showed that S-Ti had significantly lower IL-6 release from macrophages than F-Ti after 4, 12, 24, and 36 hours (Figure 3.50).

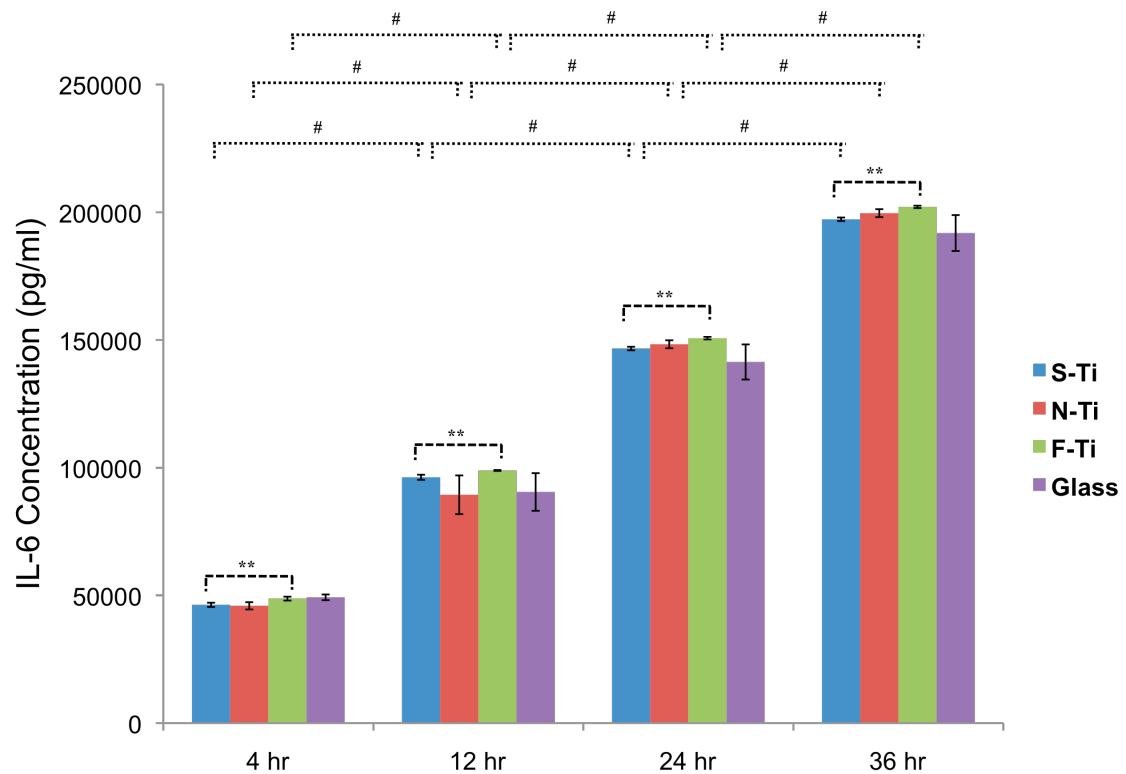


Figure 3.50 Concentration of IL-6 secreted by macrophages after 4, 12, 24, and 36 hours of culture on titanium substrates with different roughness. Seeding density was 1,000,000 cells/cm². Data=mean+/-SEM, N=3. ** $p < 0.01$ and # $p < 0.05$.

For TNF- α , it was observed that both S-Ti and N-Ti had less cytokine release from macrophages than F-Ti after 4, 12, 24, and 36 hours (Figure 3.51). However, there was no statistical difference between S-Ti and N-Ti.

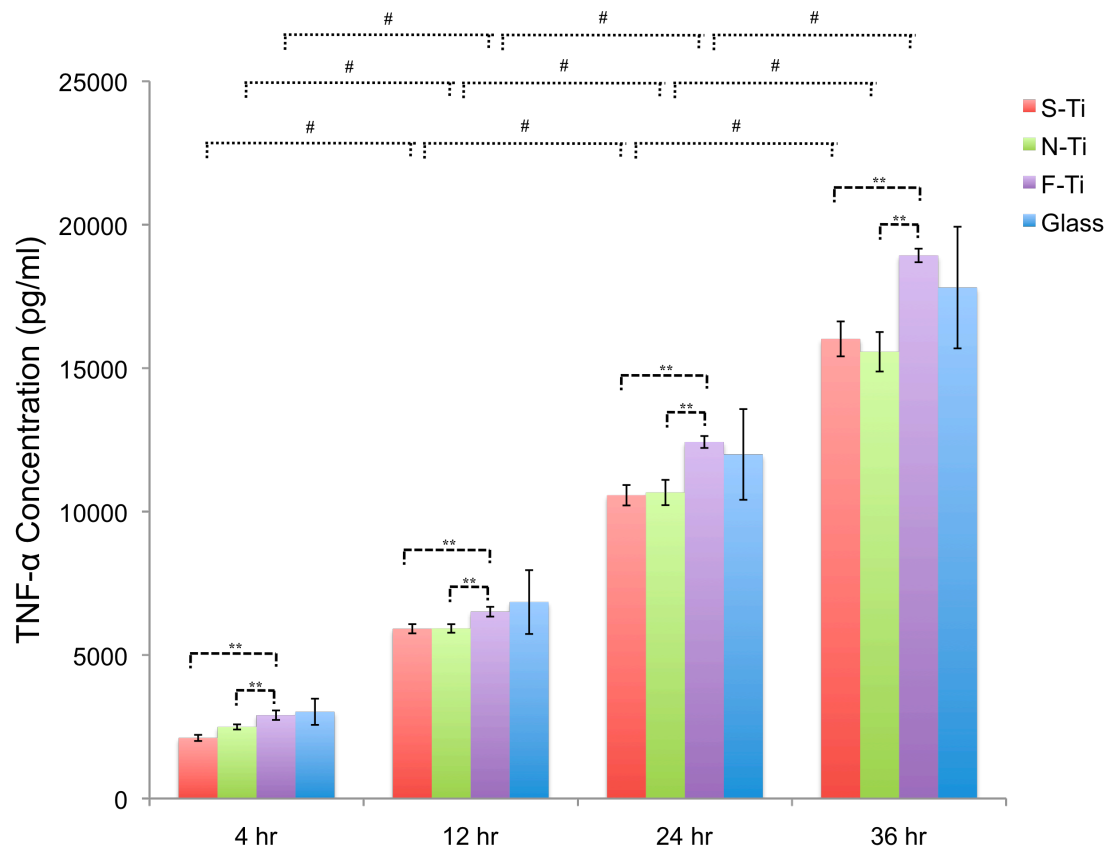


Figure 3.51 Concentration of TNF- α secreted by macrophages after 4, 12, 24, and 36 hours of culture on titanium substrates with different roughness. Seeding density was 1,000,000 cells/cm². Data=mean+/-SEM, N=3. ** $p < 0.01$ and # $p < 0.05$.

3.3. The Vessel Flow Model in a Home-made Flow System

3.3.1. Coating Stability Test

As stated in the above in vitro assays, vascular cell (endothelial and smooth muscle cells) and immune cell (macrophages and monocytes) functions varied depending on titanium coatings of different roughness and surface energy. These studies were conducted under static conditions and provided valuable information on the relationship between cell responses and titanium surface properties. As a further step, a more complex cell culture system involving laminar flow was employed in the current investigation. This model can better simulate the vascular stent environment in the human body and serve as a useful evaluation tool before we move into in vivo animal studies.

The normal shear stress in human vascular vessels is about 15 to 50 dynes/cm². To test the durability of e-beam deposited titanium coatings (which could be applied to current metallic vascular stent surfaces) on glass, a shear stress value a little over the lower threshold, 20 dynes/cm², was chosen assuming normal vessel conditions. The flow chamber was run for 24 hours and then each coating was examined under SEM. The figure below revealed that these e-beam titanium coatings all maintained their original morphology under flow (Figure 3.52).

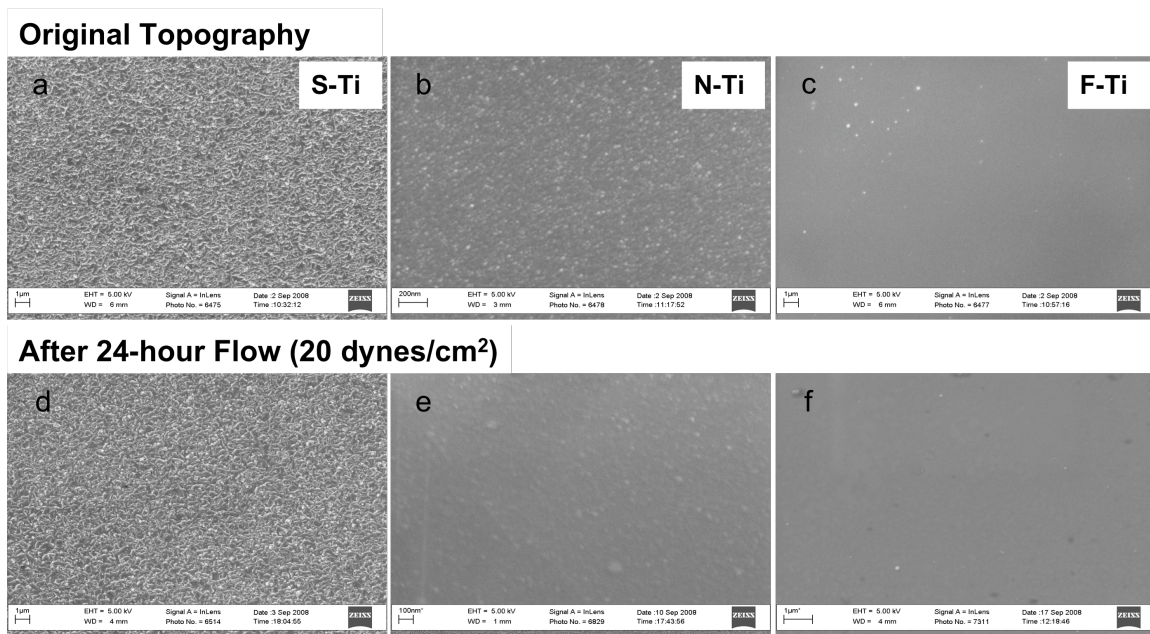


Figure 3.52 SEM images of (a, d) Submicron-rough (S-Ti), (b, e) Nano-rough (N-Ti), and (c, f) Flat titanium (F-Ti) coatings before and after exposure to flow conditions for 24 hours. The shear stress was set at 20 dynes/cm². Scale bars in (a), (c), (d) and (f) are 1 μm; in (b) and (e) are 100 nm.

3.3.2. Dynamic RAEC Adhesion Test Under Laminar Flow

As discussed in the Introduction, when vascular stents are inserted into blood vessels, they can be covered by endothelial cells or by immune cells. The static studies reported indicated that submicron-rough titanium had the greatest affinity with endothelial cells while nano-rough titanium was second. It is very interesting to determine whether or not this is the same under flow conditions. To confirm this, all four substrates (S-Ti, N-Ti, F-Ti, and glass) were first cultured in endothelial cell culture medium for 36 hours at which point they were fully covered with a layer of endothelial cells. Then, each of these substrates was transferred to the custom flow

chamber system and cultured for a day. At the end of that period, each substrate was stained and observed under a fluorescence microscope. Figure 3.53 showed that S-Ti retained the endothelial cell layer. In comparison, N-Ti generally kept the endothelial cell layer intact but some of the cell to cell networks were interrupted after 24 hours of laminar flow. F-Ti was the worst and showed less endothelial cells remaining on the surface after flow. Quantitative analysis (Figure 3.54) of the RAEC layer coverage under flow for 24 hours showed that more than 90% of the S-Ti area and more than 80% of the N-Ti area were still covered by RAEC. In contrast, only 40% of the F-Ti area was covered by RAEC under the same conditions.

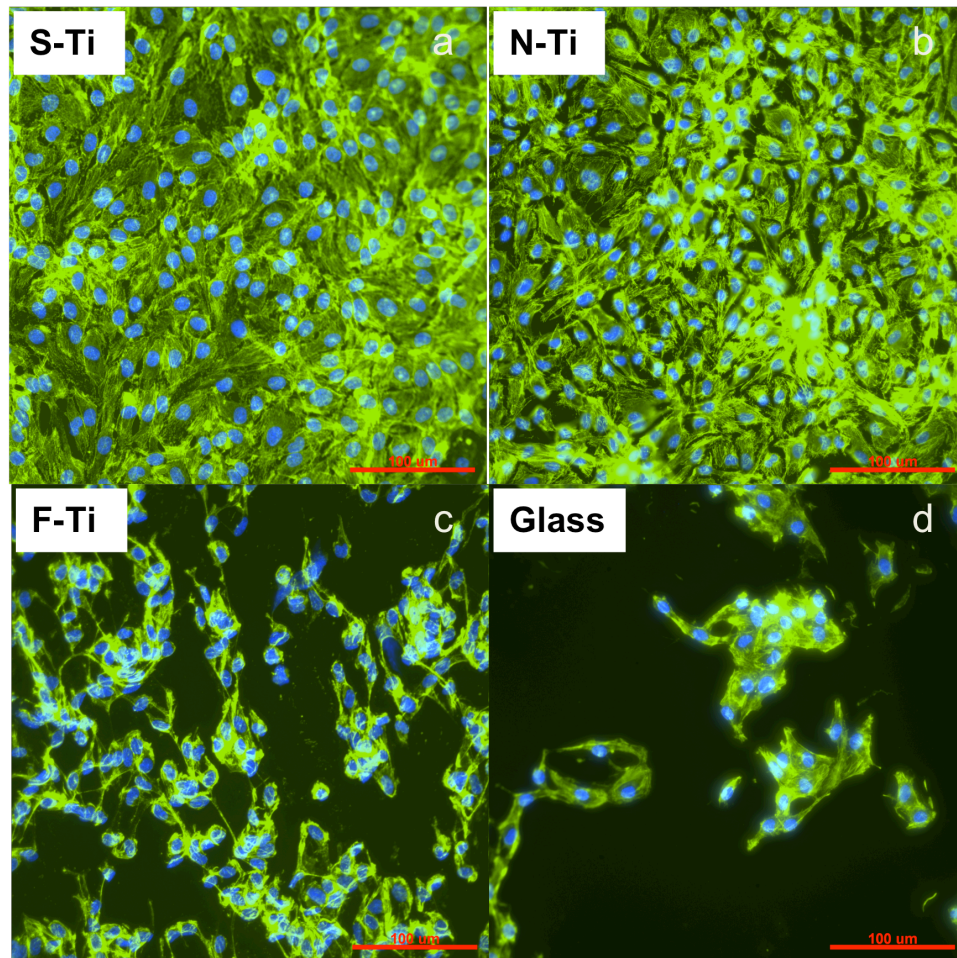


Figure 3.53 Double stained endothelial cells remaining on (a) sub-micron rough (S-Ti), (b) nanorough (N-Ti), (c) flat (F-Ti) titanium surfaces, and (d) glass after 24 hour of laminar flow. Shear stress was kept at 10 dynes/cm². All substrates were pre-cultured with endothelial cells for 36 hours to form a complete layer of endothelial cells.

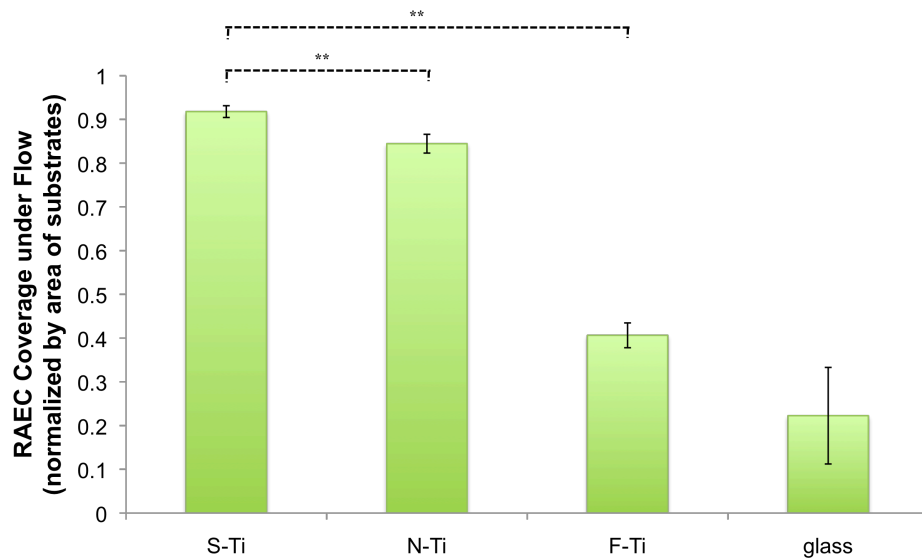


Figure 3.54 RAEC Coverage on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) surfaces under flow after 4 hours, showing that the area RAEC covered on both S-Ti and N-Ti was twice as large as on F-Ti. The spreading area was normalized by the area of the substrates. All error bars are mean $\pm$ SEM; N=3; ** $p < 0.05$.

3.3.3. Dynamic Monocyte Adhesion Test Under Laminar Flow

Monocytes can attach to the endothelium, migrate into the sub-endothelium, and finally lead to inflammatory tissue damage. In this study, human monocyte attachment was studied under shear stress at 5 dynes/cm². This condition simulated a diseased blood vessel with lower shear stress. All substrates were pre-coated with endothelial cells to allow monocyte attachment. Results showed that F-Ti had the highest monocyte attachment after 4 hours, N-Ti was the second, and S-Ti had the least (Figure 3.55). Specifically, S-Ti and N-Ti had only about 40% and 50% of monocytes adherent with respect to F-Ti.

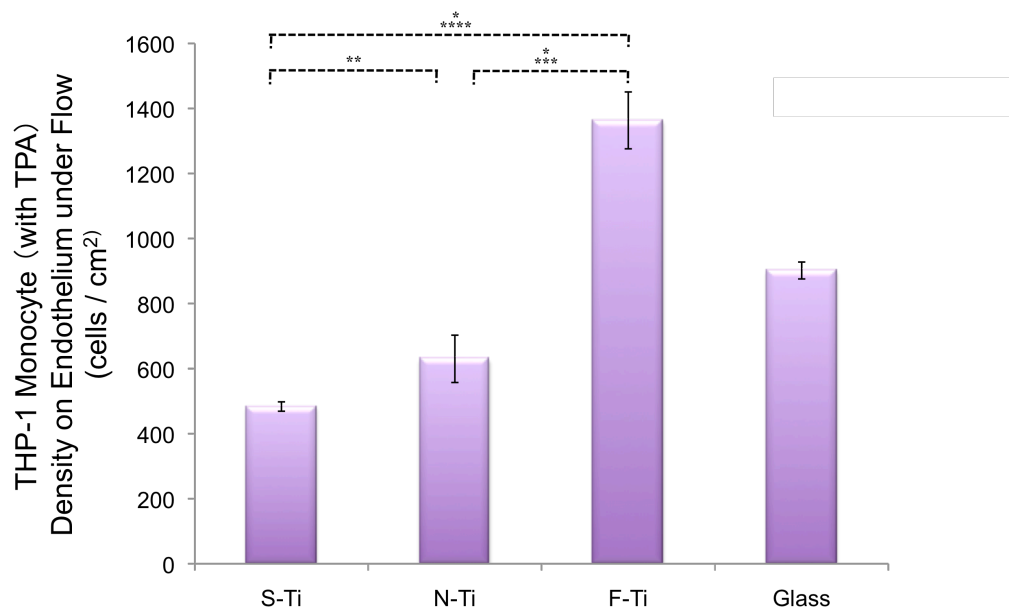


Figure 3.55 THP-1 monocyte adhesion on S-Ti, N-Ti, F-Ti, and glass under flow conditions. All substrates were pre-coated with a layer of endothelial cells and the shear stress was kept at 5 dynes/cm² for 4 hours. Cell density in the culture medium is 50,000 cells/ml. N=3, Data = Mean $\pm$ SEM. ** $p < 0.05$, **** $p < 0.005$ and ***** $p < 0.001$.

3.4. Osteoblast Responses

3.4.1. Osteoblast Adhesion Titanium Substrates with Different Roughness

Human osteoblasts (HOB) adhesion on aligned patterns clearly showed more adhesion on sub-micron structured patterns (S-Ti: dark patterns in Figures 3.56a-b) compared to flat titanium (F-Ti: bright patterns compared to S-Ti in Figure 3.56a-b). SEM images showed various HOB adhesion on both aligned S-Ti and F-Ti regions (Figures 3.56c-e). Some adherent HOB preferred attachment on S-Ti or in between the area of S-Ti and F-Ti (Figure 3.56d). Statistical analysis also showed very high selective adhesion probability of HOB on S-Ti (more than 80%) compared to

nanorough surfaces (N-Ti, more than 60%) versus HOB adhesion on F-Ti (Figure 3.56f).

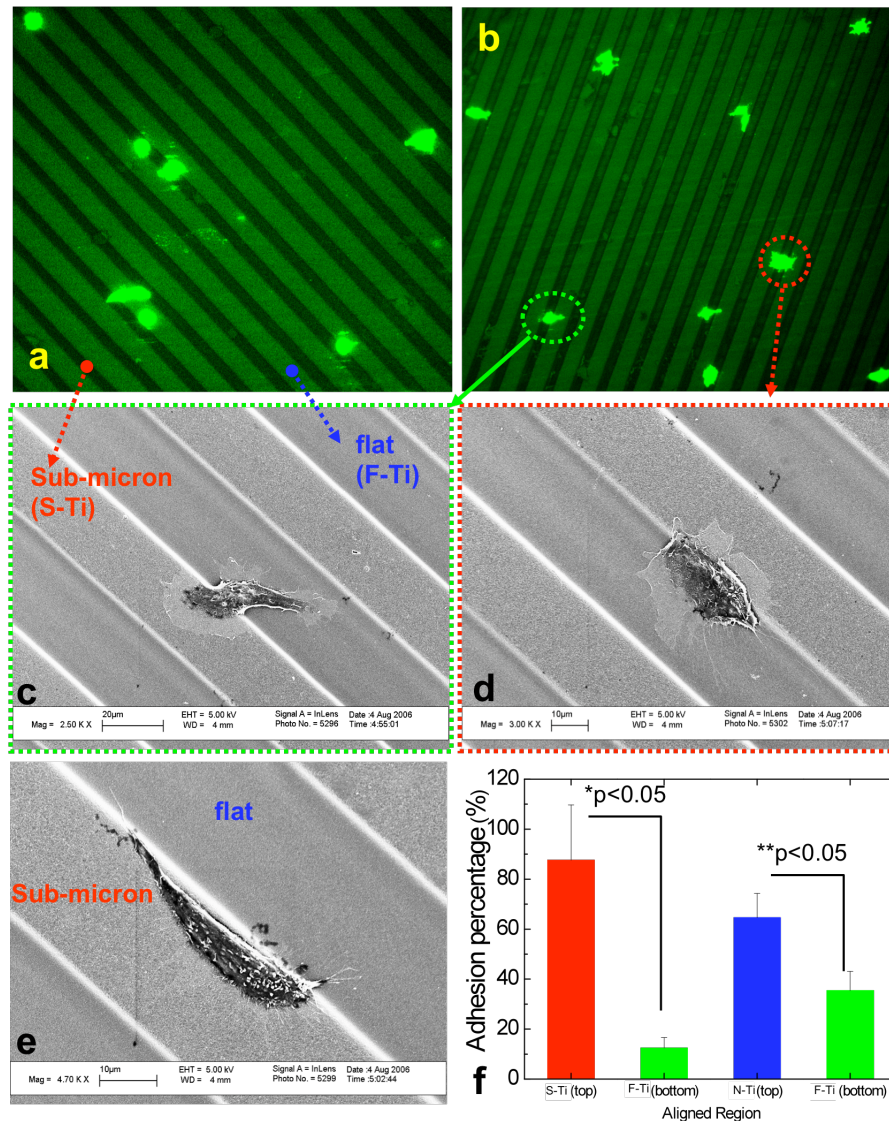


Figure 3.56 Bone cell (HOB) adhesion on aligned patterns. Fluorescence live cell images, (a) and (b), showed that osteoblasts adhered more on sub-micron rough surfaces (S-Ti) than flat titanium (F-Ti) and, thus, provided evidence of selective responses of bone cells on sub-micron regions. The SEM images (c), (d) and (e) indicated different cell adhesion. (f) HOB adhesion density analysis showed that both nano and sub-micron density led to selective adhesion compared to flat surfaces. Greater adhesion probability on sub micron roughness than nano surfaces was observed. All error bars are mean $\pm$ SEM; N=3; $*p < 0.01$, and $**p < 0.05$.

CHAPTER 4. DISCUSSION

4.1. Comparison of Micro/Nanofabrication Techniques Used in the Current Study

In this study, several different techniques were employed to fabricate micro/nano surface features on several vascular stent materials. These included cold compaction, lithography, and electron-beam deposition. Since all techniques provided promising data for the modification of vascular stents, the following sections compare and contrast each of these processes.

First, the main advantage of cold compaction is that the equipment is easily accessed and it is a relatively quick method to create submicron/nano structured surfaces. The present study used compaction of pure titanium and titanium alloys (TiNi or nitinol). The resulting surface features were particulate and heterogeneous, which limits the compaction process toward creating certain patterns (e.g., grooves and pores) which are of great interest to researchers. In addition, bulk properties of the compacts (such as strength) are major concerns when using stents that expand during implantation. Further heat treatments after cold compaction

would also be necessary to strengthen the compact for such applications, at which time the submicron to nanometer particles may coalesce during heat processing losing the uniqueness of the nanoscale properties elucidated in this study.

For this reason, the present study explored additional methods to fabricate submicron to nanoscale stent features which may be more applicable to industry. In particular, lithography, which has been used in the microelectronic field, was studied. The major advantage of lithography is that one can achieve nanoscale surface features of complicated patterns on stents. However, lithography is a delicate process which requires multiple steps and, thus, long processing times. It is definitely a disadvantage to commercialization and making products since it is expensive and time-consuming. Even in the research setting, lithography is sometimes not realistic due to facility limitations and cost considerations.

Thus, this study focused a lot of attention on a brand new nanotechnology process to the vascular stent field: e-beam deposition. E-beam deposition (with masks) is a good alternative to lithography-based techniques and cold compaction when submicron and nanoscale patterns are needed on a stent surface. In the present study, linear patterns with widths of 30 μm were successfully fabricated by e-beam deposition on glass with a help of

a copper grid mask. It is important to note that the size of patterns can be decreased into the nanoscale if a nanoscale mask is used (of course, the size limit of the mask is finally determined by lithographical techniques). However, since the masks can be used repeatedly during the e-beam deposition method (and it usually takes much less time and cost much less than lithography methods), this study demonstrated that e-beam deposition may be more appropriate for the vascular stent industry. Moreover, this study demonstrated that even non-patterned stent surfaces modified by e-beam deposition outperformed current stent surfaces, thus, minimizing the need for such masks created by lithography.

Besides patterning capability, e-beam deposition also has the ability to easily modify surface roughness (with or without patterns and horizontal and vertical) at the submicron or nanoscales. This was accomplished in this study by adjusting deposition parameters (including deposition rate and deposition thickness). This was especially meaningful for the present study because the role of surface roughness and surface energy on vascular cell responses was studied without changing vascular stent chemistry.

E-beam deposition is also more versatile than the above methods since, as a physical vapour deposition method, it can be widely used to deposit various coatings on different substrates. The coatings can be pure

titanium or a specific titanium compound. The underlying substrates can also be versatile (such as titanium, stainless steel, or cobalt-chrome) which are three materials currently used for vascular stents. As mentioned earlier, Medtronic currently sells a coated stainless steel vascular stent with titanium-nitride-oxide using a physical vapour deposition method but does not control nanometer roughness as accomplished here.

Overall, e-beam deposition is a strong tool for researchers and the vascular stent industry to fabricate coatings of various patterns and varied roughness. It also has great potential for industry to improve cytocompatibility properties of current products in the market with limited concerns in cost and efficiency.

Some of the attributes of the three methods used in this thesis are listed in Table 4.1.

Table 4-1 Comparison of three fabrication techniques used in the present study.

Technique	Cold compaction	TIDE lithography	E-beam deposition with mask
Time	Short	Long	Medium
Cost	Low	High	Medium
Bulk properties such as strength	Weak	Not a concern	Not a concern
Particle size/surface roughness	100 nm	Very smooth	Controllable (up to tens of nanometers)
Pattern capability	No	Yes	Yes
Pattern size	none	Hundreds of nanometers	Tens of microns (mask dependent)
Applicable to stent	No	Yes	Yes

4.2. Using E-beam Deposition to Create Different Roughness and

Patterns: Mechanisms and Surface Properties

As stated, the objective of the present study was to create the next generation of bare metal vascular stents, which have optimal surface properties (such as surface topography, surface roughness and surface energy) to improve endothelial cell function and inhibit smooth muscle cell hyper-proliferation that could result in restenosis. It is very important to note that surface properties (such as surface roughness and surface energy) have great influence on vascular cell responses via influencing initial protein adsorption as demonstrated in the present study. The RMS values of the nano/sub-micron patterned titanium samples created by the

TIDE process were nearly zero according to AFM analysis, which means that these substrates only contained ultra-smooth titanium surface features. To study the effects of surface roughness/surface energy on vascular cell functions, e-beam deposition was therefore used here to systematically study various stent surface roughness values.

Material characterization results demonstrated that the expected flat, nano and sub-micron titanium surface features were obtained by e-beam deposition in this study. First, a slow e-beam deposition rate ($0.5\text{-}1\text{ \AA/sec}$) generated flat surfaces of titanium (F-Ti) on cover glass. AFM sectional analysis indicated no structural variation in both micron and nano scales (RMS values equaled zero). The depth analysis also showed negligible depth (vertical) hits. These results indicated that there were no meaningful surface features for both the vertical and lateral scales for the flat titanium surfaces.

In comparison, high e-beam current densities led to higher deposition rates ($20\text{ \AA/sec}$) and generated tiny surface features (with deposition thickness of 50 nm) at the nano scale. Interestingly, more distinct surface features at the nano-scale were observed than at the sub-micron scale. Sectional analyses clearly showed visible nano surface structures of titanium (N-Ti: structures were $30\text{-}40\text{ nm}$ in the lateral and $2\text{-}6\text{ nm}$ in the vertical scales). Since both vertical and lateral structures were less than

50nm, all the surface roughness contributed from these features was truly at the nanometer dimension.

In contrast, a high e-beam deposition rate (20 Å/sec) with a thickness of 1µm generated rough titanium surface features (structures were 100-110 nm in the lateral and 40 nm in the vertical scale) at the sub-micron scale (S-Ti). Drastic differences of surface roughness (both lateral and vertical) were found between the sub-micron titanium surfaces (S-Ti) and the nano-scale titanium surfaces (N-Ti). However, at the nanoscale, S-Ti was relatively smooth compared to N-Ti in the lateral scale. This clearly demonstrated that sub-micron scale surface features (larger than 100 nm in lateral width) were dominant on S-Ti surfaces (but vertical structures were still less than 100 nm).

For F-Ti, it was considered that titanium atoms bombarded by an electron gun initially nucleated, formed islands on the substrates and finally achieved even coverage at a thickness of 50 nm (Figure 4.1 b). It is proposed here that a slightly different deposition mechanism occurred during the fabrication of N-Ti. Specifically, an increased deposition rate imposed some difficulty for the system to achieve good layer-by layer structures. Instead, during the formation of multilayer structures, the lateral and vertical differences between each island were enlarged and finally appeared to be nanoparticulate in structure (Figure 4.1 a). The reason for

the altered surface structures on S-Ti (compared to N-Ti) can be explained using the following model (Figure 4.1a). At the beginning of the e-beam deposition process, many layers of titanium nanoparticles were coated onto the surface just as in the formation of N-Ti. However, since S-Ti was much thicker (1 μm compared to 50 nm of N-Ti), two effects became more significant as deposition time increased. The first was an effect based on coalescence driven by a reduction of surface energy, i.e., the consumption of small islands and formation of larger features. In this light, the lateral feature size on the S-Ti surface grew into sub-micron scale features. Second, as the coating grew thicker, some favorable feature orientations grew faster than others and finally produced a much larger vertical variation of surface structures than N-Ti (but still in the nanometer scale).

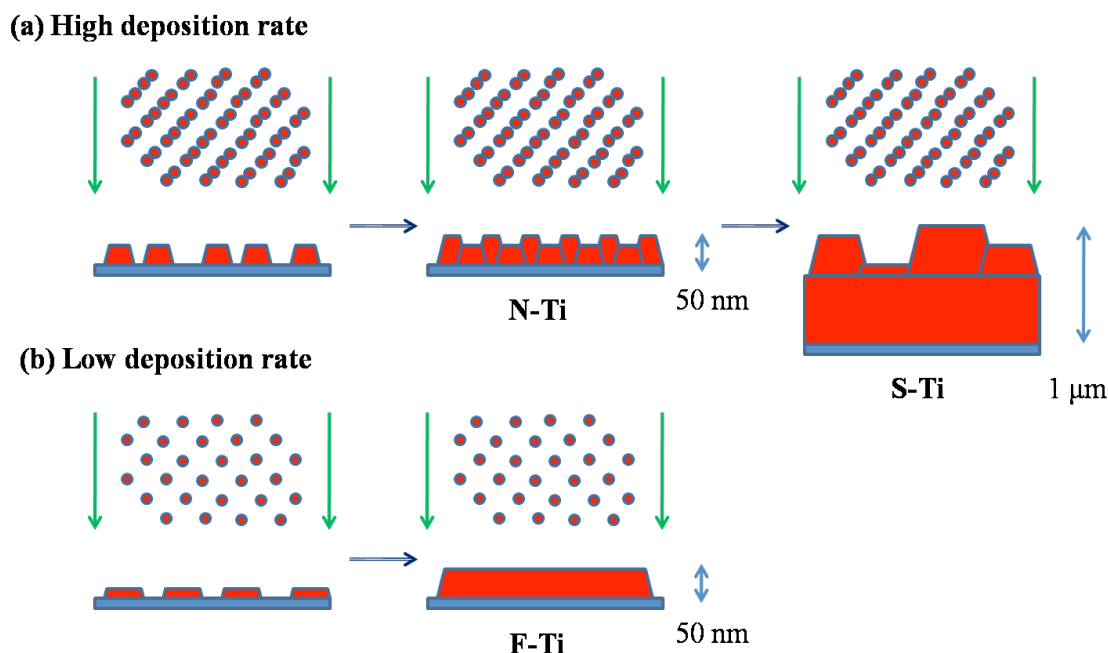


Figure 4.1 Schematic using e-beam deposition to create to N-Ti, S-Ti, and F-Ti surfaces by altering deposition rate. High deposition rate in (a) is 20 Å/sec while low deposition rate in (b) is 0.5-1 Å/sec. Small particles in the schematic indicate titanium atoms. F-Ti is flat titanium, N-Ti is nano-rough titanium and S-Ti is submicron rough titanium.

The three different titanium surfaces (F-Ti, N-Ti, and S-Ti) possessed different feature sizes and surface roughness values, which were all fabricated by adjusting just two parameters during e-beam deposition (deposition rate and deposition thickness). This relationship with resulting surface roughness is shown in Figure 4.2. For F-Ti to N-Ti, the deposition thickness remained at 50 nm while the deposition rate increased from 0.5-1 Å/sec to 20 Å/sec, respectively. The RMS roughness increased from near 0 to 1 nm, for F-Ti and N-Ti, respectively. This was achieved by increasing the current density from 50 mA/cm² to 130 mA/cm². For N-Ti to S-Ti, the deposition rate was kept constant at 20 Å/sec while the

deposition thickness increased from 50 nm to 1 μm . The RMS roughness increased to 14 nm for S-Ti compared to 1 nm for N-Ti.

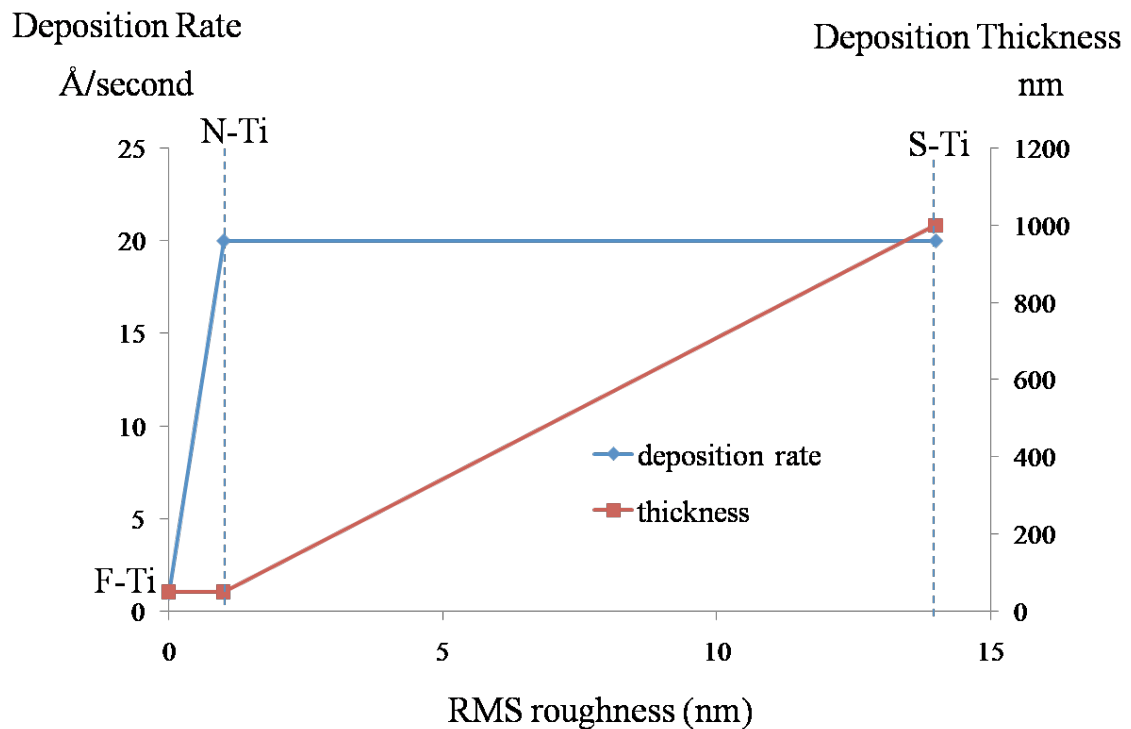


Figure 4.2 Relationship between e-beam deposited surface roughness and e-beam deposition parameters (specifically, deposition rate and deposition thickness).

Besides creating specific roughness on titanium surfaces, e-beam deposition in the present study also generated micron-patterned titanium surfaces. Although surface patterns did not reach nano/sub-micron scales in this study due to the dimensions of the grids used, this could be accomplished in the future since e-beam deposition indeed provides a faster and more inexpensive way to fabricate highly controllable titanium surface features for enhancing vascular cell functions than lithography.

Moreover, FESEM images showed that the e-beam deposition method developed in this study successfully generated two different surface features of aligned patterns, specifically, a combination of F-Ti (flat) and N-Ti (nano) after deposition of 50nm thick titanium.

The results from contact angle analysis (F-Ti: less than 45°; N-Ti: less than 32°; S-Ti: less than 20°) indicated that the small nano-sized surface features on titanium (RMS values less than 2 nm, 40 nm lateral width, and 4 nm vertical depth achieved on 50 nm titanium thin films) altered water contact angles by more than 10° compared to F-Ti surfaces. This result is very intriguing since small changes in surface area (0.7 % increment between N-Ti and F-Ti) led to a 10% increase in surface energy between N-Ti and F-Ti. As for S-Ti, although sub-micron surface roughness did not generate such a large increment in surface energy with respect to altered surface area (30% increase in surface energy with a 6% increase in surface area between S-Ti and F-Ti) as nano titanium surface roughness did (10% increase in surface energy with a 0.7% increase in surface area between N-Ti and F-Ti), RMS values as measured by AFM for sub-micron features drastically increased (more than 10 nm) due to an increased vertical depth compared to nano titanium surfaces.

4.3. Nanostructured NiTi Created by Cold Compaction Methods for Regulating Vascular Responses

NiTi has good shape-memory and superelastic properties which make it one of the most ideal biomaterials for vascular stent applications. As discussed above, the major challenge in vascular stent development is to inhibit or reduce inflammation and thrombogenicity as well as limit conditions that promote neointimal tissue growth. Moreover, stent surfaces are needed that increase the affinity for endothelial cells to form a protective monolayer on the vascular stent surface.

In this study, sub-micron to nanostructured surface features on NiTi were investigated for improving vascular stent applications. Increased cell adhesion on fine grain ($<60\text{ }\mu\text{m}$) compared with coarse grain NiTi substrates ($<100\text{ }\mu\text{m}$) was observed here. Endothelial cells adhered (after 5 hours) and proliferated (after 1 day) more on NiTi with greater sub-micron and nanoscale surface roughness; such approaches may eliminate the need for anti-thrombogenic and anti-inflammatory drug-eluting stents since this would possibly lead to greater vascular stent endothelialization. In longer-term cell experiments, cells did not grow well on the larger grain size NiTi compacts since the coarse particles created large voids on the surface. However, the presence of a greater amount of homogeneous interparticulate voids on the fine grain NiTi (and hence, greater porosity at the nanometer scale), seemed more beneficial for endothelial cell

adhesion and proliferation leading to the formation of an endothelial cell monolayer as early as 5 days.

In preparing the NiTi substrates, heat treatments were not used for two reasons. First, heat treatments will result in brittle compacts which cannot be used for vascular stent applications. During heat treatments, NiTi is also disposed towards forming a heterogeneous surface oxide layer which makes it vulnerable to corrosion. At room temperature, NiTi is known to oxidize and form a TiO_2 film on the surface. If this TiO_2 film is uniform across the surface, it may be beneficial for increasing endothelial cell functions [171]. It is uncertain how the preparation of the NiTi substrates in liquid nitrogen may have modified this TiO_2 layer. EDS analysis did show that the surface chemistry was very similar for all NiTi substrates; a property important for us to control in order to delineate sub-micron to nanoroughness as a key property towards promoting endothelialization. NiTi compact synthesis was performed by compressing particles under cold temperatures within a liquid nitrogen bath. Previous studies [172, 173] suggested that lowering the temperature during compression can achieve a greater percentage of strain or deformation of the NiTi particles. Compressing at higher pressures will increase the austenitic transformation temperature range, and compacts will remain intact at standard cell culture temperatures of 37 °C. Compacts with smaller grain size have higher transformation temperatures as a result of increased

local elastic energy due to the presence of more grain boundaries [174].

Although there are potentially numerous ways to create sub-micron to nanostructured NiTi, this study employed simple powder metallurgy techniques, and in doing so, it sets the stage for other nanotechnology-based techniques. In summary, the outcome of the present study is consistent with the results of other studies involving nanophase materials [116, 134, 175].

This common phenomenon of improved performance using nanostructured materials has drawn much curiosity towards a scientific rationale. An explanation for this phenomenon was given by Klابلunde *et al.*, who claimed that nanophase materials have higher numbers of atoms at the surface compared with bulk, greater areas of increased surface defects (such as edge/corner sites and particle boundaries), and larger proportions of surface electron delocalization. These properties have since been shown to modulate initial protein interactions that control subsequent cell functions [144].

4.4. Surface Topography (Nano/Sub-micron Titanium Patterning) Created by TIDE Lithography and E-beam Deposition for Regulating Vascular Cell Functions

4.4.1. Vascular Cell Function on TIDE Created Patterned Surfaces

This study compared vascular cell morphology, adhesion, and proliferation on patterned titanium surface features (with dimensions ranging from 100 μm to 750 nm created by the TIDE process) to random nanostructured surface features. Results demonstrated improved endothelial cell function and cell orientation on nano/sub-micron patterned titanium surface features when feature size decreased. Moreover, the smaller the pattern dimension was, the better the endothelial cells elongated into a morphology that is similar to the natural vascular endothelium, in which endothelial cells align with each other in the same direction with the blood stream.

Although the mechanism of improved endothelial cell adhesion and spreading on nano/sub-micron patterned substrates has not been completely elucidated, numerous studies have attributed improved endothelial cell responses on nanosurfaces to a physical effect, that is, changes in the alignment and shape of cells were related to cell function [126]. For example, Choudhary *et al.* created random nanostructured titanium surface features by using a uniaxial hydraulic press to press titanium nanoparticles (250-750 nm) into titanium compacts. The *in vitro*

studies indicated that vascular stents with random nanostructured surface features invoked vascular cell responses promising for improving vascular stent applications [116]. Palmaz *et al.* reported that the endothelial cell migration rate doubled on nitinol patterns of microscopic grooves (1, 3, 15, and 22 μm) compared to smooth metal surfaces, hypothesizing that such micron patterns may reduce time to endothelialization of stents and, thus, the risk of in-stent restenosis and late thrombosis [130]. They attributed this increase to a physical effect, that is, changes in the alignment and shape of cells were related to a simple cell conformation process. Other studies have also demonstrated that cell shape and orientation are related to cell gene expression [131]. In addition, as was mentioned, nanopatterned surface features resemble the interface between circulating blood in the lumen and the rest of the arterial wall better than random nanostructured surface features.

However, there is some contradiction concerning the influence of surface patterns on cell alignment. While this has been largely untested for nanomaterials and associated nanopatterns, it has been extensively studied for conventional materials. For example, some studies indicated that the degree of cell alignment increases with groove size [176], but other studies showed that the larger the concentration of ridges on the grooved surface, the greater the effect on cell morphology [130]. So the influence of the dimension of nanopatterns is another important subject to

be further studied on these novel substrates. But here, this study provided the first evidence of promoted endothelial cell function on Ti nanopatterns prepared via the TIDE process.

In addition, since as demonstrated here there was a contribution of nanopatterns to endothelial cell shape and orientation (which are associated with cellular responses to implantable materials), these nano or micro machined surfaces can be applied to other implantable materials (such as for improving bone prostheses where the preferred orientation of calcium containing mineral and collagen is vital). Faster formation of bone-like cell multilayers have already been observed on microgrooves (150 μm) compared to smooth surfaces [177]. With the help of nano and micromachining techniques, Ti-based bone prostheses can be easily coated with micropatterns of nanomaterials to enhance their performance and possibly result in the next generation of anisotropic bone juxtaposed to an implant surface. In this manner, this study highlighted an important manufacturing process that can improve metals used in a number of implants to perform better.

4.4.2. Vascular Cell Function on E-beam Deposition Created Patterned Surfaces

Titanium patterned surfaces were also created by e-beam deposition. As discussed before, the dimensions of the patterns fabricated by e-beam deposition could only achieve as low as 30 μm because of currently

limited availability of commercial masks. However, it indeed provides an alternative more economical and efficient way to make titanium patterns on stents. Moreover, different surface roughness could be created on the same titanium patterns by e-beam deposition so that the effects of surface roughness and surface energy could be investigated under the same conditions. In this study, two different titanium patterns were created: a combination of sub-micron rough (S-Ti) and flat titanium (F-Ti) on the upper and bottom layers of the patterns, respectively, of 50 nm in height and a combination of nanorough (N-Ti) and F-Ti of 1 μm in height on the upper and bottom layer of the patterns, respectively.

On the first patterns (S-Ti and F-Ti), results showed that more than 80% of RAEC selectively adhered onto sub-micron titanium patterns and RAEC f-actin filaments tended to spread more on the sub-micron features compared to flat regions. On the second patterns (N-Ti and F-Ti), results showed 65% of RAEC selectively adhered onto nano surface featured titanium patterns compared to flat surfaces. In addition, RAEC responses were also tested on control patterns with similar height layers (50nm and 1 μm in height) with the same surface properties (both layers were flat) to determine if the selective adhesion of RAEC came from altered surface properties (surface roughness and surface energy). RAEC showed no preferential adhesion on the top and bottom layers. All the results clearly demonstrated that the selective adhesion of RAEC (on S-Ti and N-Ti) was

due to the presence of nano and sub-micron surface features, not due to height differences between the two layers.

In summary, it was suggested that titanium patterns created by e-beam deposition might outperform TIDE-fabricated patterns in that if spacing dimensions of masks are within micrometer range ($\geq 30 \mu\text{m}$), the patterns could combine various roughness and serve as “sensors” to investigate the effects of surface roughness and surface energy on cell responses simultaneously. On the other hand, if spacing dimensions of masks are within sub-micron to nanometer range ($<1 \mu\text{m}$), the submicron/nano patterned titanium surfaces could enhance endothelial cell responses as discussed before. Moreover, optimal surface roughness could be combined with nanopatterns by e-beam deposition to further improve cell responses.

4.5. Surface Roughness and Surface Energy for Regulating Vascular Endothelial Cell Functions on E-beam Deposition Created Surfaces

Figure 4.3 showed the correlation between the adhesion probability of RAEC and various surface features with respect to altered width and depth on e-beam deposited titanium surfaces. Depending on the surface feature width and depth values, titanium surface structures were defined as flat, nano and sub-micron surfaces (F-Ti, N-Ti and S-Ti). The results of RAEC adhesion after 4 hours showed that RAEC density increased 50%

on nano titanium surface features compared to flat (F-Ti) titanium surfaces. Also, RAEC adhesion on sub-micron structures (S-Ti) increased 200% compared to flat titanium. In addition, RAEC increased 40% on submicron features compared to nano featured titanium. A majority of S-Ti features in the lateral dimension were larger than 100 nm although vertical depths of sub-micron titanium structures (S-Ti) were below 100 nm. Since altered lateral widths of nano surface features were almost ten times higher than the altered vertical structures, an increase in lateral width was considered as a meaningful factor for altered cell adhesion. Especially, it is impressive that altered lateral nano dimensions (about 40 nm) led to drastic changes in surface energy (10% increment from a just 0.7% increased surface area compared to flat surfaces) and induced greater cell adhesion (50%). This might be due to altered titanium nano topographies (increased lateral width of surface features) inducing more spreading (greater wettability or surface energy) with enhanced cell adhesion than flat titanium surfaces with identical chemistry [178, 179]. It was noticed that altered vertical structures (about 4 nm) did not lead to a drastic increase in surface energy and cell adhesion because increased vertical depths led to lower surface energy and decreased cell densities, as observed by previous studies [179-181]. Also, altered vertical depths (4 nm) were negligible compared to altered lateral dimensions (40 nm). However, a more rigorous study needs to focus on understanding the altered stent surface properties with respect to subsequent cell adhesion.

Importantly, in this analysis, it was clearly identified that small nano surface titanium roughness (due to nano scale surface features) resulted in a drastic increase in surface energy (an important factor for cell adhesion) that led to the subsequent improvement in RAEC attachment (more than 50%) [182, 183].

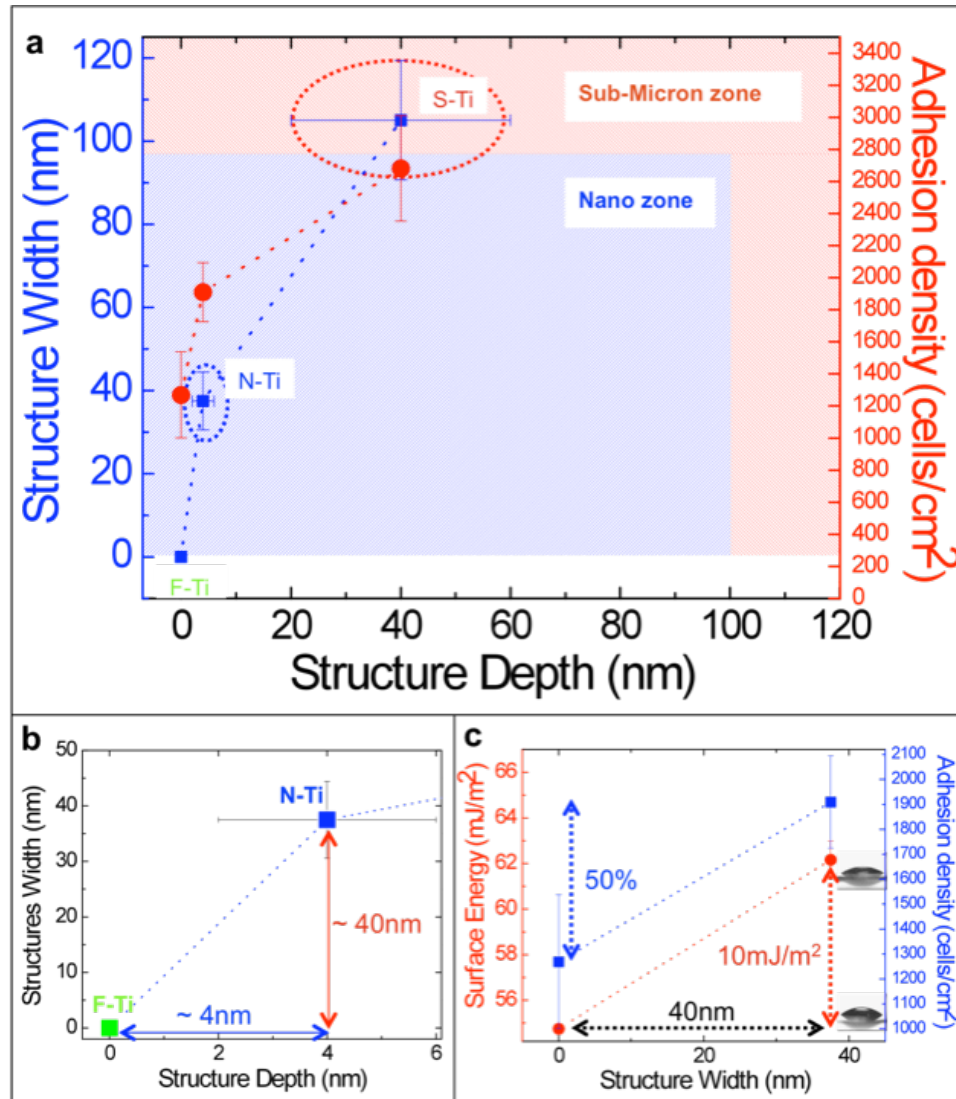


Figure 4.3 Correlations between lateral width and depth versus RAEC adhesion: Importance of nano surface structures with respect to surface energy and cell adhesion. (a) RAEC adhesion on nano surface structures (less than 50nm for both vertical and lateral scales) increased about 50% compared to flat (F-Ti) surfaces. Also, RAEC adhesion densities on submicron structures (S-Ti) increased about 200% compared to flat titanium. The cell adhesion difference between nano (N-Ti) and S-Ti was 40% (more cells on sub-micron compared to nano structures). Although vertical depths in sub-micron structures (S-Ti) were below 100nm, a majority of S-Ti features in the lateral dimension showed structures larger than 100nm. Criteria of sub-micron and nano features were determined by the current definition of nanometer less than 100nm. (b) Vertical depth showed negligible differences between flat and nano titanium (4nm) while lateral widths increased about 40nm which is almost ten times greater

compared to the altered depth. (c) The importance of lateral nano surface features was stressed with respect to surface energy and cell adhesion. The 40nm lateral structures led to both greater surface energy (8mJ/m^2) and greater cell densities (50% increment for bone and endothelial cells).

To further investigate the effects of surface energy on endothelial cell responses, critical surface tensions of sub-micron, nano and flat titanium surface features were measured by means of a Zisman plot in this study. Critical surface tension of a given surface is defined as surface tension below which a liquid completely wets the surface. Figure 4.4 represents correlation of critical surface tension of a solid with its relative biological interactions. If surface critical tension of a biomaterial is in area “A”, the material has minimal cell attachment and cell spreading. A biomaterial, whose surface critical tension is within area “B”, has good bioadhesion with surrounding biological environment, that is, it promotes cell attachment and spreading. As shown in Figure 4.4, the average surface critical tensions of F-Ti, N-Ti and S-Ti are 33, 37 and 41 dyne/cm, respectively. All these tension values are within the intermediate region between area “A” and “B”, where biological interactions of these substrates are enhanced with increased surface critical tensions. Therefore, Figure 4.4 clearly shows S-Ti has the best bioadhesion of all the substrates as it has the highest surface critical tension. N-Ti also promote cell responses on them when compared with F-Ti. These results further confirm the important role of surface energy in controlling cell functions.

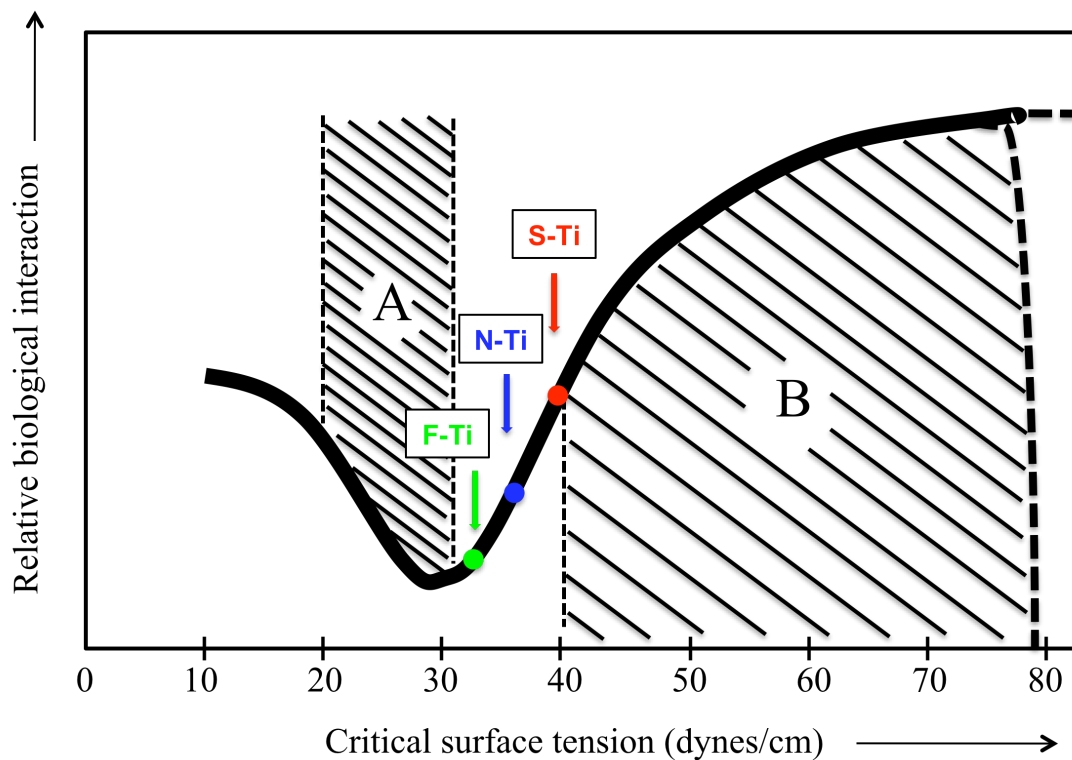


Figure 4.4 Relationship between the biological interactions and the critical surface tension of a biomaterial. Area “A” represents a non-adhesive zone; area “B” represents biomaterials with good bioadhesion to their biological environment (Adapted and redrawn from [184]).

In addition, NO_x and endothelin-1 synthesis by RAEC for up to 24 hours were investigated in this study. The release of NO_x and endothelin-1 play important roles in regulating vascular tone. That is, vascular hemostasis is maintained by vasodilators such as NO_x and vasoconstrictors such as endothelin-1 to control vessel dilation and constriction, respectively. Both of these factors are released by endothelial cells. Moreover, NO_x can also

prevent platelet aggregation and inhibit smooth muscle cell proliferation while endothelin-1 promotes platelet aggregation.

In this study, the secretion of NO_x by RAEC on S-Ti after 24 hours was higher than the other substrates (nano and flat). On the other hand, the release of endothelin-1 remained similar on all the substrates. Thus, the ratio of NO_x/endothelin-1 synthesis was greatly enhanced on S-Ti compared with N-Ti and F-Ti (Figure 4.5), which suggests that an increased balance of NO_x/endothelin-1 on S-Ti might lead to limited adhesion of blood cells (such as platelets) and decreased smooth muscle cell proliferation to limit thrombosis development. In addition, with the help of increased surface roughness and surface energy (as for S-Ti), the increased release of NO_x from endothelial cells on S-Ti could enhance vasodilation bioactivity, keeping the endothelium in a healthy state. Thus, results of this study demonstrated that submicron titanium structures created by e-beam deposition had the best cytocompatibility properties for endothelial cells. On the other hand, nano-rough titanium had the highest efficiency for both increasing surface energy and increasing endothelial cell adhesion when focusing on the altered width of surface features (about 40 nm).

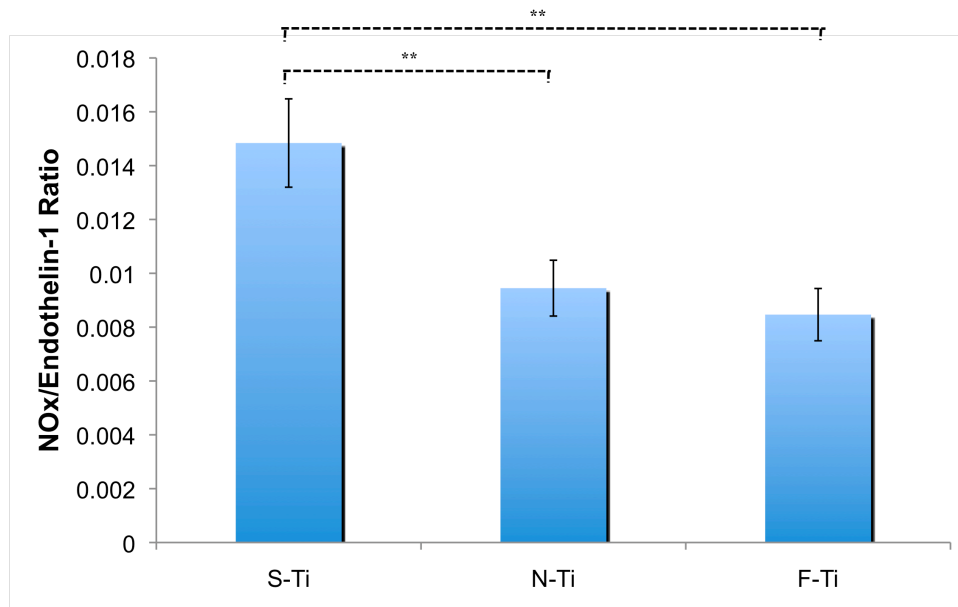


Figure 4.5 Ratio of NOx to endothelin-1 released by RAEC after 24 hours on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) surfaces. All error bars are mean $\pm$ SEM; N=3; $**p<0.05$.

4.6. Comparison of Vascular Endothelial Cell and Smooth Muscle Cell

Functions on E-beam Deposition Created Surfaces with Various

Roughness and Surface Energy

As discussed in the literature review, smooth muscle cell proliferation plays an important role in the development of atherosclerosis and in-stent restenosis. In response to endothelium dysfunction, smooth muscle cells increase their proliferation rate in contrast to a normally quiescent state. The hyperproliferation of smooth muscle cells, together with the accumulation of lipid and lipid-filled macrophages, leads to the formation of in-stent restenosis. Therefore, it was necessary to study the effects of surface roughness and surface energy of stent materials on vascular smooth muscle cells and compare their function with endothelial cells in a

competitive culture model. Proliferation studies of RAEC, RASMC and co-culture of both cells were conducted on S-Ti, N-Ti and F-Ti for 1, 3 and 5 days. Results of this study showed that after 1, 3 and 5 days, while more RAEC adhered on S-Ti and N-Ti than F-Ti, the RAEC proliferation rate (estimated by MTS assays) on both substrates was greater than on F-Ti, resulting in a faster formation of a monolayer of endothelial cells. On the other hand, when comparing the influence of surface roughness from S-Ti and N-Ti on cell behavior, results of this study indicated that S-Ti was more desirable for RAEC attachment and N-Ti was better for RAEC proliferation. Unlike RAEC, RASMC showed no differences on the various titanium substrates tested here after 1 day of culture. After 5 days of culture, RASMC density on F-Ti was the highest because of a slightly higher proliferation rate compared to the other substrates. Such results suggested that in contrast to RAEC, both S-Ti and N-Ti might not enhance competitive smooth muscle cell proliferation. Previous studies demonstrated that smooth muscle cell functions and phenotypes (synthetic and contractile) were dependent on hydrophobicity [185]. Smooth muscle cell responses were enhanced on more hydrophobic surfaces, which was consistent with the results in this study. F-Ti had the highest hydrophobicity of all the substrates and induced higher proliferation rates of smooth muscle cells. Moreover, results of the RAEC and RASMC co-culture proliferation experiments confirmed previous results in which cells were cultured

separately, and demonstrated for the first time more RAEC than RASMC on S-Ti than any other substrate tested here (created by e-beam deposition). In contrast, there were no significant differences between the growth of RAEC and RASMC on F-Ti.

Obviously, it is important to consider why endothelial cell and smooth muscle cells behaved differently on each substrate. Coupled to this change in cell number, cell morphology was also different on these substrates. As mentioned in earlier sections, prior studies have correlated a change in cell shape and alignment to improved endothelial cell migration and modified cell gene expression [186-188]. This study found that the aspect ratio and spreading of RAEC increased on S-Ti and N-Ti compared with F-Ti. At the same time, RASMC morphology was not influenced by underlying surface roughness, which might be due to size differences between RASMC and RAEC. According to the fluorescence images of these two cells, RASMC (length of more than 100 μm) were much larger than RAEC (length of about 10 μm). It is possible that RASMC are not as sensitive as RAEC to sense the underlying sub-micron to nanosize particles. Moreover, RASMC functions were enhanced with decreased surface hydrophilicity [185], which could also explain the improved RASMC proliferation on F-Ti to S-Ti and N-Ti. Besides the effects of surface roughness or topography on RAEC and RASMC adhesion and proliferation, these surface properties might also affect other

functions of cells (such as collagen and elastin production). These initial changes in cell morphology may have led to the overall higher synthesis of proteins like collagen and elastin by RAEC cultured on S-Ti and N-Ti compared to F-Ti. Since chemistry was the same between all substrates in this study [15], these results indicated that Ti surface roughness (at the nanometer and sub-micron level created by e-beam deposition) influenced RAEC functions and, thus, should be the focus of future investigations.

Thus, all of these results collectively indicated the role of nano and sub-micron surface features (roughness) on endothelial cell functions.

Including the results from a previous study highlighting the role of nano surface roughness on polymers for promoting cell functions [189], this study suggested again an important design criteria for future vascular stents, that is, the application of sub-micron rough surfaces to improve functions of endothelial cell functions, not those of smooth muscle cells.

4.7. Protein Adsorption

After stent implantation, injury due to the implantation procedure might change the composition of the extracellular matrix surrounding vascular cells similar to other wound healing processes [190]. This new provisional healing matrix would include proteins like vitronectin, fibronectin, von Willebrand factor, fibrinogen and thrombin, which are all important in regulating endothelial cell adhesion, proliferation and gene expression [191, 192]. Among them, both vitronectin and fibronectin are major cell

adhesion molecules due to their Arg-Gly-Asp (RGD) sequences [148, 149]. They are involved in cell adhesion, spreading as well as migration with specific integrins such as $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_1$ and $\alpha_{II}\beta_3$ [148, 149].

In this study, the protein source used in the cell experiments was bovine serum, which contains similar quantities of fibronectin and vitronectin, but the overall adsorption of vitronectin on all the three substrates (S-Ti, N-Ti and F-Ti) was much larger than that of fibronectin after 4 hours. Although both S-Ti and N-Ti had higher vitronectin and fibronectin adsorption than F-Ti, when comparing S-Ti and N-Ti, vitronectin adsorption on S-Ti was significantly higher than on N-Ti while fibronectin adsorption on N-Ti was higher than on S-Ti. Some studies [193] have demonstrated that under normal cell culture conditions, vitronectin could be successfully adsorbed to substrates in the presence of other proteins in serum and promote cell adhesion and spreading. On the other hand, fibronectin adsorption to substrates is interrupted due to the inhibitory effects of other serum proteins. These phenomena could partly explain the overall enhanced adsorption of vitronectin over fibronectin on all the substrates when using the same serum. Moreover, it has been shown that vitronectin is a primary extracellular matrix protein in medium serum to dominate cell adhesion onto various substrates [193-195]. As a result, enhanced endothelial cell adhesion was observed on S-Ti compared to the other substrates because S-Ti had the highest amount of vitronectin adsorption.

Fibrinogen is one of the most abundant blood serum proteins, which adsorbs immediately to vascular stent surfaces after implantation. It induces the agglomeration of blood platelets and finally triggers thrombus formation [196]. However, fibrinogen is also involved in the adhesion and spreading of endothelial cells [149]. Dejena *et al.* revealed that human a endothelial cells adhered and spread on fibrinogen coated surfaces with similar attachment rate to that on other adhesion protein coated surfaces (such as fibronectin), while smooth muscle cells didn't attach to fibrinogen coated substrates[149]. All these results indicated that despite its critical role in thrombus formation, fibrinogen might induce endothelial cell adhesion and migration. In the present study, the highest amount of fibrinogen was detected on S-Ti, which is consistent with the results of endothelial cell adhesion, in which endothelial cell density was significantly increased on S-Ti as well.

It is important to investigate why the effective adsorption of major extracellular matrix proteins (all proteins are influenced by other proteins) improved on S-Ti. Khang *et al.* provided a model describing the linear relationship between the amount of absorbed protein and nanosurface roughness/surface energy [189]. According to the model, protein adsorption as a function of surface roughness and surface energy can be expressed as:

$$P_{adsorption}(r_{eff}, E_s) = \alpha r_{eff} + \beta E_s \quad (4.1)$$

where r_{eff} is surface roughness; E_s is surface energy; α and β are coupling constants of r_{eff} and E_s .

Considering that surface roughness is correlated to surface energy, this equation can be also described as a function of only roughness or only surface energy:

$$P_{adsorption}(E_s) = (\kappa + \beta)E_s - \beta\kappa E_{0,s} \quad (4.2)$$

where $E_{0,s}$ is the ground surface energy which is only determined by chemical interaction (when $r_{eff}=0$); $\kappa=\alpha/\beta$.

Equation (2) demonstrates that if ground surface energy is constant on all the substrates (that is, same surface chemistry as demonstrated here for the e-beam samples), protein adsorption is purely proportional to surface energy of the substrates. Figure 4.6 shows the correlation of extracellular matrix protein adsorption (vitronectin and fibrinogen) with surface energy obtained on the three titanium surface features (sub-micron, nano and flat titanium created by e-beam deposition). The curves of vitronectin adsorption and surface energy on S-Ti, N-Ti and F-Ti have the same trends, which mean that vitronectin adsorption is proportional to surface

energy on these substrates which was controlled by changing surface roughness. These results demonstrated the importance of surface roughness and surface energy on protein adsorption for vascular stent applications. Greater surface energy led to greater amounts of adsorbed proteins followed by greater endothelial cell adhesion.

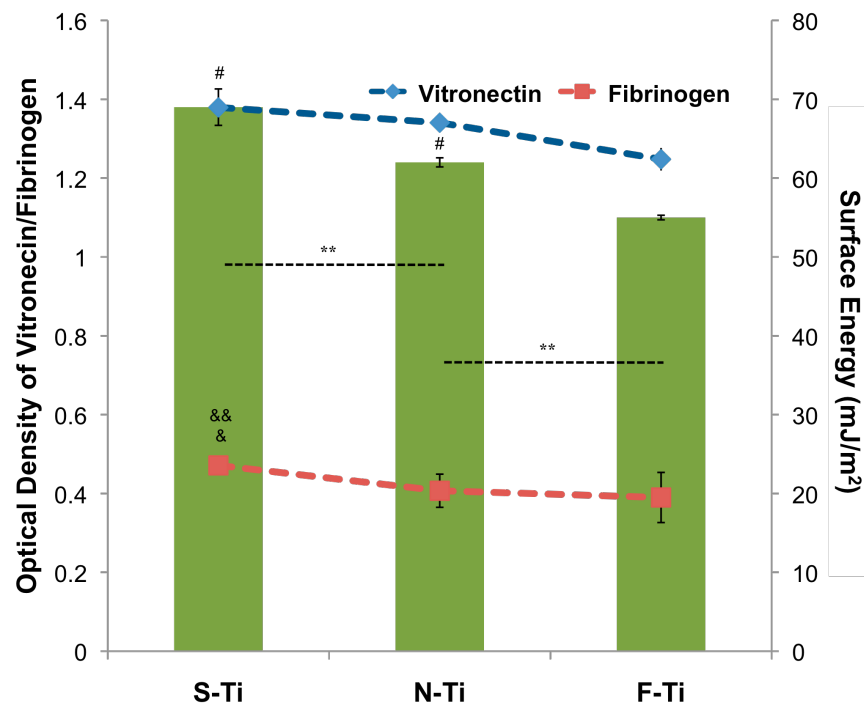


Figure 4.6. Protein adsorption (vitronectin and fibrinogen) proportional to surface energy on submicron (S-Ti), nano (N-Ti) and flat (F-Ti) surfaces. Flat (54mJ/m²), nano (62mJ/m²) and submicron (69mJ/m²) titanium had increased surface energy. Data=mean+/-SEM, N=3. ** $p < 0.01$, # $p < 0.01$ (compared to F-Ti), & $p < 0.01$ (compared to F-Ti) and && $p < 0.05$ (compared to N-Ti).

4.8. Role of Sub-micron and Nano Surface Structures on Endothelial Cell Migration

As discussed before, implantation of a vascular stent can significantly decrease restenosis, however, the process may lead to in-stent restenosis due to an unrecovered endothelium and smooth muscle cell migration. To restore an intact endothelium, not only should one promote endothelial cell adhesion and proliferation, but also one should promote endothelial cell migration. Previous studies confirmed that improved endothelial cell migration accelerated endothelialization of stents, resulting in a reduction in thrombosis [170]. In this study, a time-lapsed endothelial cell migration assay (12, 24 and 96 hours) was conducted on flat, nano and sub-micron titanium surfaces created by e-beam deposition to investigate the optimal surface roughness for promoting endothelial cell migration.

Figure 3.39 shows the migration displacement of RAEC on the substrates after each time point according to fluorescence images. The migration distance was significantly increased by 46% on S-Ti and 22% on N-Ti when compared with F-Ti after 24 hours. After 96 hours, as migration speeds of RAEC on S-Ti and N-Ti were enhanced (as determined by the slopes of the curves between 24 hours and 96 hours), the increments of migration distances increased by 56% on S-Ti and 25% on N-Ti compared to F-Ti. RAEC migrated the most quickly on S-Ti of all the substrates.

It is interesting to compare these results with those of RAEC adhesion. DiMilla *et al.* proposed that, to trigger cell locomotion over substrates, cell-substratum adhesion strength is one of the most important controlling factors [197]. Previous studies indicated that, from both mathematical modeling and experiments, cell migration rates have a biphasic dependence on the concentration of adhesive ligands and cell adhesion strength. As the concentration of extracellular matrix proteins is altered, cell migration speed increases as the concentration increases. Maximal speed is achieved at an intermediate concentration of adsorbed extracellular matrix proteins [197, 198]. In this study, the effective adsorption of overall extracellular matrix proteins and adhesion of RAEC were both enhanced on S-Ti compared with the other substrates, while on the other hand, RAEC migration was also the best on S-Ti. These results suggest that the surface concentrations of extracellular matrix proteins on all the substrates were below the intermediate level, which allowed for increased endothelial cell adhesion and migration behavior with increased adsorbed proteins.

To further investigate the migration of RAEC on different substrates, the focal adhesion protein vinculin was analyzed in this study by fluorescence microscopy. Protrusion of cell lamellipodia is controlled by the formation of focal adhesion on the leading edge of the cell and disassembly on the rear edge, thus, focal adhesion is one of the most important regulators of cell

migration. As discussed before, the focal adhesion distribution of RAEC on S-Ti, which formed small clusters on the substrates, was different from the homogeneous distribution on F-Ti. Previous studies showed that the small-cluster-formed cells could promote cell mobility and spreading, which might also explain faster RAEC migration on S-Ti and on N-Ti than on F-Ti [119, 199]. In addition, it was observed that the focal adhesion clusters were mainly on the edges of protruded filopodia on S-Ti and N-Ti, confirming the strong mobility of RAEC on these substrates.

4.9. Immune Responses

Macrophages, as the primary cell involved in acute inflammation after implantation of vascular stents, plays a key role in determining stent biocompatibility. Thus, investigating how macrophages respond to prospective biomaterials is necessary for predicting their efficacy. In this study, IC-21 macrophages were cultured on the substrates with different roughness created by e-beam deposition. Lipopolysaccharide (LPS), as an inflammatory activator was treated in one group of the experiments considering that LPS is prevalent inside the body even under aseptic conditions. The results of 12 and 24-hour adhesion tests showed that whether treated with LPS or not, macrophage density decreased on S-Ti and N-Ti, especially on N-Ti, compared to F-Ti.

Previous studies showed that pro-inflammatory functions of macrophages were induced on titanium substrates produced by acid etching with rough surface features [200]. However, the source of roughness was not investigated. Therefore, it was unclear whether the roughness came from nanostructure or sub-micron structure. The results of this study investigated the effects of different kinds of roughness (sub-micro and nano roughness) on IC-21 macrophage functions. Besides macrophage density assays, their morphologies were also studied here. Results of this work (Figure 3.45) showed that the macrophage spreading was dependent on surface roughness. Although the cell density on S-Ti was higher than that on the N-Ti, the average cell area on S-Ti was the smallest of all the substrates, which might suggested that among the adherent macrophages on S-Ti, less cells became polarized compared with the other substrates.

Furthermore, to study the effects of surface roughness on macrophage polarization, the expression of inflammatory mediators, i.e., cytokines, was investigated here. The release of three major pro-inflammatory cytokines, IL-1 β , IL-6 and TNF- α , was measured for up to 48 hours. Without LPS, the expressions of IL-1 β , IL-6 and TNF- α were undetectable on all the substrates. In the presence of LPS, specifically, the expression of each cytokine was the highest on F-Ti. Along with macrophage adhesion and spreading, results demonstrated that, flat titanium surface features, which

didn't contain any nanometer or sub-micron roughness, might work together with LPS to affect the activation of IC-21 macrophages.

Comparing S-Ti and N-Ti, the expression of IL-1 β on S-Ti was slightly higher than that on N-Ti (although not significant), while the concentrations of IL-6 and TNF- α on N-Ti were similar on both substrates. These results suggest both S-Ti and N-Ti decreased pro-inflammatory cytokine synthesis by activated IC-21 macrophages.

Paul *et al.* studied the topographical control on human macrophages by micro- and nanostructured (poly)-vinylidene fluoride (PVDF) textures. It was reported that microstructured but not nanostructured PVDF triggered the activation of macrophages in a different way than LPS [201]. These results were consistent with what were observed in the present study. That is, N-Ti didn't enhance the activation of IC-21 macrophages in the presence of LPS. Liu-Snyder *et al.* reported decreased IC-21 macrophage adhesion and proliferation on greater nanometer surface rough alumina surface features created by cold compaction [202].

The adhesion and differentiation of THP-1 monocytic cells were also investigated in this study since monocytes differentiate into macrophages. THP-1 monocytes differentiated into macrophage-like adherent cells after activation with phorbol 12-myristate 13-acetate (TPA). THP-1 monocytes

are often used with vascular endothelial cells to provide models of atherosclerotic development in numerous studies [170]. In this study, THP-1 monocytes stimulated by TPA adhered directly onto S-Ti, N-Ti and F-Ti for 4 hours in one set of the experiments. In another set of the experiments, activated THP-1 monocytes were cultured on a monolayer of endothelial cells grown on the various titanium substrates. After 4 hours, monocyte density decreased on N-Ti compared with the other two substrates, which was consistent with the results of IC-21 macrophage adhesion. However, the results of monocyte adhesion to endothelial monolayers showed that the lowest cell density was detected on S-Ti. This might indicate that the underlying titanium surface features of S-Ti changed the functions of endothelial cells so that their adhesiveness for monocytes was reduced. Previous studies [203] proved the important role of NO_x in deactivating monocyte adhesion to endothelial cells. In this study, it has been shown that when interacting with S-Ti, endothelial cells generated the highest amount of NO_x among all the substrates, which could lead to a decreased adhesiveness of endothelial cells and increased resistance of endothelial cells to monocyte adhesion.

4.10. Endothelial Cell and Monocyte Adhesion Under Flow

Since the previous cell experiments were conducted under static conditions, it would also be of value to determine endothelial cell adhesion and migration in flow conditions which simulate the *in vivo* stent

environment. If endothelial cells can rapidly adhere and proliferate on sub-micron to nanorough titanium surfaces, there will be greater protection of the vascular stent surface from any adverse inflammatory and thrombogenic responses. After vascular stents are implanted into blocked arteries, they are exposed directly to blood stream. Clinical studies showed that vascular stents could prevent balloon-endothelium contact so that endothelial cell damage could be reduced and vascular healing accelerated on stent surfaces [204]. However, another question was proposed here, that is, which surface properties of vascular stents further influence endothelial cell regrowth and endothelium integrity. To answer this, a home-made flow system with pulsatile flow was established in this study to provide a reliable method for investigating the adhesion strength of vascular cell under flow.

As discussed before, under static conditions, RAEC adhesion was significantly increased on sub-micron titanium surfaces (S-Ti) when compared to the other substrates (nano and flat). Results also showed a strong adhesion strength of RAEC on sub-micron titanium surface with the highest surface energy. With the help of the home-made flow system, RAEC monolayers with underlying titanium roughness were placed under flow conditions (10 dynes/cm^2) for 24 hours. Under a fluorescence microscope, it was observed that S-Ti retained the integrity of endothelial cell monolayers very well. On the other hand, the cell layers on N-Ti were

partially intact, but many cells were isolated from surrounding cells due to the flow of cell culture medium. F-Ti showed much less remained endothelial cells on the surface, indicating a lack of endothelial cell adhesion strength to the flat titanium surface. Quantitative analysis of RAEC coverage on the substrates confirmed the observations from fluorescence images, which showed that more than 90% of the S-Ti area and more than 80% of N-Ti area were still covered by RAEC after exposure to flow for 24 hours. Only 40% of the F-Ti area was covered by RAEC under the same conditions.

The results obtained in this study were also consistent with previous studies under flow conditions. Martines *et al.* demonstrated that under dynamic conditions, endothelial cells preferred to attach to nanopitted PMMA surface features (100 nm) created by e-beam lithography after 1-hour exposure to flow [205]. Therefore, it could be suggested under both static and dynamic conditions, surface roughness might have strong control on endothelial cell functions and increased surface roughness might enhance endothelial cell adhesion strength.

THP-1 monocyte adhesion onto endothelial monolayers under flow conditions was also investigated in this study. Interestingly, it was found that under both static and dynamic conditions, the effects of titanium surface roughness and surface energy on monocyte-endothelial cells

interactions were the same, that is, monocyte adhesion to the endothelium decreased on S-Ti compared to N-Ti and F-Ti and on F-Ti, the highest monocyte density was observed. Sub-micron surface roughness might change the adhesiveness of the endothelial cells to titanium surfaces, which further led to the reduction of monocyte adhesion to endothelial cell layer.

4.11. Future Directions

In the present study, cold compaction, lithography, and e-beam deposition was successfully used to fabricate nano and submicron titanium surfaces in this study. E-beam deposition was also used to fabricate patterns on titanium surfaces. A number of *in vitro* investigations, including vascular cell responses, immune cell responses, and flow chamber simulation, indicated that such e-beam deposited titanium has great potential for better vascular stent surfaces. Of course, there is a lot room to explore the use of e-beam deposition for vascular implants and it would clearly be beneficial for patients if such stents can be brought to the next level. Some future directions to make this happen are discussed below (Figure 4.7).

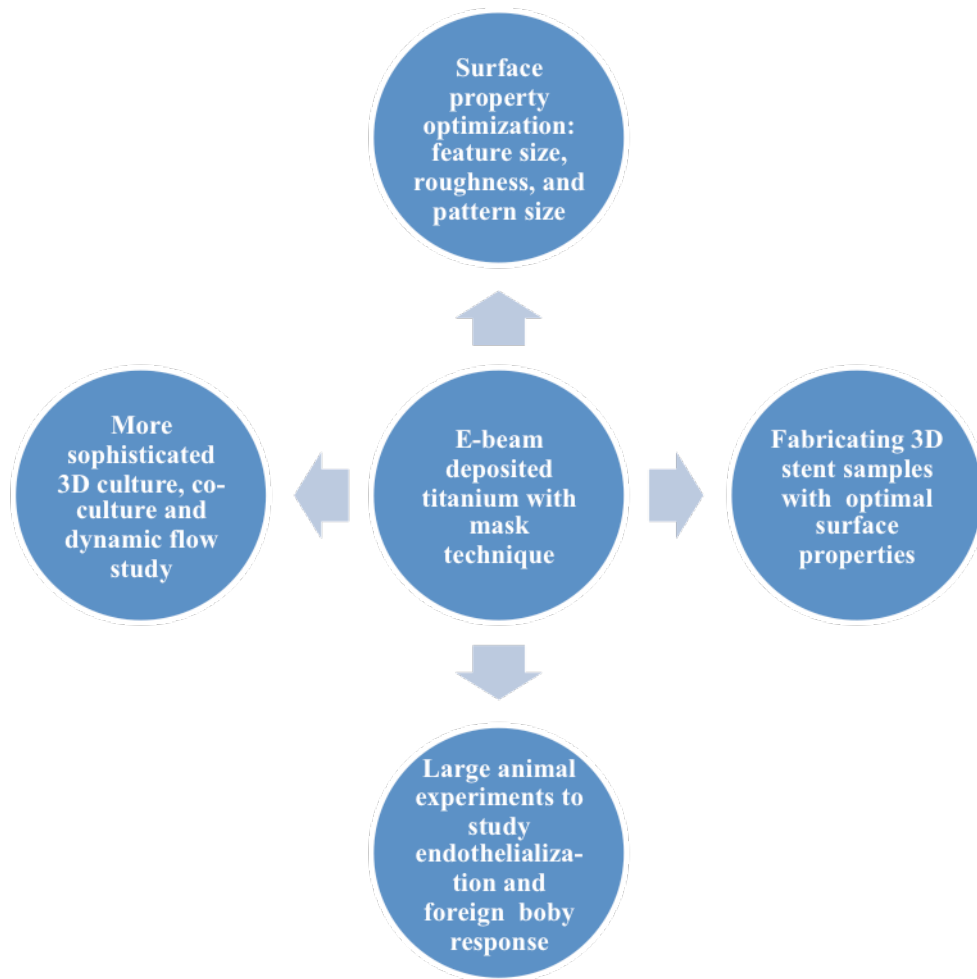


Figure 4.7 Future directions of the present study focusing on developing the next generation vascular stents (without drugs) using e-beam deposition method.

1. Optimize feature size and roughness of titanium surface as well as pattern sizes.

The present study created general titanium surface features at the nanoscale (N-Ti) and submicron scale (S-Ti), which had RMS roughness values around 2 and 14 nm, respectively. According to the results of vascular cell and immune cell responses in this study, it is

considered that a titanium surface with properties between S-Ti and N-Ti would be an ideal candidate to improve the overall performance of stents. This would require some extra work to optimize these surface parameters on titanium. Since only one linear pattern width (30 μm) was used in the present study, much smaller pattern sizes (by using masks with smaller pattern sizes) down to submicron and nanoscale should be evaluated in the future. Existing evidence from TIDE samples already confirmed that decreased pattern size could enhance the endothelialization process. With combined optimal surface feature size/roughness and surface patterns, this new candidate titanium surface could be used for further studies such as for actual 3D stent fabrication and in vivo experiments.

2. Use actual 3D stent samples in future in vitro/ex vivo studies.

In the present study, flat titanium coatings deposited on glass were tested for all in vitro experiments. Thus, while this is common for 2D cell culture systems which do not accurately reflect the actual 3D stent environment to better simulate the natural environment, 3D stent samples should be used in future studies. To do this, titanium coatings can be deposited onto flat titanium meshes using optimal coating parameters and appropriate masks. Then, the mesh could be folded to form a 3D stent shape and used for in vitro experiments. Or the titanium coatings could be deposited on flat titanium surfaces by

rotating the stent. It is important to note that each side of the stent, inner and outer, can be coated using different conditions. Specifically, the inner surface should be treated to both maximize endothelialization and minimize foreign body responses while the outer surface should focus on decreasing immune response [206-208].

3. Study cell responses under more sophisticated cell models: 3D cell culture, co-culture and dynamic flow conditions.

As mentioned above, conventional in vitro cell culture models (2D and static) are not good enough to reproduce many of the key factors found in the natural environment. It is well known that cell-cell interactions, cell-matrix interactions as well as diffusion and transport phenomenon can be remarkably different under 3D culture, competitive culture, and flow culture conditions [208]. Therefore, results from the current in vitro study usually cannot accurately predict outcomes in animal experiments. In other words, this limits the significance of in vitro studies to serve as better screening/evaluating tools before doing animal studies.

An important effort of the present study is to employ some of the more complicated cell culture models, including co-culture and dynamic flow, to better understand different cell behaviors in vitro. As a next step, when 3D stent-like samples are used, a 3D culture model is needed

and should be combined with other complicated cell culture models like co-culture and dynamic flow. To do this, the rectangular, 2D flow chamber system used in the present study should be modified to be a cubic chamber to hold 3D stent samples (Figure 4.8). Various cells (including endothelial cells, smooth muscle cells, and immune cells) can be selectively added into the culture system. For example, endothelial and smooth muscle cells can be co-cultured in this dynamic flow chamber, whose results can then be compared to the static study conducted in the present study. Additionally, chemical factors (such as LPS or TPA) can be added as stimuli into the flow chamber system if needed.

Besides, the system will allow studying pre-coated stent samples in a dynamic situation. For instance, to study the influence of inflammation on endothelialization, titanium stents pre-coated with an endothelial monolayer could be co-cultured with macrophages. Although the present study focused on bare-metal stents, it is also possible to study drug-eluting stent in this system.

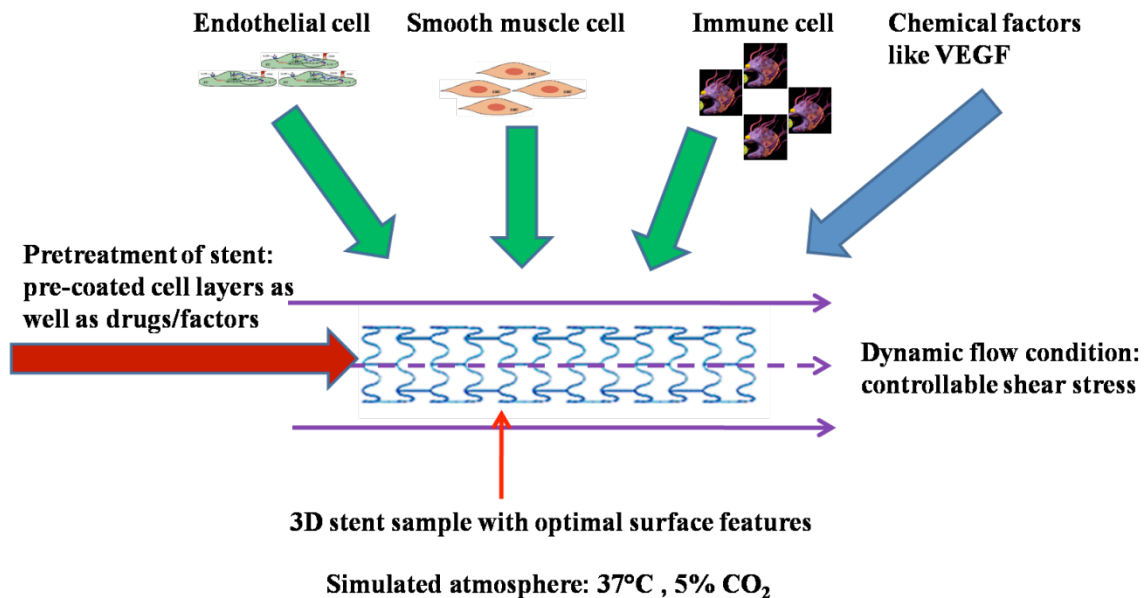


Figure 4.8 Schematics of a cell culture system to study vascular stents combining 3D culture, co-culture and dynamic flow conditions. VEGF is vascular endothelial growth factor.

4. Large animal study.

When the above-mentioned *in vitro* models provide successful stent candidates (starting with S-Ti and N-Ti studied here), animal models should be used. To test the biocompatibility of vascular stents, an ovine (sheep) model should be used [209, 210]. E-beam deposited stents and control stents should be implanted into sheep iliac arteries.

Animals should be then sacrificed after 3 months. Pre-, post-implantation and pre-explantation luminal diameters should be measured for each implant and implantation site. These intravascular ultrasonography and angiographic measurements should indicate luminal narrowing. Histopathology will be used to evaluate the

neointimal area. Specifically, segment slides should be prepared and stained with hematoxyline and eosin. Accumulation of immune cells (granulocytes, lymphocytes, giant cells) should be counted to evaluate inflammation response. Finally, electron microscopy should be used to examine endothelialization on all stents. Only after translating the promising *in vitro* data from this study to *in vivo*, will the next generation of vascular stents be found.

CHAPTER 5. CONCLUSIONS

The contribution of the present study to the field of vascular implantology centers on finding optimal surface properties of bare-metal vascular stents to control cellular responses using nanotechnology. Further contributions from this study to the field are outlined below:

1. Nano and micropatterned titanium surfaces consisting of periodic arrays of grooves with spacings ranging from 750 nm to 100 μm were successfully fabricated in the present study by utilizing a novel plasma-based dry etching technique (TIDE) that enables machining of titanium with unprecedented resolution.
2. Using *in vitro* models to demonstrate enhanced endothelial cell responses on nanometer-scale titanium surface feature patterns compared to larger micrometer-scale titanium surface feature patterns, as well as controls consisting of the aforementioned random nanostructured surface features. Furthermore, nanometer patterned titanium surface features induced endothelial cell alignment similar to the natural endothelium.
3. E-beam evaporation combined with a masking process was developed in this study to create different titanium roughness values including sub-micron roughness, nano roughness and flat. Moreover, with this method, as low as 30 μm patterns of titanium particles have been produced to combine different

- scales of roughness on the same substrates. Results of surface characterization demonstrated that nano (less than 50nm in both the lateral and vertical dimension) and sub-micron (larger than 100nm in the lateral dimension) surface structures drastically altered titanium surface energy (one important factor that controls cell adhesion) without the modification of surface chemistry.
4. A complete *in vitro* study with vascular endothelial cells, smooth muscle cells, immune cells (monocytes/macrophages) and osteoblasts was carried out on different titanium roughness values created by e-beam deposition and the results indicated enhanced cell responses on both nano structured and sub-micron structured titanium surfaces compared to flat (smooth) titanium surfaces.
 5. Fine grain (nano to submicron roughness) and coarse grain (micron roughness) NiTi surface features were created by cold compaction. *In vitro* studies showed that fine grain NiTi substrates with greater submicron to nanometer roughness were more effective at improving endothelial cell responses compared to coarse grain NiTi.
 6. A flow system with a parallel flow chamber and a pulsatile pump was constructed in this study. Titanium surfaces with features created by e-beam deposition were found to be stable under flow. A preliminary *in vitro* study

under flow was conducted with vascular endothelial cells and monocytes.

Results demonstrated enhanced endothelial cell and monocyte adhesion on nano structured and sub-micron structured titanium surfaces compared to flat (smooth) titanium surfaces.

7. Adsorption of major proteins (vitronectin, fibronectin and fibrinogen) on different titanium roughness values was conducted to determine the mechanism why endothelial cell responses were improved on nano/sub-micron titanium surfaces. Results demonstrated protein adsorption was correlated with surface energy. Greater surface energy on nano/sub-micron titanium surfaces leads to enhanced protein adsorption, resulting in improved endothelial cell adhesion (Figure).

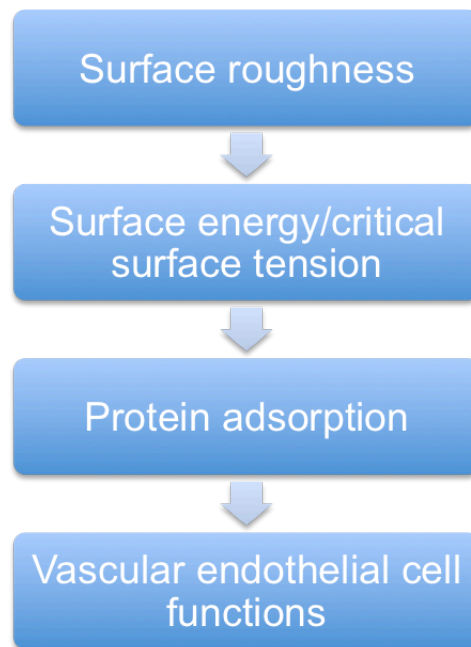


Figure 5.1 Correlation of surface energy, roughness, protein adsorption and vascular endothelial cell functions.

In summary, different titanium surface properties (including ordered surface features), different surface roughness and surface energy, were created by TIDE, e-beam deposition and cold compaction in this study. These surface properties were investigated for their ability to control vascular cell and immune responses to find the optimal surface parameters for better bare-metal vascular stents. For the first time, results demonstrated that e-beam deposition was able to control different optimal surface properties (nano to sub-micron patterned titanium) and had great potential for designing novel bare-metal vascular stents.

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