2008

Biocompatibility of Osteoblast Cells on Titanium Implants

Dilip Ayyala Somayajula
Cleveland State University

BIOCOMPATIBILITY OF OSTEOBLAST CELLS ON TITANIUM IMPLANTS

DILIP AYYALA SOMAYAJULA

Bachelor of Technology in Chemical Engineering

Jawaharlal Nehru Technological University

April, 2003

submitted in partial fulfillment of requirements for the degree

MASTER OF SCIENCE IN CHEMICAL ENGINEERING

at the

CLEVELAND STATE UNIVERSITY

May, 2008

This thesis has been approved

for the Department of Chemical and Biomedical Engineering

and the College of Graduate Studies by

Thesis Chairperson, Dr.Joanne M. Belovich

Department & Date

Dr.S.N.Tewari

Department & Date

Dr.Ronald J.Midura

Department & Date

This thesis is dedicated to my parents Krishna Murthy and Surya Kumari
and my wife Deepti

ACKNOWLEDGEMENTS

I sincerely thank my research advisor Dr. Joanne M Belovich, for her continuous support and guidance during my research and while preparing this report. I would like to thank Dr. Ronald Midura and Dr. Surendra Tewari for giving me valuable suggestions while conducting experiments and allowing me to use their laboratory.

I also take this opportunity to thank Mrs. Sharon Midura for helping me learn aseptic techniques in cell culture. I specially thank Mr. Pradeep Kodumuri for helping me all the way preparing titanium samples and imaging them. I appreciate support and enthusiasm provided by my laboratory colleagues.

BIOCOMPATIBILITY OF OSTEOBLAST CELLS ON TITANIUM IMPLANTS

DILIP AYYALA SOMAYAJULA

ABSTRACT

Adhesion and proliferation of UMR 106-01 osteoblast cells were studied on various surface modified titanium materials such as polished, sandblasted, anodized and alkaline treated. Anodization of polished surface in Hydrofluoric acid developed nano-tubes, while NaOH treatment produced spongy microporous morphology. Test samples were coated with non-adhesive protein bovine serum albumin and compared with fibronectin coated specimens. The adhesion study lasted for 4 hrs, where osteoblast cells were cultured in serum free medium. Polished titanium, anodized titanium and NaOH titanium have shown similar percentages of cell adherence. The proliferation study lasted for 48 hrs, where cells were initially allowed to adhere to the surface in serum free medium for 4 hrs, followed by a medium change to 10% fatal bovine serum. The specific growth rate after 48 hrs in culture on the polished surface was found to be comparable to the tissue culture plastic, which exhibited a high growth rate. No significant difference was found in cell numbers between polished, anodized and NaOH-Ti, but each has varying cell orientation on the surface. Fluorescence images stained with alkaline phosphatase revealed that polished surface had cells flattened to the surface with short filapodia. Anodized surface had cells uniformly distributed across the surface where as NaOH-Ti displayed cells in colonies. Cells were found bonding to the surface of NaOH-Ti firmly using their filapodia as an anchoring agent. These results suggest that NaOH-Ti provides support in initial hours of implantation and bolsters cell proliferation. All together this process may help to better integrate titanium implant surfaces.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Titanium is considered as a wonder metal, the glamour metal and the metal of promise[1] by many researchers and orthopedic surgeons. Due to its properties such as corrosion resistance, its inert nature, its ability to adsorb proteins readily onto its surface, and low cytotoxic leachables, titanium has been considered as a very good biocompatible material for surgical implantation for years. Its applications lie in orthopedic surgery, maxillofacial and oral surgery, neurosurgery, and cardiovascular surgery. Worldwide the dental implant market is estimated to be $1.2 billion[2] (US) and expected to grow at a rate of 15% yearly. The market for orthopedic implantation is expected to be $8.7 billion (US) presently and anticipated to grow to $11.6 billion[3] (US) in 2012.

A good implant is expected to be osseointegratable and osteoconductive in nature. Titanium is found to be the best biomaterial, which stands out with its extraordinary properties, compared to stainless steel, ceramics, and other plastics. Both the commercially pure titanium and its alloys show better corrosion resistance than any other material when contacted with human bone, body fluids, and soft tissue. Titanium is 40%

lower in density than stainless steel; at the same time has good fracture and wear resistant properties. However no material even titanium has been completely free of adverse reactions in either humans or animals.

The search of the best biomaterial for implantation is not complete with the material selection. It is found that cell interactions are highly dependent on surface topography (micro/nano porous structure) and surface chemistry[4]. No direct relationship between these two parameters was found yet. It would be advantageous to learn such physico-chemical characteristics of surfaces, which would help in developing osteoblast friendly biomaterials.

Extensive research work is ongoing to understand osteoblast cell interaction with titanium materials. As soon as an implant is placed in a human body, the first event that happens is the interaction of the implant material with the body fluids or blood plasma. Blood plasma makes up to 55% of the total volume of the blood. Blood plasma is 90% water and the remaining 10% consists of proteins such as albumins, globulins, and fibrinogens. Serum albumin occupies a maximum proportion of the plasma proteins. It is believed that albumin is the first protein (Meyer et al.[4]), which might be interacting with titanium surface after implantation. Albumin is a non-adhesive protein i.e., it does not support attachment (Yunzhi et al.[5]).

Many extracellular proteins such as collagen, thrombospondin, fibronectin, vitronectin, and osteopontin, have been shown to augment bolster the cell attachment on implant

materials. It is due to this reason that there are numerous papers available on the interaction of osteoblast cells on micro/nano porous textured titanium surfaces using adhesive protein coating. However, it is not always feasible both economically as well as aseptically to produce titanium implants with such adhesive protein coating. So, the present research work concentrates on developing a titanium surface where no adhesive protein coating is necessary, yet can support osteoblast attachment. Such study would simulate a situation where cells interact with only surface texture rather than proteins during the initial attachment phase.

The current research study uses the findings from the Mata et al.[6] work with UMR 106-01 osteoblast cells on the surface of chlorotriflouroethylene coverslips with bovine serum albumin (BSA) as a protein coating. Interestingly it was found that cells were able to adhere to the surface in the presence of BSA and suggests that surface texture can also promote cell attachment.

The aim of this thesis is to develop a titanium surface that promotes cell adhesion and proliferation. In this work, titanium alloy (Ti-6Al-4V) was polished and then treated with NaOH or anodized in HF. Some samples were polished and sandblasted and then treated with NaOH or anodized in HF. The experimental setup was developed in such way that cells interact only with the titanium surface. This study is unique as the titanium alloy specimens were coated with a non-adhesive protein serum albumin, which simulates in vivo implantation. Cell numbers were obtained by DNA assay and cell morphology determined using fluorescence microscopy.

Results associated with this work will enable the assessment of surface modification procedures of titanium in order to qualify their use with implants. Implant loosening is considered to be a serious issue in orthopedic surgeries. It is believed that replacement of a hip joint replacement is very complicated and not as successful as first time operation of hip replacement. This work significantly helps in developing an implant surface, which is more biocompatible, bioadhesive and osteoconductive. Such material would help arrest loosening after implantation as well as stop osteolysis.

CHAPTER II

BACKGROUND

2.1 Bone

Bone is a dynamic tissue and is made up of several other tissues such as osseous tissue, cartilage, dense connective tissue, nervous tissue, and epithelium and adipose tissue. Eighteen percent of human body weight (wet weight) is made up of bones. Bone stores minerals such as calcium and phosphorous and whenever necessary it releases them into the bloodstream to maintain blood serum mineral balances.

Osseous or bone tissue contains abundant extracellular matrix whose composition is about 25% water, 25% collagen fibers and rest is crystallized mineral salts (The percentages mentioned depends on age of the bone). The most abundant mineral is calcium phosphate ($Ca_3(PO_4)_2$), which combines with calcium hydroxide ($Ca(OH)_2$) to form calcium hydroxyapatite. Such formed calcium hydroxyapatite crystals combine with other mineral salts such as calcium carbonate ($CaCO_3$) and ions such as Mg^{++}. These minerals are deposited in the framework formed by collagen fibers of the extracellular

matrix; they crystallize and eventually tissue hardens. This process of calcification is initiated by bone forming cells called osteoblasts.

2.2 Bone Morphology

Ossification (bone formation) happens in two ways, intramembranous ossification and endochondral ossification. Four types of cells exists which help in bone formation, resorption and maintenance. They are osteogenic cells, osteoblast cells, osteocytes, and osteoclasts (Figure 1).

Osteogenic cells are located along the inner portion of periosteum, in the endosteum, and within the blood vessels. These cells are derived from mesenchyme, the tissue from which all connective tissues are formed. Osteogenic cells undergo cell division and develop into osteoblast cells.

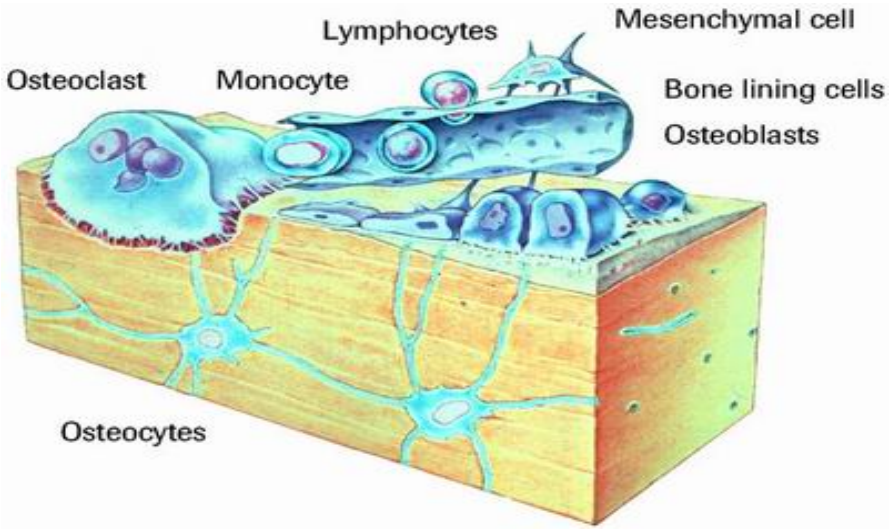

Figure 1. Diagram shows osteoblast, osteoclasts and osteocytes cells.
Image source: http://www.medicalook.com/human_anatomy/organs/Bone_cells.html.

Osteoblast cells are also known as bone building cells. They secrete collagen type-1 fibers and other minerals necessary for bone formation. Osteoblast cells secrete extracellular matrix containing calcium hydroxyapatite and get trapped to become osteocytes. Osteocytes help in maintaining the bone by exchanging the nutrients and wastes with blood stream. They do not undergo any further cell division. They respond to mechanical and electrical signals in the bone. Osteoclasts are multi-nucleated cells, derived from fusion of as many as fifty monocytes/ macrophage precursors and are located in the bone marrow and along the endosteum. They release lyzosomal enzymes and acids, which digest the bone matrix. These cells help in resorbing fractured or damaged bone.

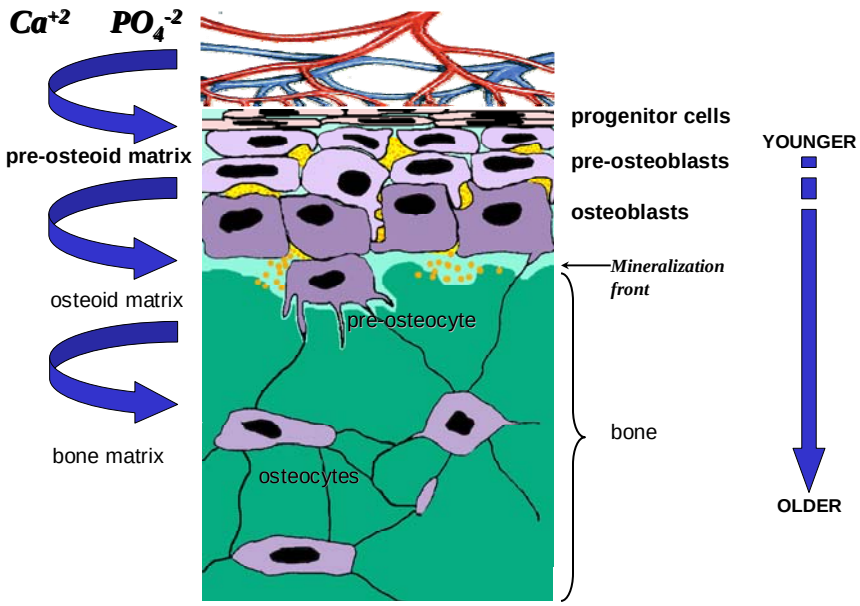

Figure 2. Osteoblast mineralization.
Image source: Lecture notes of Biomineralization by Dr.Ronald. J. Midura.

2.3 Titanium Implantation

A summary of different stages in bone healing and remodeling is provided by Setti et al.[24]. Neutrophils and macrophages are the cells that first arrive at the surface after titanium implantation. Neutrophils are produced in bone marrow and they play an important role in inflammation. Macrophages are usually in the resting state until activated. Activated macrophages remove unnecessary material from the site of inflammation. Osteoprogenitor cells migrate to the site of implantation and differentiate to form osteoblast cells, which further differentiate and mineralize. Fibroblasts are attracted towards the implantation site by cytokines released by the macrophages and they then encapsulate the titanium material into the bone. Different stages in bone healing/ remodeling are summarized in table 1.

Table 1. Stages of Bone Healing and Remodeling
Reproduced table from: Setti S. Rengachary.M.D. Bone morphogenetic proteins: basic concepts. Neurosurg Focus 13(6), December 2002, p 1-6.

Stages of bone healing and remodeling	
I: Induction	Formation of hematoma at fracture site: release of growth factors & cytokines
II: Inflammation	Recruitment of inflammatory cells, macrophages, & fibroblasts to the injury site
III: Cartilage formation	Mitosis of mesenchymal cells and differentiation of chondrocytes; hypertrophy of chondrocytes & calcification; deposition of extracellular collagenous matrix; local angiogenesis
IV: Woven bone formation	Differentiation of osteoblasts, mineralization of EM
V: Lamellar bone formation	Bone resorption, remodeling, formation of lamellar bone & hematopoietic marrow

2.4 Cell Culture Models

Various cell lines have been used as *in vitro* models of osteoblast cells for testing biocompatibility of titanium implants, specifically with regard to cell adhesion and proliferation. They are: osteosarcoma cell lines, intentionally immortalized cell lines, non-transformed clonal cell lines, and primary cultures (bone marrow stromal cells, intramembranous bone or periosteal-derived cells). The research work presented here employs UMR 106-01 osteoblast cells, an osteosarcoma cell line that requires a substrate to adhere and to survive. It is believed that if a surface failed to attract adherent osteoblast cells onto its surface, then such an implant would fail to integrate in to bone.

2.5 Various Factors Influencing Cell Activity

Table 2. Different Growth Factors Involved in the Bone Generation and Remodeling
Table source: Setti S. Rengachary.M.D. Bone morphogenetic proteins: basic concepts. Neurosurg Focus 13(6), December 2002, p 1-6.

TABLE 2

Growth factors and cytokines involved in the generation of new bone and remodeling

BMPs
TGF-β
PDGF
insulin-like growth factor SI & II
epidermal growth factor
fibroblast growth factor
vascular endothelial growth factor
Turner necrosis factor

BMP- Bone morphigenetic protein, TGF-Transforming growth factor, PDGF-Platelet-derived growth factor.

Table 2 show different growth factors involved in the generation of new bone. There are numerous other parameters, which also influence the osteoblastic phenotype expression. A few important ones are culture medium, culture time and number of passages in culture. β-glycerophosphate ascorbate and dexamethasone also influence the in vitro cell behavior.

Recently two vital factors were identified that affect bone remodeling both in vitro and in vivo. One factor is an osteoclast differentiation factor known as RANKL also known as TRANCE or osteoprotogerin ligand (OPGL) and the other factor is osteoprotogerin (OPG). Zreiqat et al.[7] research work on human bone derived cells showed that surface modification of Ti alloy (Ti-6Al-4V) with peptides such as Arg-Gly-Asp (RGD), RGE and cystine affected cellular mechanisms. They found increased expression of m-RNAs for osteocalcin, pro-collagen Iα1, and alkaline phosphatase on RGD-coated Ti-6Al-4V compared to uncoated Ti alloy. Proteins such as osteocalcin, type I collagen, and bone sialoprotein are markers of osteoblastic differentiation. RGD-coated titanium alloy has shown higher levels of proteins than RGE-and cystine-coated titanium alloy surface. Zreiqat et al. also found that Ti-6Al-4V coated with peptide such as RGD, RGE, cystine reduced OPG protein production but increased RANKL m-RNA expression. These results suggest that surfaces modified with peptides had an effect not only bone formation but also on bone resorption bone through osteoclast formation.

2.6 Surface Topography and Chemistry of Titanium

Surface topography has profound impact on osteoblast cell attachment, proliferation, and differentiation. Bren et al.[8] work confirms that surfaces with nano-scale roughness have greater influence over osteoblast differentiation than micro-scale roughness. Keller et al.[9]. showed that osteoblast attachment to titanium is directly related to the surface roughness. Keller et al.[10] also determine that core-binding factor alpha subunit 1 (Cbfa1) and BSPII gene expression are influenced by surface microtopography. Cbfa1 is a transcription factor that regulates osteoblast differentiation while BSPII gene expression is important for mineralization. Degasne et al.[11] work on human osteoblast-like cells (Saos-2) indicates that surface roughness and presence of adhesive proteins such as fibronectin or vitronectin are important for cell attachment and proliferation on titanium.

Surface chemistry has also been found to influence the implant selection in terms of protein and cell adhesions. Klinger et al.[12] confirmed that electrostatic interactions are involved in the adsorption of human serum albumin to on commercially pure titanium. Divalent calcium ($Ca+^{2}$) and magnesium (Mg^{+2}) increase the adsorption of albumin on titanium. pH of the environment is another controlling parameter for albumin adsorption. Fibronectin and vitronectin are found to be good cell adhesive proteins as they contain Arg-Gly-Asp (RGD) sequence, which is specific to cell adhesion. Bren et al. research work suggests that a high surface free energy of the material corresponds to a high differentiation rate of osteoblast cells.

2.7 Surface Modification of Titanium

Surface modification is necessary for titanium in order to use it as an implant material. Bare titanium after manufacturing is prone to oxidation, contamination, and plastic deformation. It is non-uniform and poorly defined. Material with such features cannot be used as an implant. The other reason to modify the surface of titanium is that cells respond to the surface chemistry and surface texture or morphology. Good surface modification treatments retain the extraordinary physical properties as well as bolster tissue integration and bone growth. Liu et al.[13] has provided a summary of different mechanical, chemical and physical surface modification methods, in Table 3

Table 3. Titanium Surface Modification Methods
Table source: Liu et al.[13]

Overview of surface modification methods for titanium and its alloys implants

Surface modification methods	Modified layer	Objective
Mechanical methods		
Machining Grinding Polishing Blasting	Rough or smooth surface formed by subtraction process	Produce specific surface topographies; clean and roughen surface; improve adhesion in bonding
Chemical methods		
Chemical treatment		
Acidic treatment	<10 nm of surface oxide layer	Remove oxide scales and contamination
Alkaline treatment	~1 μm of sodium titanate gel	Improve biocompatibility, bioactivity or bone conductivity
Hydrogen peroxide treatment	~5 nm of dense inner oxide and porous outer layer	Improving biocompatibility, bioactivity or bone conductivity
Sol–gel	~10 μm of thin film, such as calcium phosphate, TiO_2 and silica	Improve biocompatibility, bioactivity or bone conductivity
Anodic oxidation	~10 nm to 40 μm of TiO_2 layer, adsorption and incorporation of electrolyte anions	Produce specific surface topographies; improved corrosion resistance; improve biocompatibility, bioactivity or bone conductivity
CVD	~1 μm of TiN, TiC, TiCN, diamond and diamond-like carbon thin film	Improve wear resistance, corrosion resistance and blood compatibility
Biochemical methods	Modification through silanized titania, photochemistry, self-assembled monolayers, protein-resistance, etc.	Induce specific cell and tissue response by means of surface-immobilized peptides, proteins, or growth factors
Physical methods		
Thermal spray		
Flame spray Plasma spray HVOF DGUN	~30 to ~200 μm of coatings, such as titanium, HA, calcium silicate, Al_2O_3, ZrO_2, TiO_2	Improve wear resistance, corrosion resistance and biological properties
PVD		
Evaporation Ion plating Sputtering	~1 μm of TiN, TiC, TiCN, diamond and diamond-like carbon thin film	Improve wear resistance, corrosion resistance and blood compatibility
Ion implantation and deposition		
Beam-line ion implantation PIII	~10 nm of surface modified layer and/or ~μm of thin film	Modify surface composition; improve wear, corrosion resistance, and biocompatibility
Glow discharge plasma treatment	~1 nm to ~100 nm of surface modified layer	Clean, sterilize, oxide, nitride surface; remove native oxide layer

The present research study employs four surface modification methods from the Table 3. They are i) mechanical polishing, ii) sandblasting, iii) anodization and iv) alkali (NaOH) treatment. Polishing titanium provides very smooth surface and the roughness factor value range in nano-scale. Performing any other surface treatment on such nano-smooth surface will develop micro- and nano-texture.

The following reactions involved in anodizing titanium surface:

at the anode (titanium): $Ti \leftrightarrow Ti^{+2} + 2e^-$

at the Ti oxide/electrolyte interface:

$2H_2O \leftrightarrow 2O^{2-} + 4H^+$ (oxygen ions react with titanium to form oxide)

$2H_2O \leftrightarrow O_2$ (gas) $+ 4H^+ + 4e^-$ (O_2 gas evolves or stick at the electrode surface)

at both interfaces: $Ti^{+2} + 2O^{2-} \leftrightarrow TiO_2 + 2e^-$

The chemical reactions involved in NaOH treatment of titanium are shown here,

partial dissolving of titanium dioxide takes places with NaOH

$TiO_2 + NaOH \rightarrow HTiO_3^- + Na^+$

simultaneously hydration of titanium takes place,

$Ti + 3OH^- \rightarrow Ti(OH)_3^+ + 4e^-$

$Ti(OH)_3^+ + e^- \rightarrow TiO_2 . H_2O + ½ H_2 \uparrow$

$Ti(OH)_3^+ + OH^- \leftrightarrow Ti(OH)_4$

Negatively charged hydrates are produced on further hydroxyl attack on hydrated TiO_2 ,

$TiO_2 . H_2O + OH^- \leftrightarrow HTiO_3^- . nH_2O$.

Na^+ ions react with negatively charged hydrates and produce a sodium titanate hydrogel layer.

Material scientists have been working to find a surface modification method that induces not only the initial cell attachment, but also allow cells to proliferate and mineralize. Andriana et al.[14] cultivated mice osteoblastic cells on three different chemically treated commercially pure (CP) titanium. Their procedures include acid treatment such as hydrochloric acid/sulfuric acid (average roughness (Ra) 2.78 μm), acid treatment plus anodic oxidation with phosphoric acid (Ra 3.04μm), and acid treatment plus thermal oxidation and also immersion in sodium fluoride solution (Ra 2.21μm). Acid treatment creates a rough surface and anodic and thermal oxidation increases the thickness of oxide layer on the surface. Performances by these treated titanium surface were evaluated by means of cell attachment, proliferation and differentiation. It was found that cell differentiation and proliferation were higher in acid treated and acid treated plus thermal oxidation for 21 days in culture, compared to control sample (glass) and acid treatment plus anodic oxidation. Cells attached in greater number on control sample and acid treatment plus anodic oxidation surface of titanium than the any other treatments.

Zhao et al.[15] used MG63 osteoblast-like cells (osteosarcoma cells) on titanium, determining that cells are sensitive to submicron scale features. Morphology of cells remained similar on smooth and anodized surface, but showed elongated structure on etched surfaces. Surface treatments for titanium include polished, acid etched with HCl and H_2SO_4, anodized using H_2SO_4, and sandblasted. Cell numbers were found to be higher on polished surface followed by anodized and acid etched. Osteocalcin and

prostaglandin E2 (PGE2) expression were affected in a reverse manner. Transforming growth factor- β1 (TGF-β1) expression was increased greatly by acid-etched followed by anodized and polished titanium surfaces.

Batailon et al.[16] used MC3T3-E1 osteoblast cell line to determine any effect of surface roughness of Ti-6Al-4V on cell adhesion and proliferation. They found that the smoother the surface, the lower the cell adhesion but higher the cell proliferation. Sandblasting and polished (80, 1200 & 4000 grit polishing) Ti alloys surfaces were used for testing the cell responses. Similar response of polished surface was observed by Lee et al.[17] on neonatal rat calvarial osteoblast cells. They determined that the number of cells attached to the polished titanium surface is higher than sandblasted surface in 4% fetal bovine serum (FBS) culture medium. No difference in cell attachment was found between polished and sandblasted surface with a 10% FBS content.

2.8 Summary

Reviewing past research work, many researchers conform that polished surface (average roughness Ra $\leq$0.06μm) encourage cell attachment. It is also found that increasing surface roughness increases expression of osteocalcin, transforming growth factor, and alkaline phosphatase, which are believed to be the key proteins in osteoblast differentiation. However few others contradicted this result and showed that surface roughness induce cell attachment. It might be due to the practice of defining the surface by its modification treatment rather than surface roughness measurements. This study

concentrates on polished titanium surface with micro- and nano-topography on it. It is expected that the polished surfaces as found previously, will help in osteoblast attachment whereas micro- and nano-texture on the polished surface will promote proliferation and differentiation. Treating the polished surface with sodium hydroxide develops micro-texture on the surface and nano-texture is developed by anodization of polished titanium in HF.

CHAPTER III

METHODS

3.1 Titanium Processing and Preparation Methods

Surface modifications such as polishing, sandblasting, anodizing and alkali (NaOH) treatments were employed in this present research work. Each sample is of 25.4 mm in diameter and 4 mm in thickness.

Mechanical surface polishing: The titanium alloy (Ti-6Al-4V) surface was polished with silicon carbide grit papers starting from 240 and increasing to 400, 600, 800 and 1200. Intermittently after polishing with different sandpapers, the surface was washed with water to rinse off any particles generated while polishing. Ultrasonic cleaning in DI/ultra pure water for about 5 minutes was done after polishing to clean the surface more effectively.

Sandblasting: Some of the polished titanium alloy specimens were blasted with sand particles at a pressure of 90 psi. Ultrasonic cleaning in DI/ultra pure water for about 5 minutes was done to ensure no sand particles were left on the surface.

Anodization: Both polished and sandblasted titanium samples were anodized to produce polished and anodized titanium as well as polished, sandblasted and anodized titanium samples. Anodizing was done in 0.5% w/w HF in water for 45 minutes at room temperature (25 ^{o}C). The platinum strip serves as the cathode and the voltage was maintained at 20 V with a DC power supply. Such a treatment develops a thin titanium dioxide layer of nanotubes whose diameter range from 50-60 nm. Specimens were then cleaned ultrasonically before and also after anodization treatment. Thickness of the titanium dioxide layer so formed is directly related to the applied voltage.

Alkali treatment (NaOH): Both polished and sandblasted samples were treated with NaOH to produce polished and NaOH treated titanium as well as polished, sandblasted and NaOH treated titanium. Samples were treated with 5M NaOH solution in a Teflon beaker at 60^{o} C for 24 hours. Care was taken to maintain the titanium disc in a vertical position. Such a treatment develops a thin layer (about 1 μm) of sodium titanate hydrogel layer.

Combinations of the above described treatments were also employed for this study. Anodization and NaOH treatment was done on both polished and polished-sandblasted titanium alloy specimens.

3.2 Cleaning and Sterilization of Titanium Discs

Cleaning the surface of the titanium also has importance in this study. Titanium surface might have contaminants such as grease, DNA, and protein residues due to human contact while processing.

Titanium alloy discs after processing as described in section 3.1, were first soaked in acetone-ethanol mixture in 1:1 v/v ratio for about an hour in fume hood. Sufficient volume of the mixture is maintained in a conical flask for soaking and the solution was mixed intermittently. Ethanol and acetone are known as good cleaning reagents, which will remove any grease and dust particles. Discs were washed with filtered DI water and soaked in another conical flask containing 2% v/v detergent solution of RBS-35 (Pierce chemicals). Tap water is used to prepare the cleaning reagent. The contents of the conical flask were maintained in under negative pressure and the arrangement is as shown in Figure 3. This was done to eliminate any trapped gas molecules on the surface and maintained in such a way that no contents from the cleaning flask were drawn out.

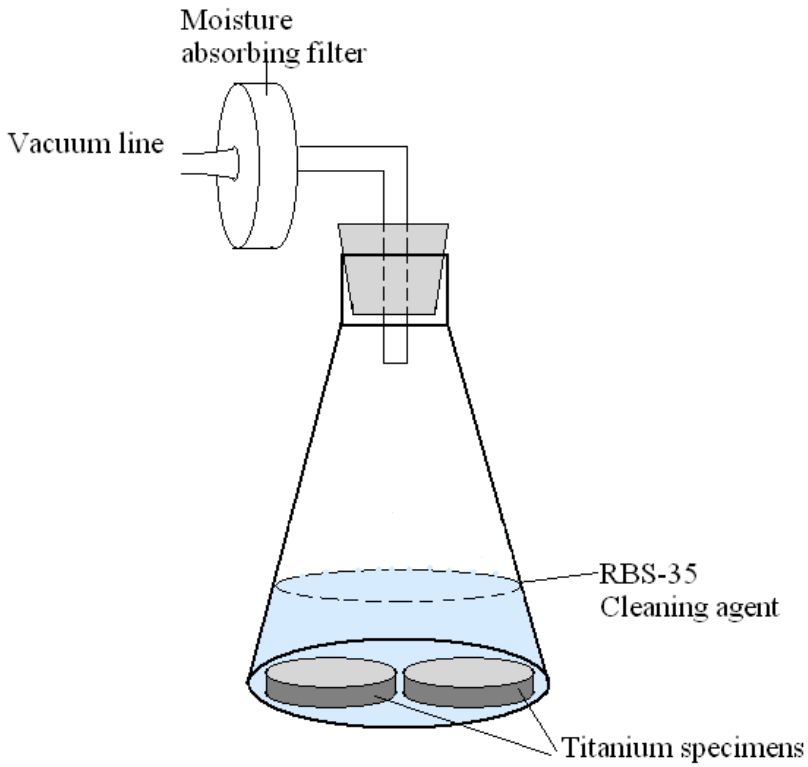

Figure 3.Titanium alloy specimen cleaning set up.

One end of the tube is connected to the flask using rubber stopper and the other end to the house vacuum with inline moisture absorbing filter. Samples were soaked for 4 hrs with intermittent swirling or tapping, in order to eliminate any trapped gas molecules. Discs were immersed in the cleaning reagent in such a way that the textured surface was always facing upwards. Afterwards, samples were washed extensively 10 times with filtered DI water and sterilized overnight by soaking in 70% v/v ethanol water mixture. Discs were always handled by gripping their sides. Steam sterilization process was not employed as this might cause change in the surface of the titanium by altering the titanium dioxide layer.

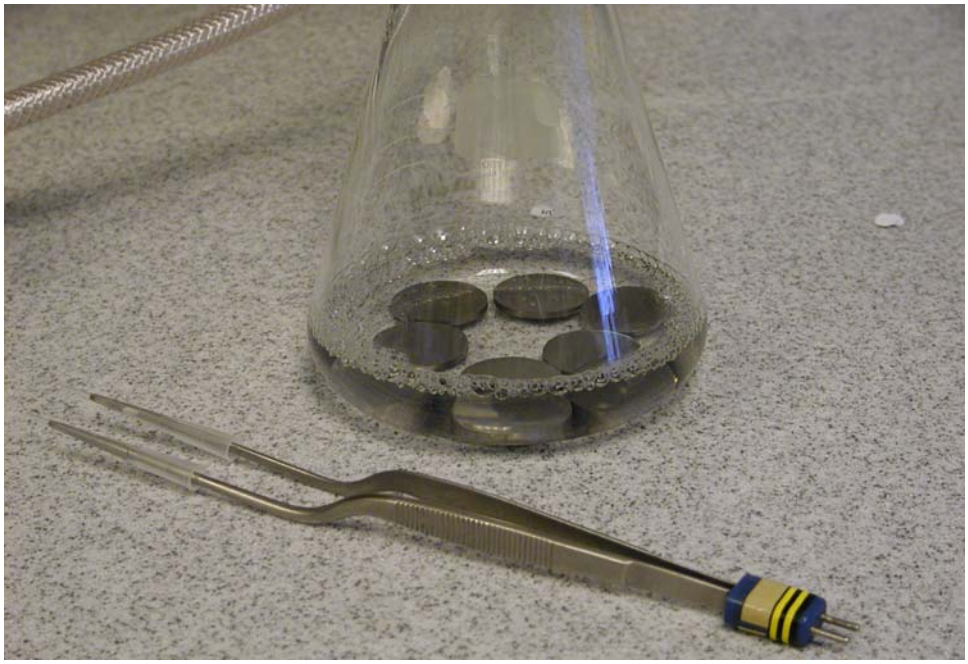

Figure 4. Image showing cleaning of titanium alloy samples in a conical flask and forceps with microtips on it used to hold the titanium discs.

3.3 Cell culture

3.3.1 Growth Medium Preparation

Growth medium consists of all the ingredients required by cells to survive and grow. 5 ml of 200 mM L-glutamine (Gibco, Invitrogen Corp.), 5 ml of 100X Non-essential amino acids (Sigma Aldrich), 10 ml of 1M HEPES pH 7 (Mediatech Inc), 200 µl of gentamicin (Sigma Aldrich) from stock of 50 mg/ml and 0.5 ml of amphotericin-B (Gibco, Invitrogen corp.) from stock of 250 µg/ml (250 µg/ml stock prepared by adding 50 mg of amphotericin-B powder to 90 ml of milliQ water) were added to 430 ml of Eagle's minimum essential medium (Mediatech Inc). 50 ml of defined fetal bovine serum (FBS) (Hyclone) was also added to the solution mixture making it 10% FBS growth

medium. While FBS supplements the necessary proteins and hormones, HEPES helps in maintaining pH of the medium and non-essential amino acids assists in protein folding. Ampotericin-B is an antifungal agent and gentamicin is an antibiotic agent. The medium was then filtered aseptically in laminar flow hood using a 0.22 μm cellulose sterile filter unit. Hanks Balanced Salt Solution (Mediatech Inc) containing 1% HEPES pH 7 buffer and growth medium were stored in +4^{o}C.

3.3.2. Bovine Serum Albumin Medium

Fetal bovine serum (FBS) contains proteins, which will allow osteoblast cells to spread and proliferate. But specific to this research study, as already discussed in the chapters above, implant materials first interact with body fluids and albumin protein, which accounts for the largest proportion of serum proteins. Simulating such a situation in vitro, osteoblast cells were allowed to adhere to the titanium surface in the presence of BSA instead of FBS. In the absence of FBS cell can survive for a short time but cannot proliferate. To prepare 0.5% BSA culture medium (Sigma Aldrich), 5 ml of 200mM L-glutamine, 5 ml of 100X non-essential amino acid, 10 ml of 1M HEPES pH 7, 2.5 g of BSA, 200 μl of gentamicin from stock of 50 mg/ml and 0.5 ml of amphotericin-B from stock of 250 μg/ml (250 μg/ml stock prepared by adding 50 mg of amphotericin-B powder to 90 ml of milliQ water) were added to 477.5 ml of Eagles minimum essential medium.

3.3.3 Thawing UMR 106-01 Osteoblast Cells

A frozen sample of UMR 106.01 cells (12.5 x 10^6 cells) suspended in 2 ml of 10% DMSO/ 90% FBS was thawed in a 37^{o}C water bath until it liquefied. The cell suspension was then added to 40 ml of growth medium with serum in a 50 ml centrifuge tube and centrifuged for 5 minutes at 100 x g in a swinging bucket rotor. Supernatant was aspirated and cell pellet was loosened by tapping on the surface of the hood. Cells were resuspended in 30 ml growth medium and the suspension was then added to a T75 tissue culture flask and incubated in humid environment at 37 ^{o}C, 5% CO_2 for 24-36hrs.

3.3.4 Splitting and Passaging UMR 106-01 Cells

Cells were observed under microscope for confluence, cell shape, pattern and any contamination. Medium was aspirated and washed with 10 ml of HBSS containing 1% HEPES to remove any traces of leftover medium. Trypsin-ethylenediaminetetraacetic acid (Sigma Aldrich) is diluted from the stock concentration of 10X to 1X using 1X HBSS solution. Cells were trypsinized by adding 10 ml of 1X trypsin and incubated for 5 minutes in the incubator. Trypsin must always be stored in refrigerator as it is deactivated at room temperature. Adding serum stops the trypsin's action of splitting the cells from the surface of tissue culture plastic. Cells with trypsin were transferred to a 50 ml centrifuge tube where already 3 ml of serum was present. Cells were mixed well and centrifuged at 100x g for 5 minutes. Supernatant was aspirated and cells after loosening were suspended in 10 ml growth medium. Cells were then inoculated at concentration of 1.5 x 10^6 cells/ml into a new T75 tissue culture flask containing 30 ml of growth medium

and incubated for 72 hrs in humidified environment of 5% CO_2 and 37 ^{o}C. This procedure of splitting the cells was repeated for every 72 hrs and the passage number recorded. UMR cell life span usually can be expected to be up to 50 passages.

3.4 DNA assay

Quantification of double stranded DNA (dsDNA) was done by Quant-iT^{TM} PicoGreen dsDNA reagent, which is a ultra-sensitive nucleic acid stain. Lysis buffer solution was prepared by adding 200 ml of 50 M formamide (Fisher Scientific), 5 ml of 1 M sodium acetate, pH 6 (Sigma Aldrich) and 5 g of sodium dodecyl sulfate (ICN Pharmaceuticals Inc.). Cells were lysed using lysis buffer solution. Cells that were to be assayed are washed with phosphate buffered saline (PBS) after the growth medium is aspirated. Lysis buffer was added in an amount corresponding to the area of the tissue culture plate used and plates were placed in an oven for 2 hrs at 60 ^{o}C. Later, lysis solution was allowed to cool down and sonicated for a minute to disrupt the cell membrane. Lysed cells were then stored at +4 ^{o}C for further analysis.

Lysed cells are diluted to either 1:20 or 1:40 using 1X Tris EDTA buffer (TE) provided with the PicoGreen reagent kit. DNA Standard solutions of concentrations 0.025 ng/ml, 0.25 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, and 1000 ng/ml were prepared using TE/lysis buffer (contains 5% v/v lysis solution and 95% 1X TE buffer). 150 µl of standards and lysed cells were pipetted out into the micro-tubes. 120 µl of PicoGreen reagent was added in to the micro-tubes and mixed well. 270 µl of sample volume is pipetted in the dark into a 96 well plate. The plate was placed in dark for 5

minutes undisturbed before running the fluorometer analysis on it. Excitation wavelength was set at 485 nm and emission at 538 nm. Concentration of DNA was produced with respect to the well in ng/ml units.

3.5 Fibronectin and BSA Coating

Positive control samples were coated with fibronectin an adhesive protein. 5 ml of 1 mg/ml fibronectin (FN) solution is prepared in PBS (without Ca& Mg^{++}). 100 µl of 1 mg/ml FN is then added to 50 ml of PBS, making a 2 µg/ml FN solution. Cleaned and sterilized titanium samples were placed in the 6-well plate with silicone wells and 943 µl of 2 µg/ml FN per well was added. After incubation for about 2 hrs at 37^{o}C, the FN solution was aspirated and the samples were washed with 1.32 ml of PBS for ten times. Post blocking with BSA was done by adding 1 ml of 0.5% BSA culture medium and incubated overnight. Test samples, after cleaning and sterilization, were coated with BSA by adding 1 ml of 0.5% BSA culture medium and incubated overnight.

3.6 Fluorescence Microscope Imaging

Fluorescence microscopy was employed to view the cell nuclei, focal contacts and cell spreading. 6-diamidino-2-phenylindoledihydrochloride hydrate (DAPI, Vector labs) was used to stain the cell nuclei and alkaline phosphatase (Vector Red, Vector labs) to stain the other parts of the cell. Staining with alkaline phosphatase indicates cell spreading. DAPI bonds[18] with natural double-stranded DNA forming fluorescent complexes

showing specific activity for adenine-thymine (AT), adenine-uracil (AU) and hypoxanthine-cytosine (IC) clusters. The alkaline phosphatase (AP) staining kit contains regents of avidin and biotinylated horse-radish peroxidase macromolecular complex. Avidin-biotin is known as an enzyme marker which readily forms complex with alkaline phosphatase. The main purpose of the AP staining is to observe the cell focal adhesion points known as filapodia, which provide better understanding of cell attachment on to the titanium surface. Fixing and staining of titanium samples with cells was done in silicone wells. They were not removed from the well until the staining procedure was complete.

3.6.1 Alkaline Phosphatase Staining

Cells to be stained were fixed with 4% v/v paraformaldehyde for about 2 hrs at +4^{o}C and then rinsed with PBS for five times to remove any excess fixative from the titanium samples. AP working substrate is prepared just before its use. 5 ml of 100 mM Tris-HCl, pH 8.2-8.5 buffer and two drops of reagent 1 from vector red assay kit were added and mixed well. Two drops of reagent 2 was added and mixed. Two drops of reagent 3 was added and mixed. Allow the stain to stay for 15-20 minutes on the surface of titanium and the stain solution is discarded into a waste container. Samples were washed with 1 ml of Tris-HCl buffer, 1 ml of 0.05% of Triton 1X100 solution and PBS solution for two times to clean the alkaline phosphatase stain traces.

3.6.2. DAPI Staining

After staining with alkaline phosphatase, cell nuclei were stained with DAPI. 5 µg/ml DAPI solution is prepared in PBS from a 1 mg/ml stock solution. Sufficient volume of 5 µg/ml DAPI was added on to the titanium surface with cells and allowed to stay in dark (DAPI dye is light sensitive) for 10-15 minutes. Used DAPI solution from sample was then discarded into a waste container and samples were washed with 1 ml PBS for 5 times. Vecta shield mounting media without DAPI (Vector labs) was dropped on to the surface of the sample and a coverslip was placed on top.

3.6.3 Fluorescence Microscopy

Samples were mounted on the fluorescence microscope and the filter was adjusted accordingly to view. Images were captured for documentation. Imagepro plus software (Media Cybernectics, MA) was used to operate the microscope. Images were taken at a magnification of 10X and later montaged to form full fields of view. Adobe photoshop was used to overlay DAPI image with AP

3.7 Sample Preparation Protocol for SEM

Cells were fixed in 2.5% v/v glutaraldehyde, 4% v/v paraformaldehyde in PBS solution for 30 minutes. Then samples were rinsed thoroughly with PBS solution twice and washed with filtered DI water. Cells were dehydrated slowly in a sequence of ethanol concentration starting with 50% v/v, 70% v/v, 80% v/v, 90% v/v, 95% v/v and 100% and

achieve critical point drying using hexamethyl disilazane reagent (Electron Microscopy Sciences). Working volume of 1 ml of ethanol was added to each of the titanium sample in silicone well.

3.8 Experiment Setup and Arrangement

Treated titanium discs are placed in a silicone rubber well of 35 mm OD and ID 25.4 mm (as shown in figure) in such a way that cells can interact only with the titanium surface.

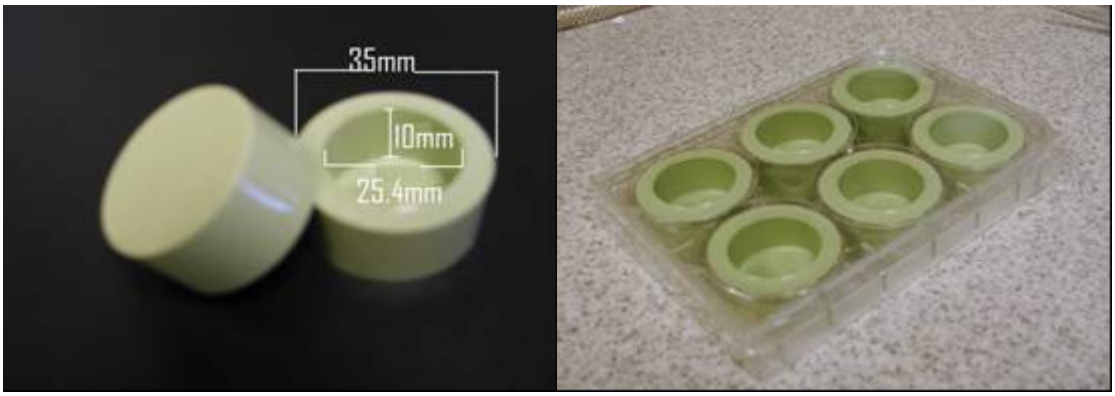

Figure 5. Silicone rubber well and six well plate.

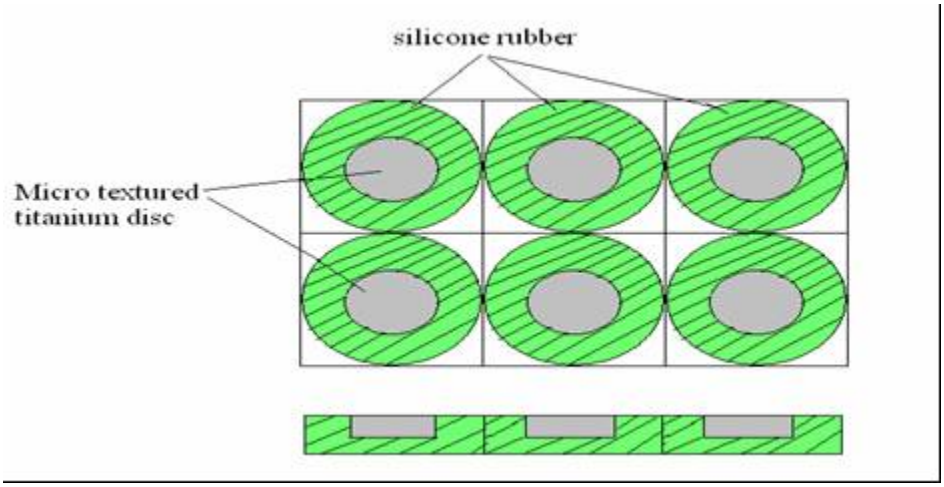

Figure 6. Experimental setup in a six well plate.

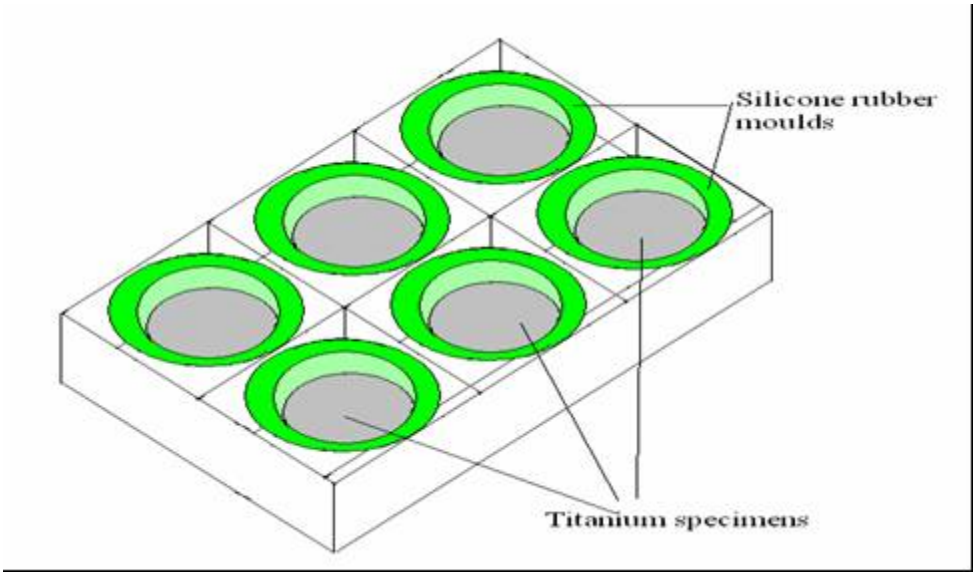

Figure 7. Isometric view of the experimental setup.

UMR 106-01 osteoblast cells are adherent cells that need some surface where they can actively attach and proliferate. Due to its surface properties silicone rubber does not allow cells to adhere to it. Steam sterilized silicone rubber wells were inserted in to the six-well

tissue culture plate in the laminar flow hood and sterilized titanium discs were inserted into the well.

3.9 Experiments

3.9.1 Cell attachment study (Phase-I)

The aim of Phase-I was to quantify osteoblast cell attachment on the surface of titanium. Six differently treated titanium samples were used in this phase. They were: 1) polished, 2) polished and sandblasted, 3) polished and anodized, 4) polished and NaOH treated, 5) polished, sandblasted and anodized, 6) polished, sandblasted and NaOH treated.

Four samples each were used, out of which, one was set up as control and rest of them were considered as test samples. The control sample was coated with FN and post blocked with BSA as described in the section 3.5 and experimental setup was arranged as described in section 3.8. Both control sample and test samples were inoculated with UMR 106-01 osteoblast cells at a very high cell density (4000 cells/mm^2) in 0.5% BSA medium and incubated for 4 hrs at 37^oC in humid environment, with 5% CO_2. After the incubation period non-adherent cells were aspirated into a centrifuge tube by collecting the medium. Discs were washed with 1 ml of PBS for 5 times and pooled to the same tube containing non-adherent cells. Non-adherent cells were then centrifuged at 100 x g for 5 minutes and the supernatant was aspirated. Both adherent and non-adherent cell

samples were mixed with 0.53 ml of lysis buffer and heated for 2 hrs at 60^{o}C. After cooling down, cells were sonicated for DNA analysis.

3.9.2 Cell Proliferation Study (Phase-II)

Phase-II was aimed at imaging the UMR 106-01 osteoblast cells after attachment (4 hours) and proliferation (48 hours), using the fluorescence microscope to view cell nuclei and spreading by staining with DAPI and AP marker respectively. One sample each for six different types of treated titanium was set up for 4 hr attachment study and one each for 48 hr proliferation study. FN coated tissue culture plastic was the control sample and all the test samples were coated with BSA, as described in the section 3.5.

4hr incubation study: Cells were cultured in the same way as Phase-I. Titanium samples were inoculated with same cell density as phase-I study (4000 cells/mm^2) in 0.5% BSA medium and incubated for 4 hrs at 37^{o}C in humid environment, with 5% CO_2. After 4 hrs cells were washed with cold hanks balanced salt solution (HBSS) and fixed with 4% v/v paraformaldehyde (Electron Microscopy Services) in PBS. Samples were stained with DAPI and AP as described in section 3.6.

48hr incubation study: In this study adherent cells after 4 hrs were allowed to proliferate for an additional 44 hr in growth medium. Cell culturing procedure remained same as 4 hr incubation study. After 4 hr cells were washed with cold HBSS and media was changed to 10% FBS growth medium. Cells were incubated for a period of 44 hrs from

the point of media change; this would provide them with an overall 48 hr period of incubation. Incubation conditions also remained same as in 4 hr period. Cells were then washed with HBSS and fixed with 4% paraformaldehyde in PBS. Samples were stained with DAPI and AP as explained in the section 3.6.

3.9.3 Cell Proliferation Study (Phase-III)

Phase-III is a 48 hr incubation period study and was aimed at obtaining quantitative data supporting Phase-II. Out of six only three of the surface treatment methods for titanium were considered for cell culture in Phase-III in order to study the effect of sandblasting and anodization specificaly. They were: 1) polished, 2) polished and anodized, and 3) polished, sandblasted and anodized. Five samples were tested for each treatment method: one FN-treated as control for DNA analysis (described in section 3.8); three BSA-treated for DNA analysis; and one BSA-treated for fluorescence microscope imaging. The experimental setup was arranged as described in section 3.8. FN-coated tissue culture plastic was a positive control. Cell inoculation density was decreased from 4000 cell/mm^2 (used in phase-I) to 500 cells/mm^2. Decreasing cell density will avoid hindered cell attachment that occurs with high cell density inoculation. All samples were inoculated with UMR 106-01 osteoblast cells and incubated at 37^oC in humid environment, with 5% CO_2. Samples that were to be analyzed for DNA were lysed with lysis buffer and samples that were to be imaged were fixed with 4% v/v paraformaldehyde in PBS.

3.9.4 Cell Attachment and Proliferation Study (Phase-IV)

Experiments in Phase-IV were setup to confirm the results obtained in earlier phases specific to polished titanium, polished and anodized titanium and polished and NaOH treated titanium. Five samples were tested for each treatment method: one FN-treated as control for DNA analysis (described in section 3.8); three BSA-treated for DNA analysis; and one BSA-treated for fluorescence microscope imaging. The experimental setup was arranged as described in section 3.8. FN-coated tissue culture plastic was a positive control. Cell inoculation density was fixed to 500 cells/mm^2 and all samples were inoculated with UMR 106-01 osteoblasts in 0.5% BSA medium. Cells were incubated for 4 hrs at 37^oC in humid environment, with 5% CO_2. After 4 hrs cells were washed with HBSS and 1ml of 10% FBS growth medium was added to each sample. Incubation was continued up to 44 hrs and then cells were washed three times with HBSS. Fix the samples with respective fixatives as per procedure discussed in sections 3.4, 3.6 and 3.7.

3.9.5 Data Analysis

Significant differences between different treated titanium materials were determined using Minitab® Version 15.1.1.0. A 2-sample t-test was performed to find the p-values for different groups of data. Results obtained were validated with student's t-test. Mean and standard deviation for the data was also calculated using Minitab®.

Equation for calculating specific growth rate:

$$\mu = \frac{\ln\left(\frac{C48}{C4}\right)}{(t - t_0)}$$

μ - Specific growth rate in FBS medium

C48 - Cell number at 48 hrs

C4 - Cell number at 48 hrs

t_0 - 4 hrs (initial time)

t - 48 hrs (final time)

3.9.6 Summary of Experiments

The following table shows summary of the experiments performed in the different phases.

Table 4. Summary of Experiments

Experiment phases	**Goal of the phase**	**Feature of the phase**	**Tools used**	**Treated titanium alloy used**
Phase-I	Cell attachment study	1) 4 hr incubation 2) Samples provided by Dr.Tewari	PicoGreen dsDNA assay	1. Polished 2. Polished and sandblasted 3. Polished and anodized 4. Polished and NaOH treated 5. Polished, sandblasted and anodized 6. Polished, sandblasted and NaOH treated

Phase-II	Cell proliferation study	1) 48 hr incubation 2) Samples provided by Dr.Tewari	Fluorescence microscope imaging	1. Polished 2. Polished and sandblasted 3. Polished and anodized 4. Polished and NaOH treated 5. Polished, sandblasted and anodized 6. Polished, sandblasted and NaOH treated
Phase-III	Cell proliferation study	1) 48 hr incubation with a cell density of 500 cells/mm^2 2) Additional step in cleaning. Soaked in 1:1 mixture of acetone and ethanol 3) No filtration of ethanol used for sterilization of Ti 4) Different BSA batch used 5) Ti surface modification by Dilip	PicoGreen dsDNA assay and Fluorescence microscope imaging	1. Polished 2. Polished and anodized 3. Polished, sandblasted and anodized
Phase-IV	Cell attachment and proliferation study	1) 4 hr and 48 hr incubation with a cell density of 500 cells/mm^2 2) Ultrasonic cleaning of Ti specimens in DI water before and after anodization 3) Different BSA batch used and confirmed effective	PicoGreen dsDNA assay and Fluorescence microscope imaging SEM imaging	1. Polished 2. Polished and anodized 3. Polished and NaOH treated

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Results

4.1.1 SEM Images of Titanium (Phase-IV)

Anodization: A scanning electron microscope (SEM) image of polished and anodized titanium is shown in figure 8(a). The image was taken at 60 KX magnification and 20 KV. The image clearly depicts the development of nano-structured pores. A high magnification SEM image of polished & anodized titanium taken at 100KX is shown in figure 8(b). Diameter of the pores were measured and found to be between 50-60 nm.

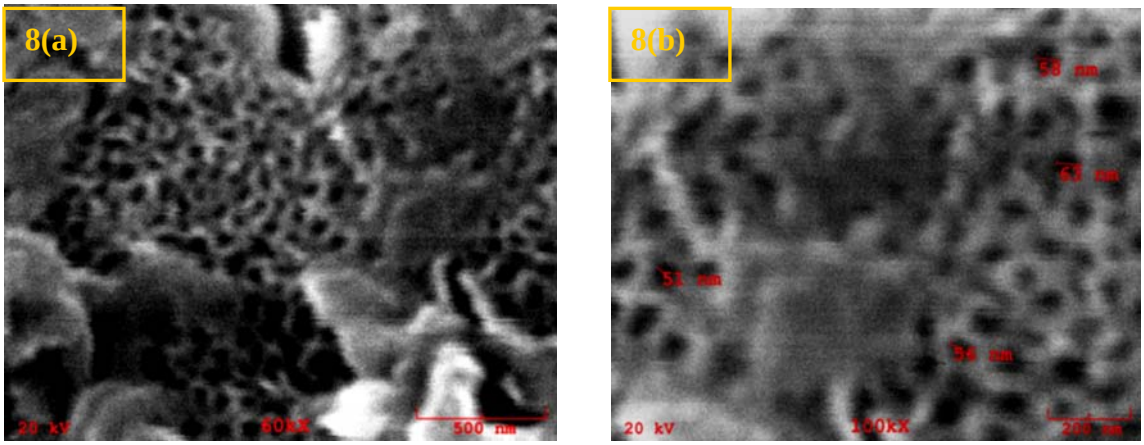

Figure 8. SEM image of polished and anodized titanium.

Alkali (NaOH) treatment: SEM image of polished and NaOH treated titanium is shown in figure 9(a). The image was taken at a magnification of 15 KX and at voltage of 20 KV. Treating with NaOH on a polished surface produces a thin layer of sodium titantate hydrogel layer. Insert area of 9(a) is believed to have similar spongy microporous morphology as in the figure 9(b).

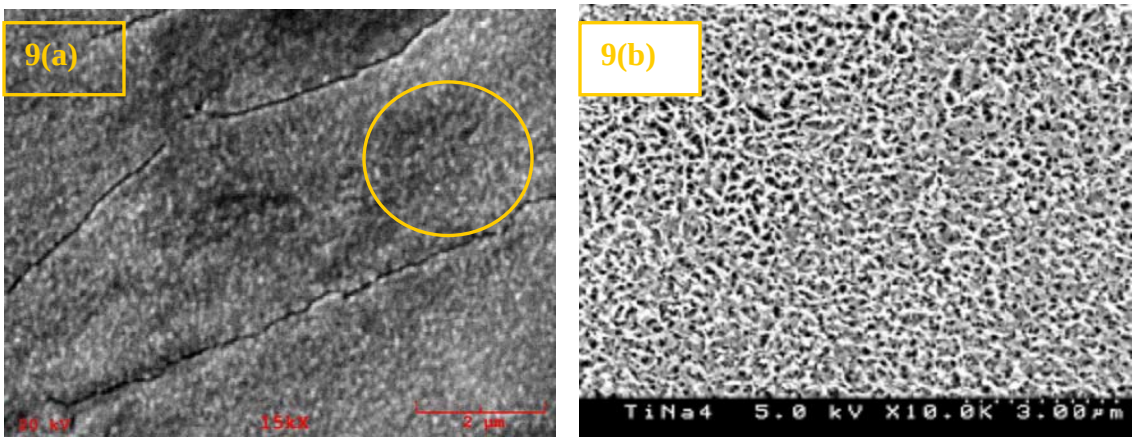

Figure 9. SEM of polished and NaOH treated titanium.

9(b) Image source: Thesis work of Kris Klingmann, Chemical & Biomedical Engineering, Cleveland State University.

4.1.2 Phase I- DNA Analysis of Osteoblast Cell Attachment

DNA analysis was done on the six differently treated titanium samples to quantify the cell attachment after for 4hrs incubation in 0.5% BSA medium. Results were obtained in percent adhesion which was calculated by taking the ratio of number of adherent cells to the sum of adherent and non-adherent cells. The plot in figure 10 illustrates the percentage cell attachment of different titanium specimens.

Results of BSA test samples illustrated in figure 10 are the average values obtained from three replicates. It is shown that coating titanium surface with adhesive protein such as fibronectin improves cell attachment than with non-adhesive protein such as BSA, except for NaOH treated titanium. BSA coated polished titanium has an average cell attachment of 12% whereas the FN coated sample has a much higher value. Interestingly no statistical difference was found in cell attachment results obtained for BSA coated test specimens between polished titanium and polished and anodized titanium. Also no statistical difference was found between polished and sandblasted titanium samples and polished, sandblasted and anodized titanium samples.

NaOH treated samples exhibited higher cell attachment than any other treated titanium. Samples coated with BSA have shown cell attachment values equivalent to FN coated discs. It was also found that no statistical difference existed for cell attachment between polished and NaOH treated samples and polished, sandblasted and NaOH treated samples. This suggests that effect of sandblasting might be not as profound as NaOH treatment.

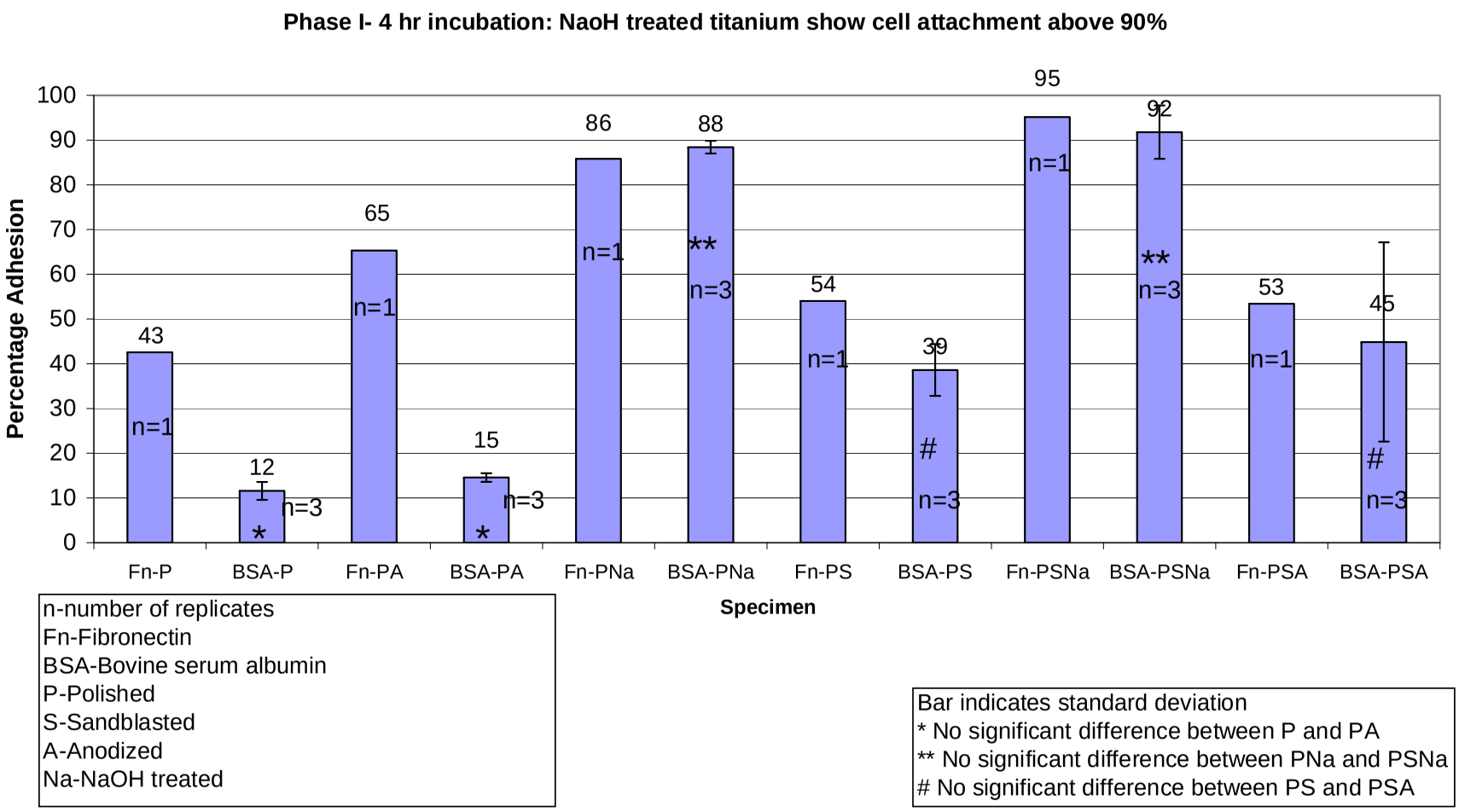

Figure 10. Osteoblast cell attachment results of phase-I for 4 hr incubation.

4.1.3 Phase-II- Images of Cell Attachment and Proliferation

Fluorescence microscope imaging was done on all six differently treated titanium materials inoculated with 4000 cells/mm^2. Cells were incubated for 4 hrs and 48 hrs (procedure discussed in the methods section). All images were taken at a magnification of 10X. An overlay image consists of both DAPI and alkaline phosphatase (AP) marker. DAPI image shows cell nuclei in blue color whereas the AP marker shows everything in the cell in red color other than cell nuclei.

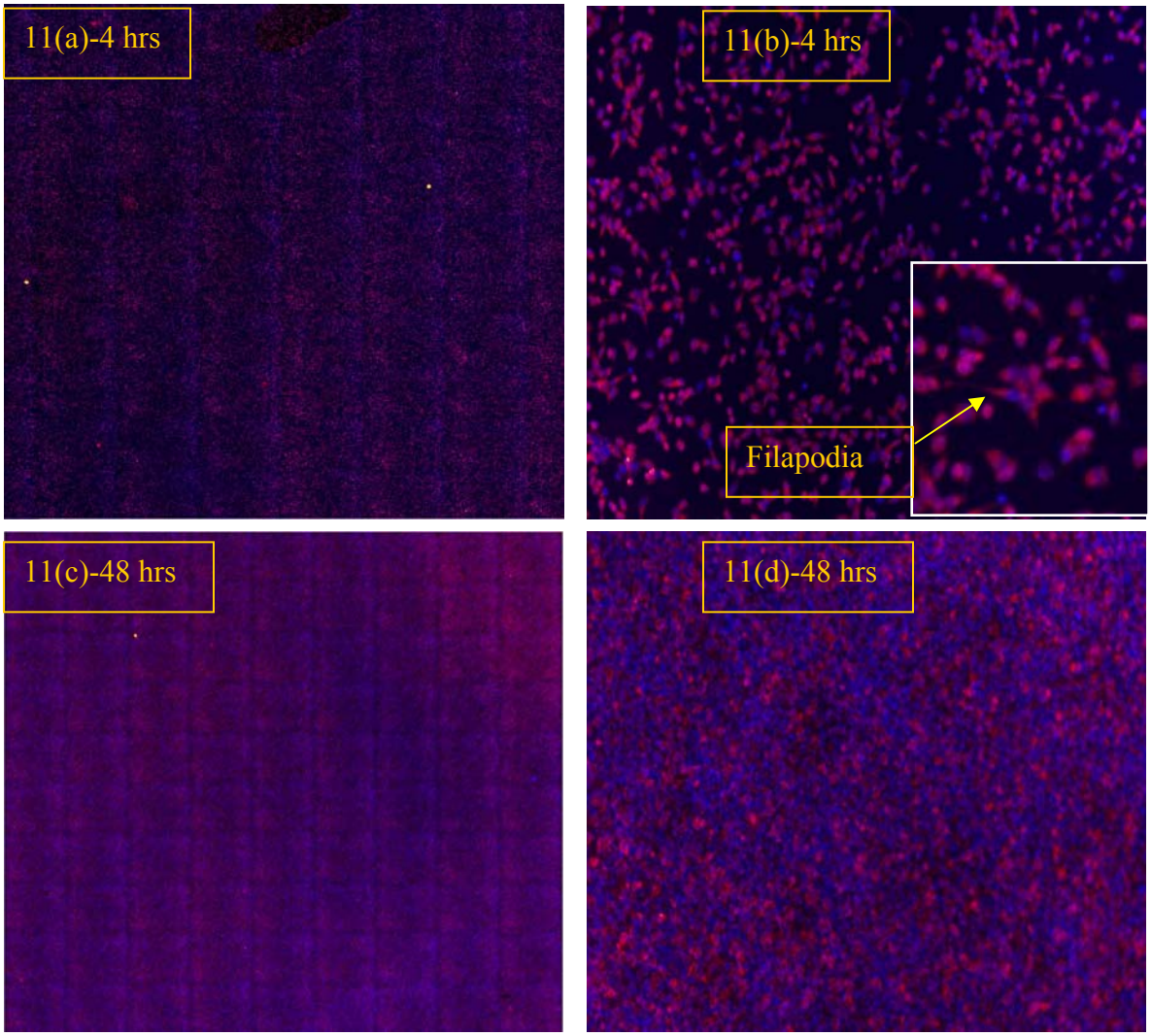

Figure 11. Overlay of DAPI and alkaline phosphatase (AP) staining of FN coated tissue culture plastic. (a) 4 hr incubation. (b) Close up of (a). (c) 48 hr incubation. (d) Close up of (c).

Images in figure 11 show osteoblast cells on FN coated tissue culture plastic after 4 hr and 48 hr incubation periods. At 4 hrs cells (figure 11(b)) formed a monolayer on the surface of the plastic and their filopodia are clearly noticed. In figure 11(d) for a given area more nuclei were found, this suggests that cells formed multi-layers.

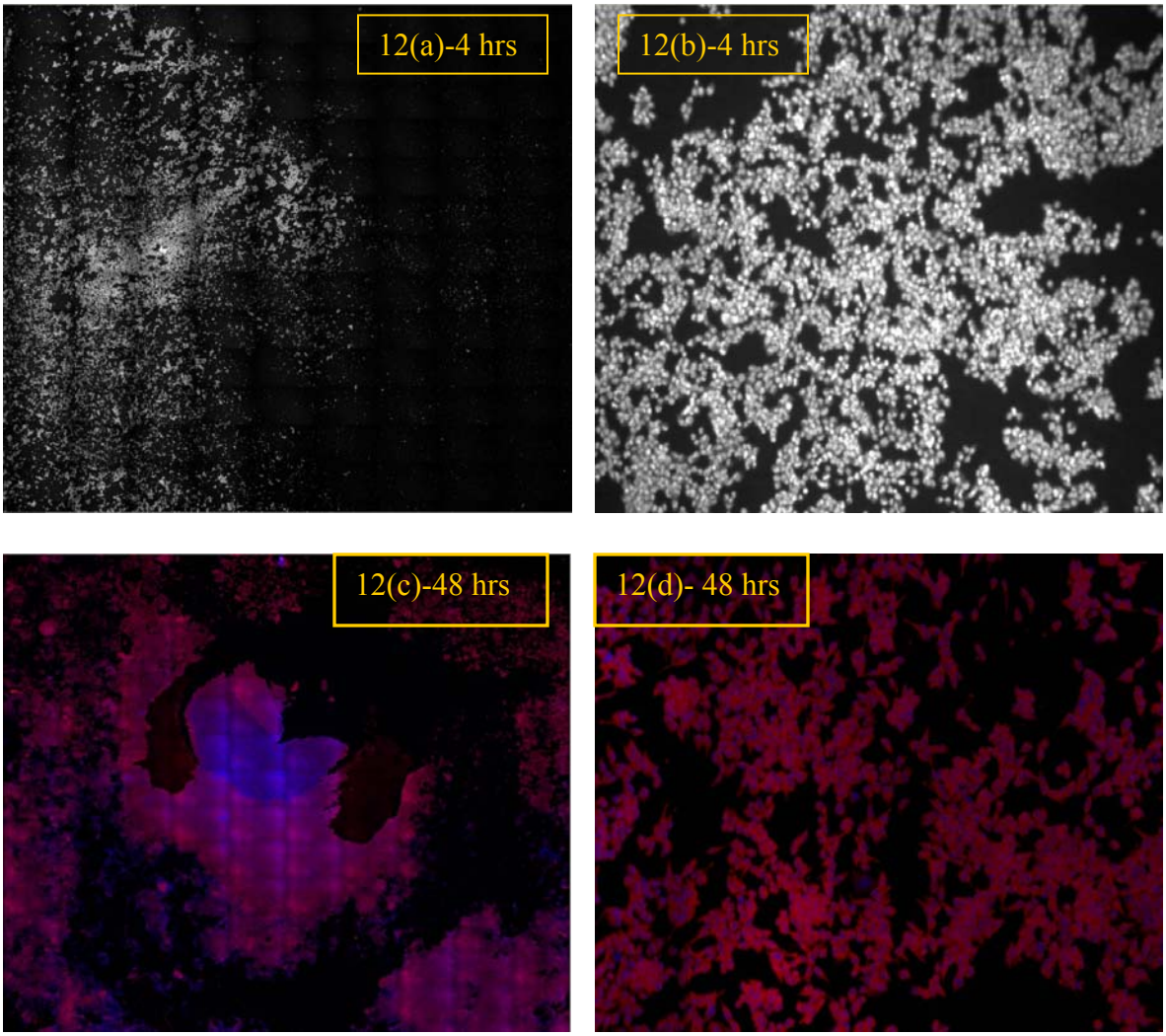

Figure 12. Fluorescent images of polished titanium. (a) DAPI staining at 4 hr incubation. (b) Close up of (a). (c) Overlay of DAPI and AP staining at 48 hr incubation. (d) Close up of (c).

Cell attachment on polished titanium surface after 4 hrs was low when compared with tissue culture plastic and not attaching at all in some areas. Images were consistent with the DNA results obtained in the phase-I. After 48 hrs cells were found proliferating in the areas where they attached.

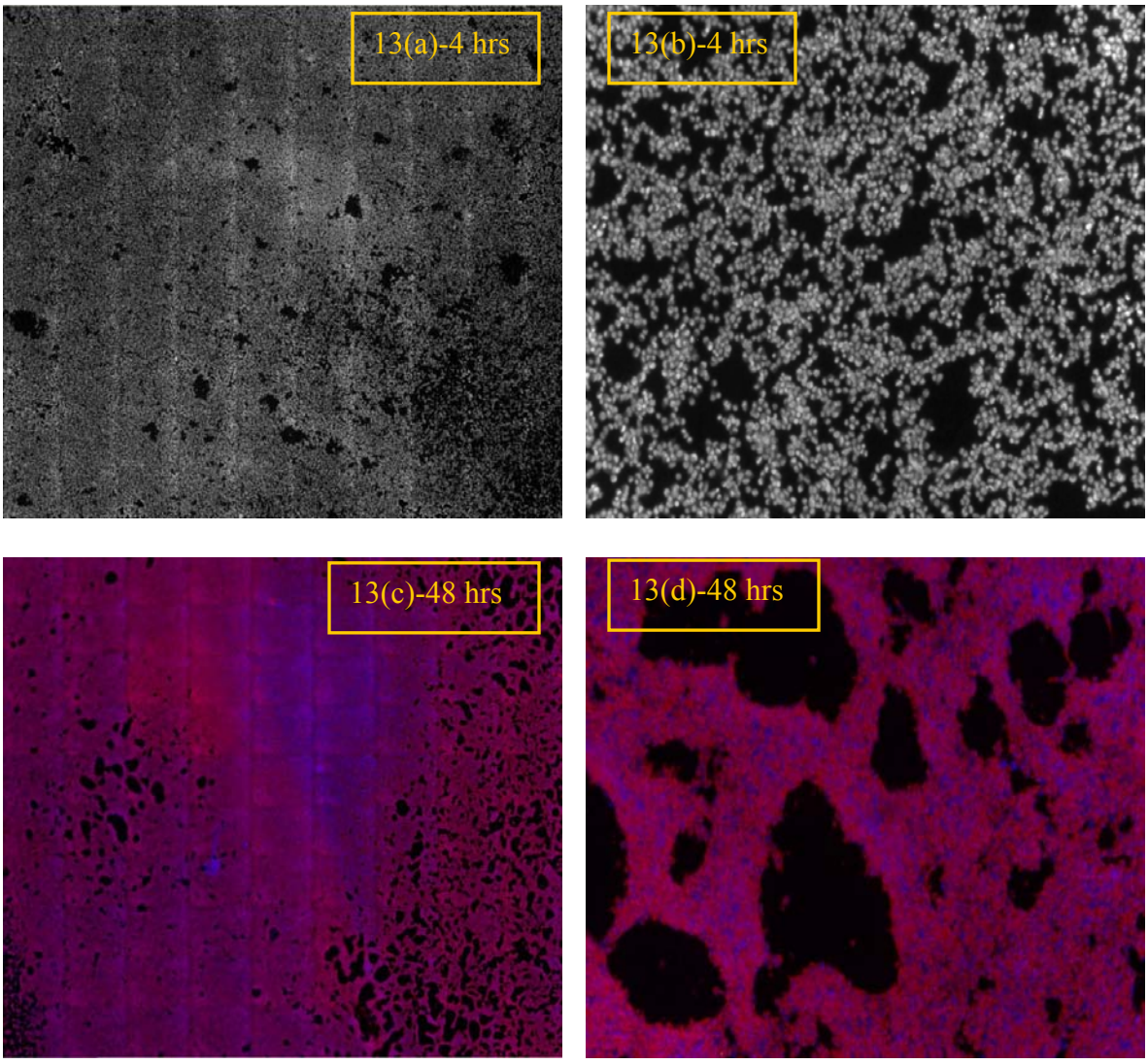

Figure 13. Fluorescent images of polished and anodized titanium. (a) DAPI staining at 4 hr incubation. (b) Close up of (a). (c) Overlay of DAPI and AP staining at 48 hr incubation. (d) Close up of (c).

Figure 13(c) suggests good cell proliferation and also multiple cell layers were noticed. In some areas pinholes were observed, where cell number was found to be meager (figure 13(d)). The reason for such pinholes is not known.

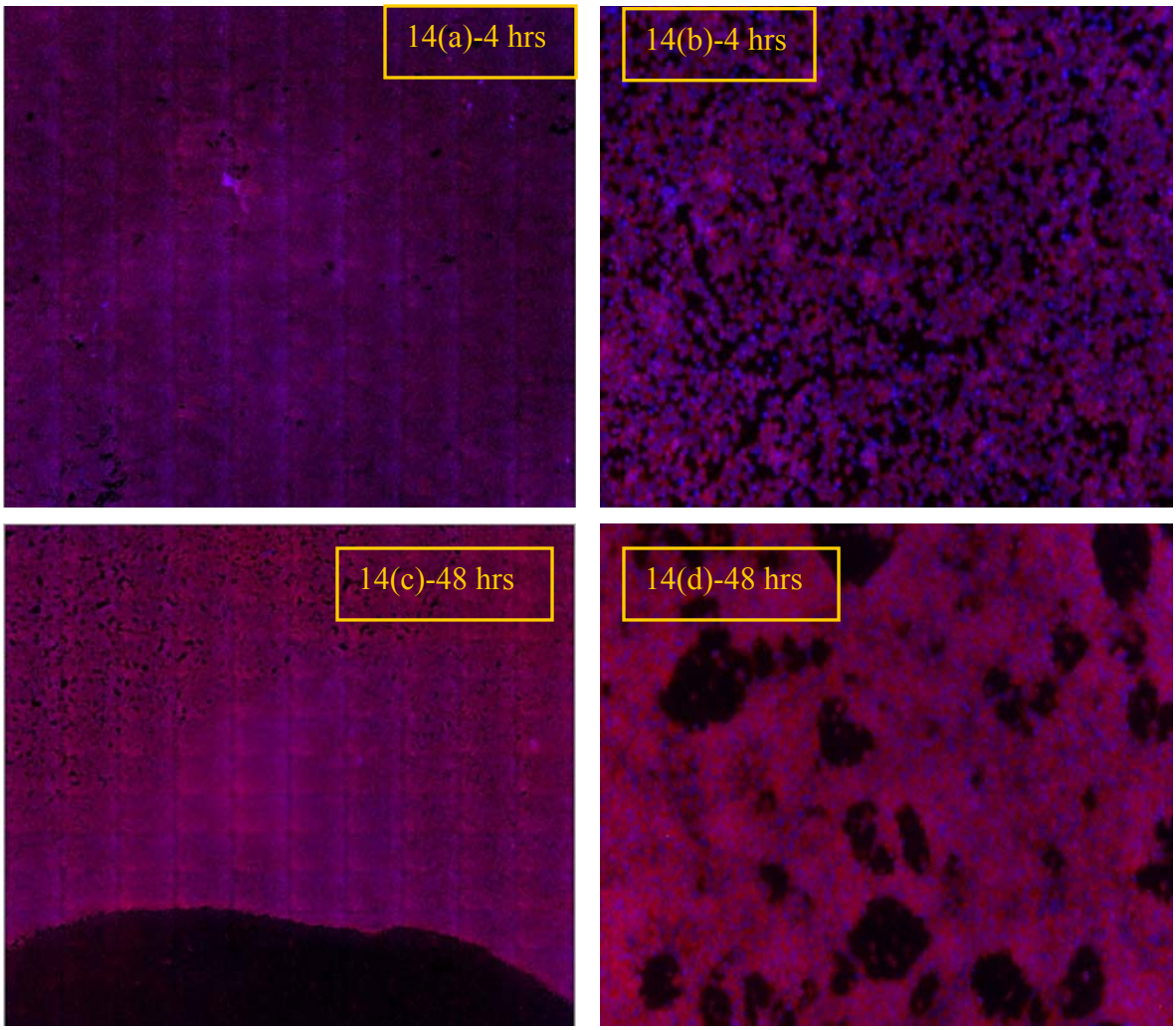

Figure 14. Overlay of DAPI and alkaline phosphatase (AP) staining of polished and NaOH treated titanium. (a) 4 hr incubation. (b) Close up of (a). (c) 48 hr incubation. (d) Close up of (c).

After 4 hrs of incubation with cells, the surface of polished and NaOH treated titanium was found covered by cells though some small pinholes are present (Figure 14(a) and 14(b)). Figure 14(d) suggests that the cells formed multiple layers. These images were found to be consistent with the DNA results obtained in phase-I.

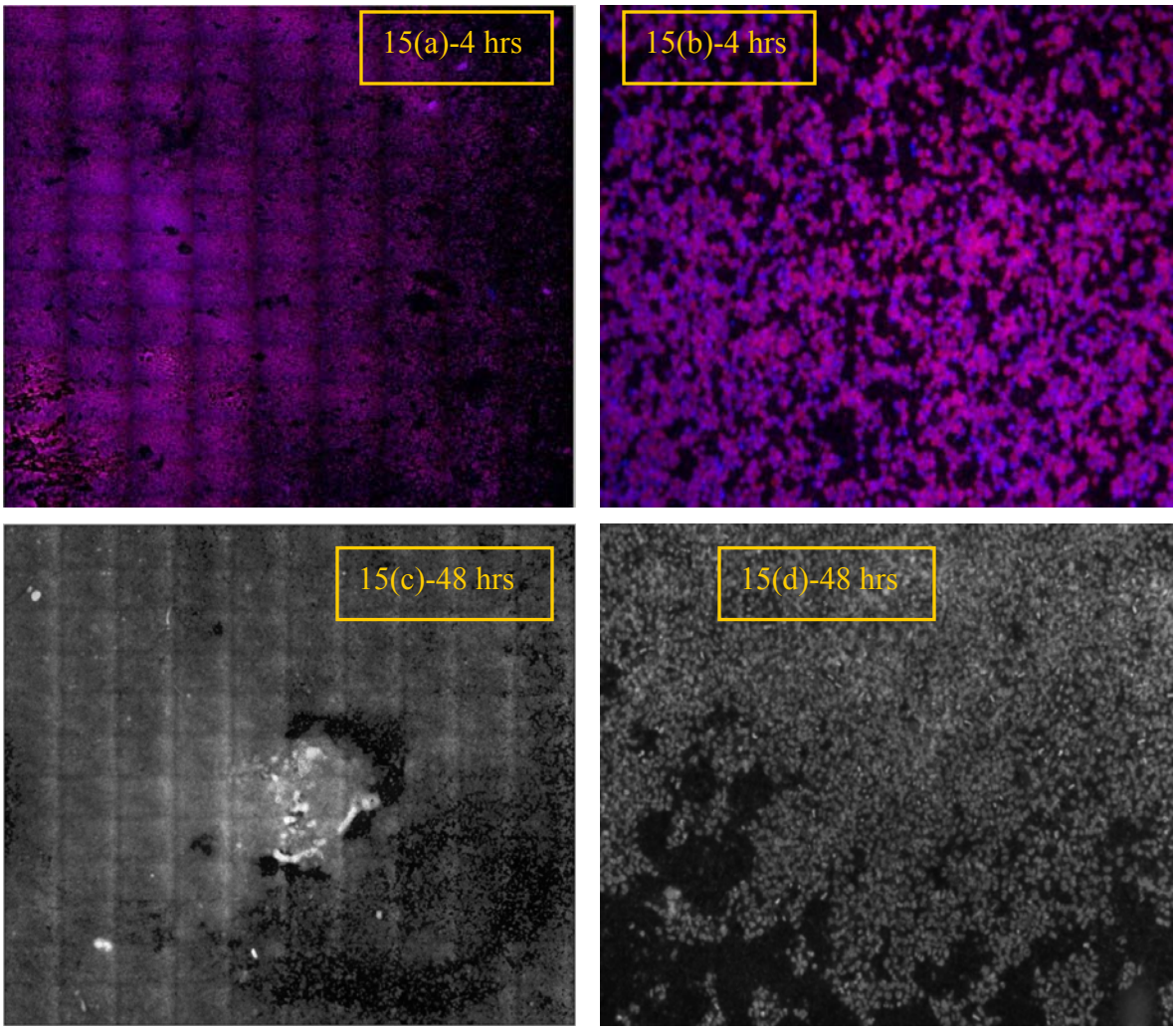

Figure 15: (a) Overlay of DAPI and AP staining polished and sandblasted titanium at 4 hr incubation. (b) Close up of (a). (c) DAPI staining of polished and sandblasted titanium at 48 hr incubation. (d) Close up of (c).

Sandblasting creates micro-texture on the surface of the titanium. Polished and sandblasting resulted in better cell adhesion and proliferation, than just polished titanium. DAPI image in the figure 15(c) suggest that cells were healthy and proliferating.

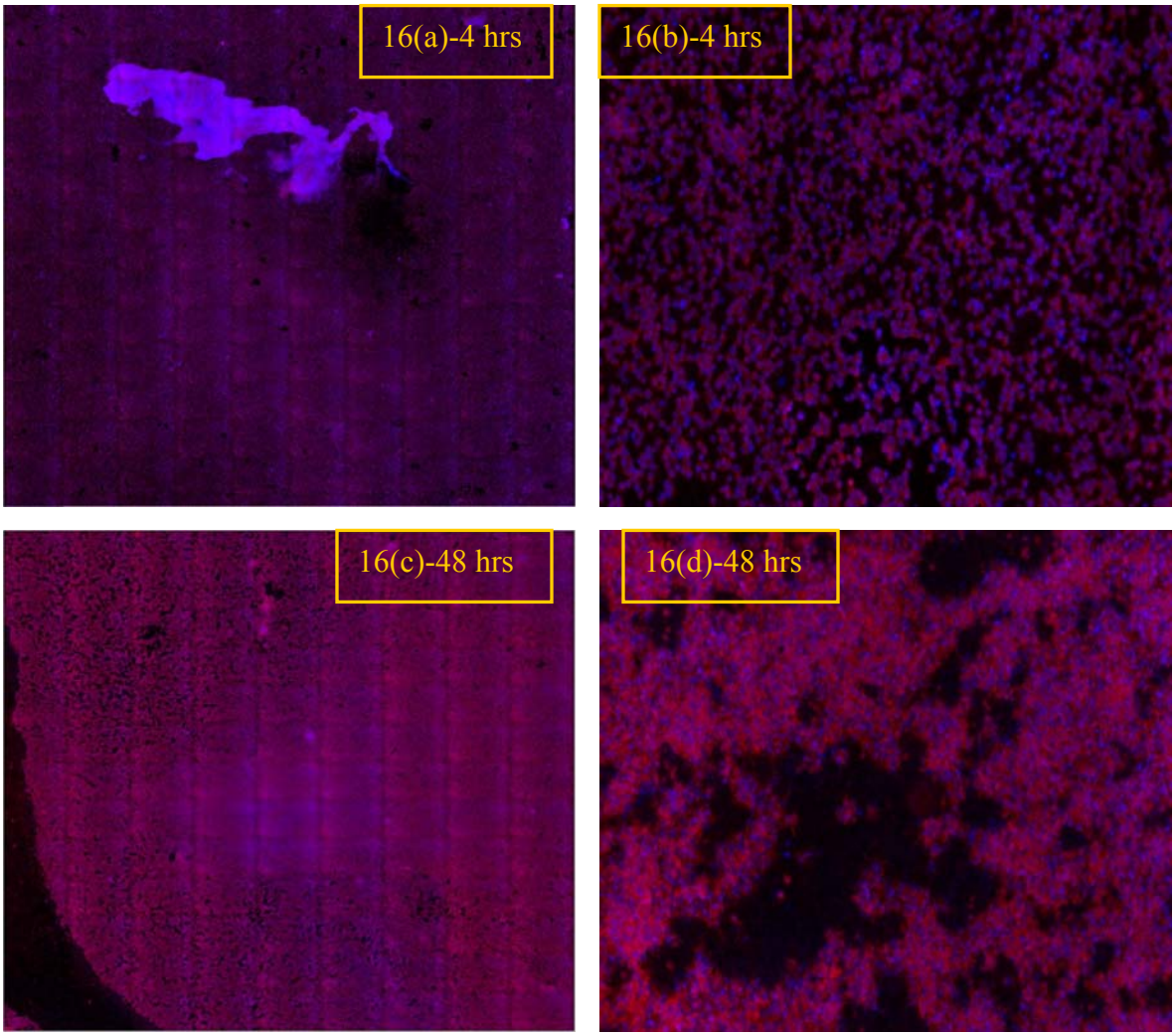

Figure 16. Overlay of DAPI and alkaline phosphatase (AP) staining of polished, sandblasted and NaOH treated titanium. (a) 4 hr incubation. (b) Close up of (a). (c) 48 hr incubation. (d) Close up of (c).

At 4 hrs, cells completely and uniformly covered the surface of the polished, sandblasted and NaOH treated titanium. This high cell attachment is consistent with the DNA results obtained in phase-I.

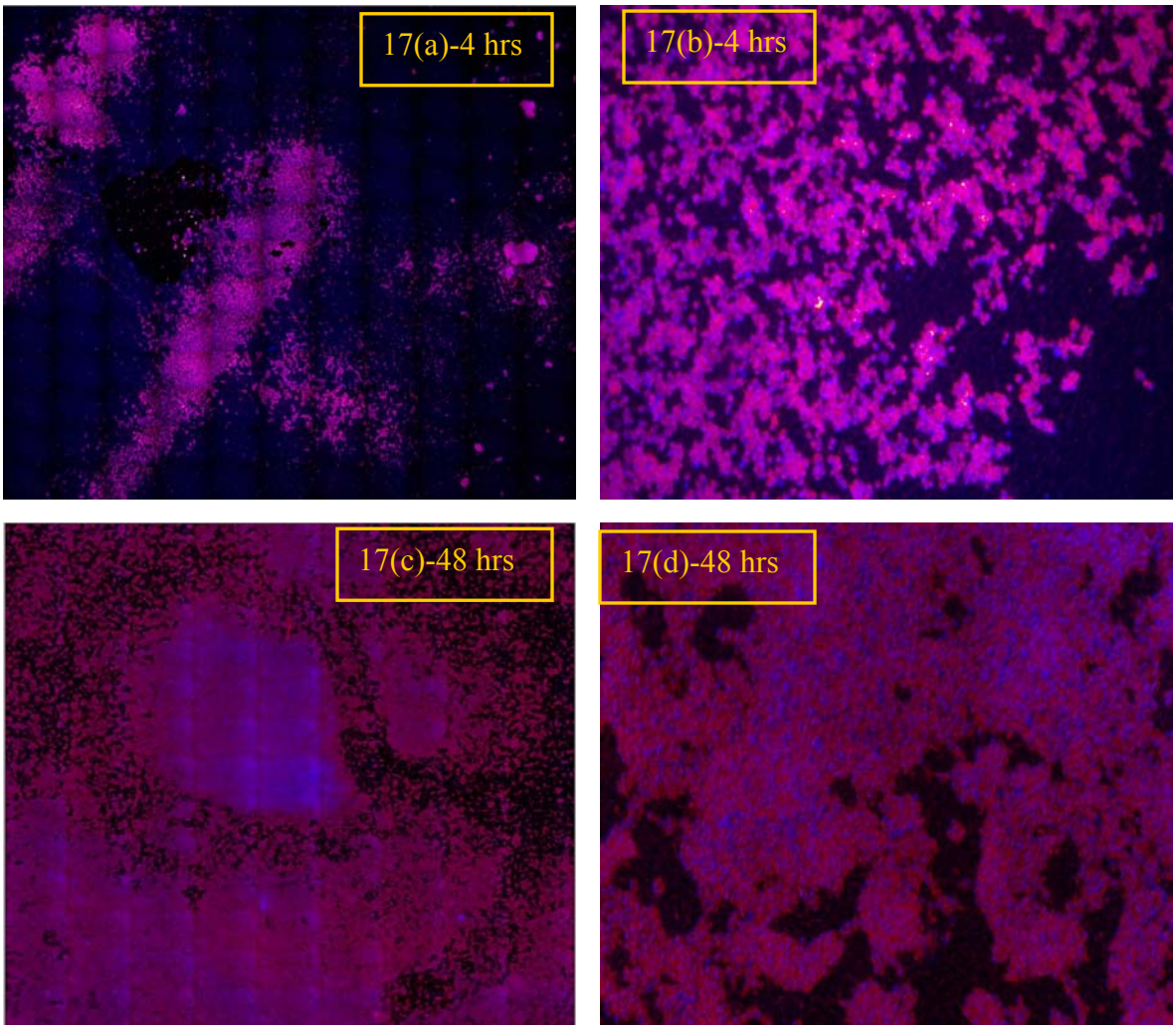

Figure 17. Overlay of DAPI and alkaline phosphatase (AP) staining of polished, sandblasted and anodized treated titanium. (a) 4 hr incubation. (b) Close up of (a). (c) 48 hr incubation. (d) Close up of (c).

At 4 hrs, figure 17(a) and (b) suggest that cells attached to the surface and formed a monolayer in isolated regions of the surface. Figure 17(c) and (d) imply that they are proliferating.

Images of polished and sandblasted (figure 15) and polished, sandblasted and anodized (figure 17) showed little difference in terms of cell attachment at 4 hrs and proliferation at 48 hrs. This analysis was found consistent with the DNA results obtained in phase-I. In the same way, not much difference was found between polished and NaOH treated titanium (figure 14) and polished, sandblasted and NaOH treated titanium (figure 16). Sandblasting titanium did not show any additional effect on attachment and proliferation with NaOH treated samples.

4.1.4 Phase-III-DNA Analysis and Images of Cell Attachment and Proliferation

DNA analysis was done on the three treated titanium samples to quantify the cell attachment at 48 hrs incubation. They are: 1) polished, 2) polished and anodized, and 3) polished, sandblasted and anodized.

4.1.4.1 48 hr Incubation Study

Results were obtained in number of cells per unit area. The graph presented in figure 18 illustrates the DNA results obtained for 48 hrs of incubation in terms of number of osteoblast cells per square millimeter of the titanium surface. No statistical difference was found between all the BSA coated samples. As expected, tissue culture plastic (positive control) showed the highest rate of cell proliferation.

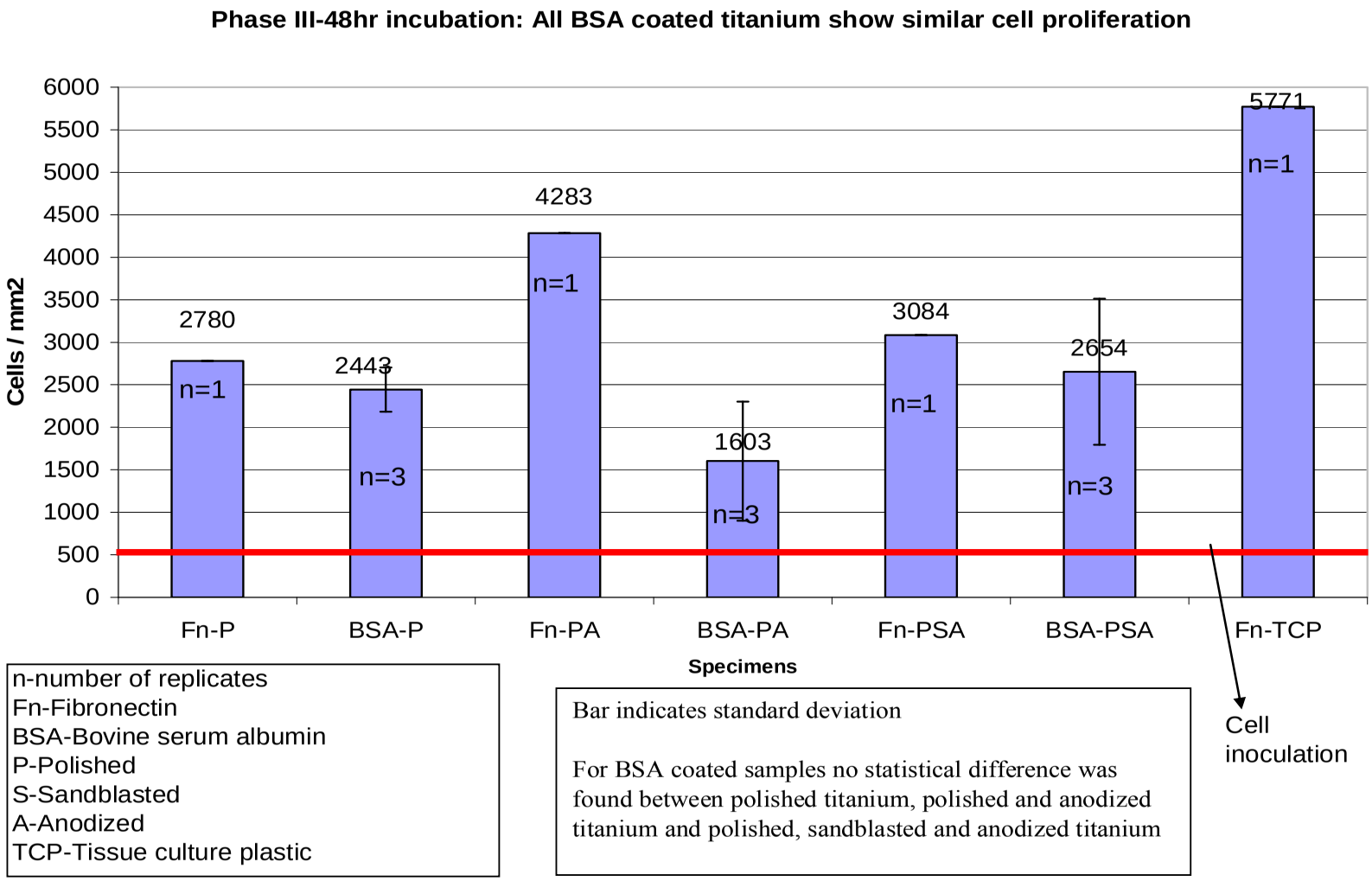

Figure 18. Osteoblast cell proliferation results of phase-III.

4.1.4.2 Fluorescence Images

Titanium samples in culture for 48 hrs were imaged and shown below in figure 19.

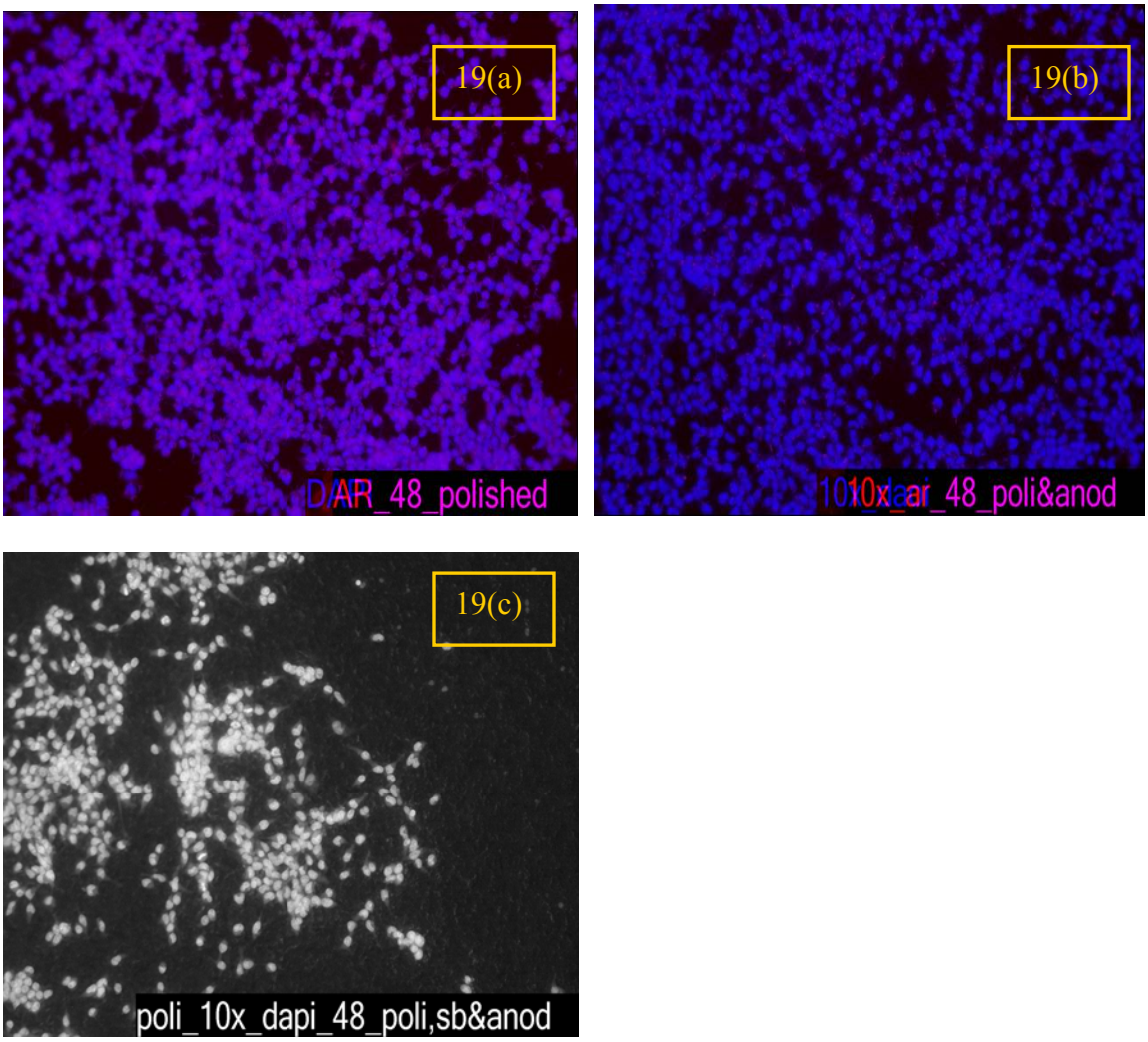

Figure 19: (a) Image showing overlay of DAPI and AP staining of polished titanium.
(b) Image showing overlay of DAPI and AP staining of polished and anodized titanium.
(c) Image showing DAPI staining of polished, sandblasted and anodized titanium.

Results of fluorescence images are shown in the figure 19. The main purpose of imaging in phase-III is to view cell proliferation of osteoblasts at lower cell inoculations i.e., 500 cells/mm^2. Osteoblasts formed a monolayer on all three titanium surfaces, compared to the multiple layers obtained in phase-II on all six treated titanium surfaces. The difference in layering is most likely due to the higher inoculation density of 4000 cells/mm^2 used in Phase I. The existence of regions on the surface where cells did not attach and proliferate is one phenomenon which was found in common among phase-II and phase-III of polished titanium, polished and anodized titanium, and polished, sandblasted and anodized titanium.

4.1.5 Phase-IV-DNA Analysis and Images of Cell Attachment and Proliferation

DNA analysis was performed on the three treated titanium samples to quantify the cell attachment at 4 hrs and 48 hrs incubation. They are: 1) polished, 2) polished and anodized, and 3) polished and NaOH treated titanium

4.1.5.1 4hr Incubation Study

Results were obtained in percent adhesion which was calculated by taking the ratio of adherent cells to the sum of adherent and non-adherent cells. The graph in figure 20 illustrates the percentage cell attachment of osteoblast on titanium.

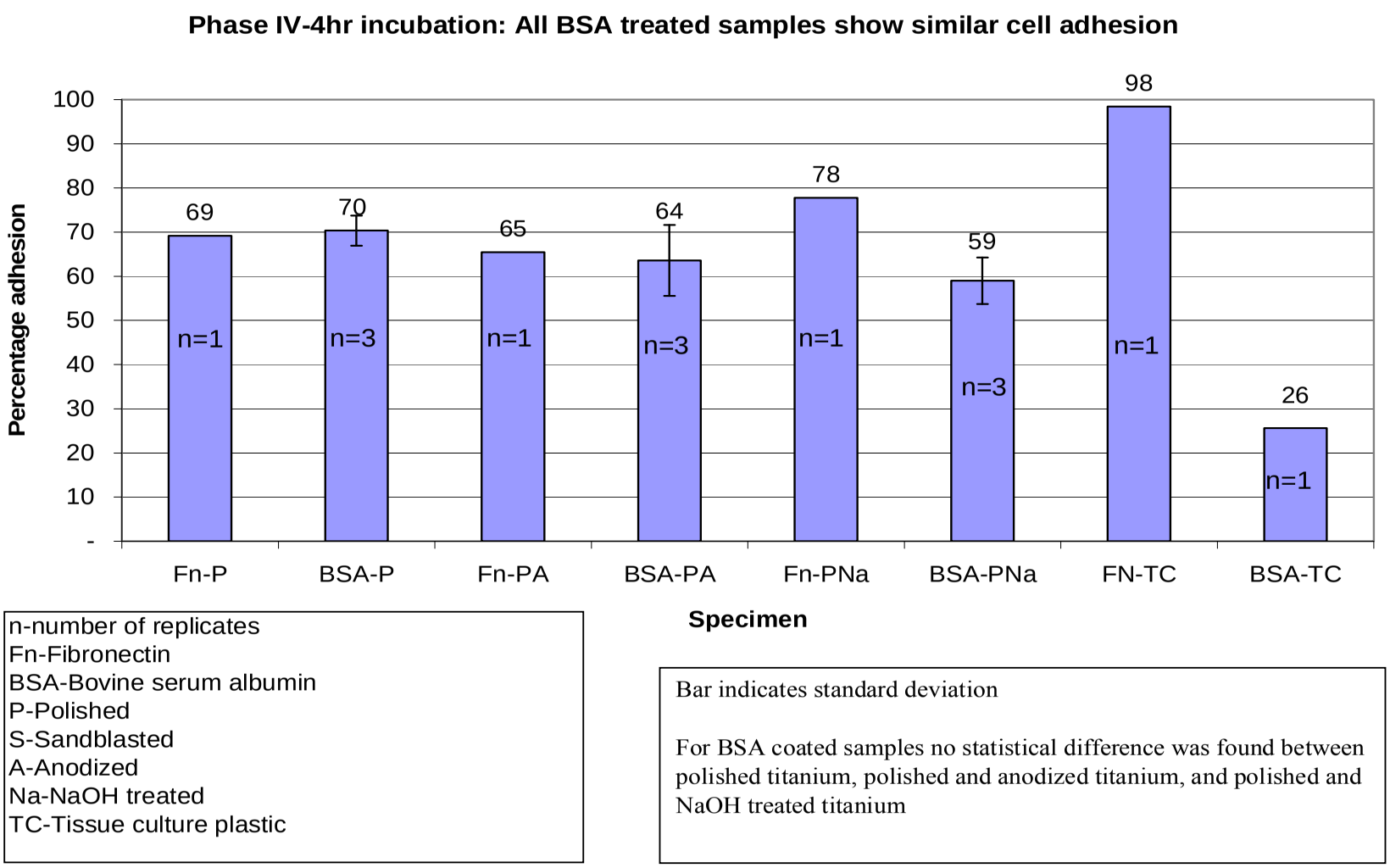

Figure 20. Osteoblast cell attachment results of phase-IV for 4 hr incubation.

For each sample, cell attachment was the same for both FN and BSA treatment, in contrast to the expected result exhibited by the control (tissue culture plastic). This suggests that irregardless of the surface protein coating (either adhesive or non-adhesive) osteoblasts interact with surface texture. Tissue culture plastic with fibronectin coating has shown cell adhesion as high as 98% whereas BSA coated sample has exhibited 26% cell attachment. No statistical difference was found between polished titanium, polished and anodized titanium, and polished and NaOH treated titanium.

4.1.5.2 48 hr Incubation Study

Results were obtained in number of cells per unit area. The graph presented in the figure 21 illustrates the DNA results obtained for 48 hrs of incubation in terms of number of osteoblast cells per square millimeter of the titanium surface. From statistical analysis it was found that no difference exists between polished, polished and anodized, and polished and NaOH treated titanium samples. The other interesting result is that there exists a difference between FN and BSA coated surfaces whereas no noticeable difference was found in 4 hr incubation period. BSA coated tissue culture (TC) plastic which has shown cell attachment of 26% at 4 hrs incubation proliferated to 5015 cell/mm^2 where as FN coated TC plastic value stands at 5476 cell/mm^2.

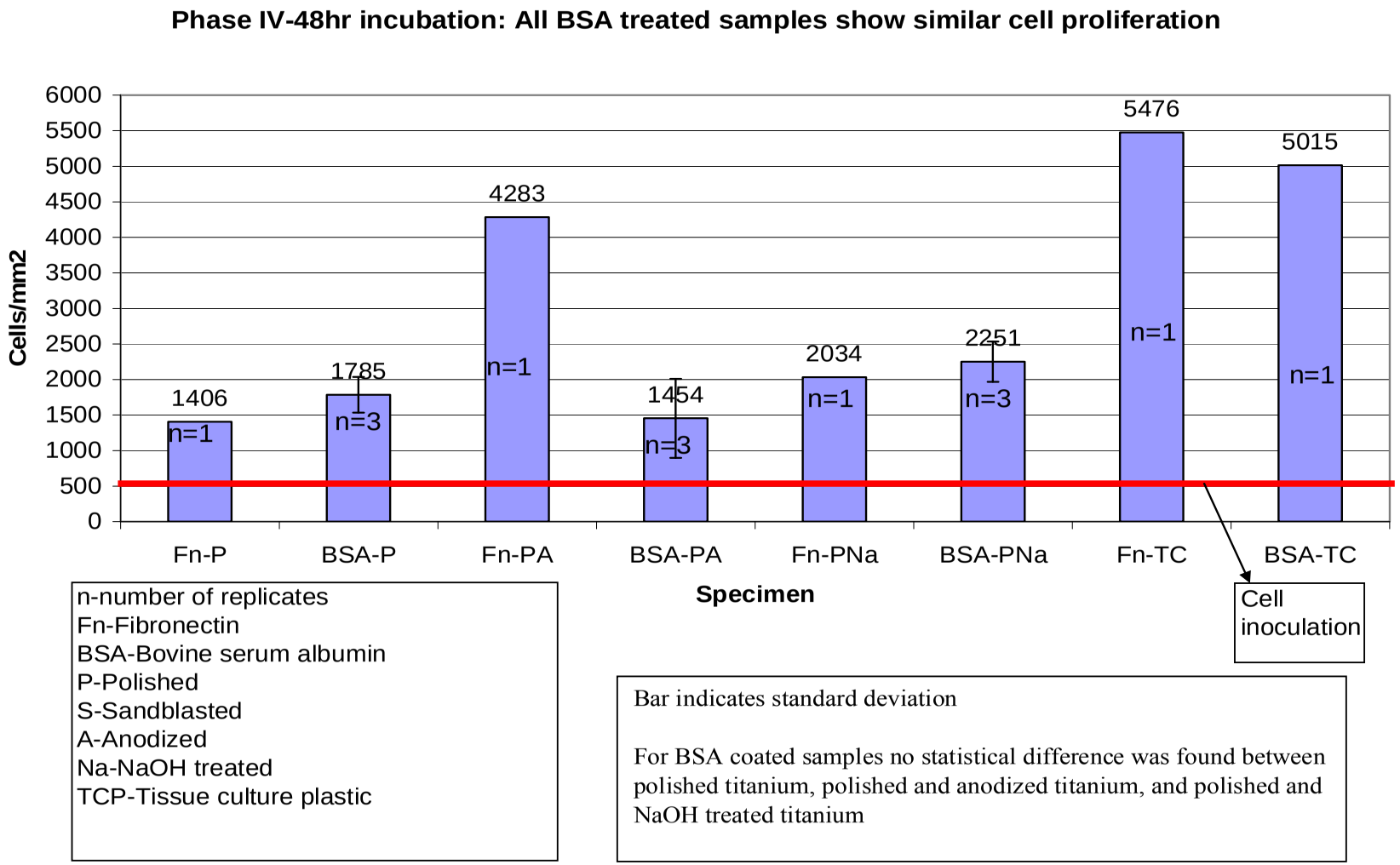

Figure 21. Osteoblast cell proliferation results of phase-IV for 48 hr incubation.

4.1.5.3 Fluorescence Images

Fluorescence images for titanium samples in culture for 48 hrs were imaged and are shown below in the figure 22.

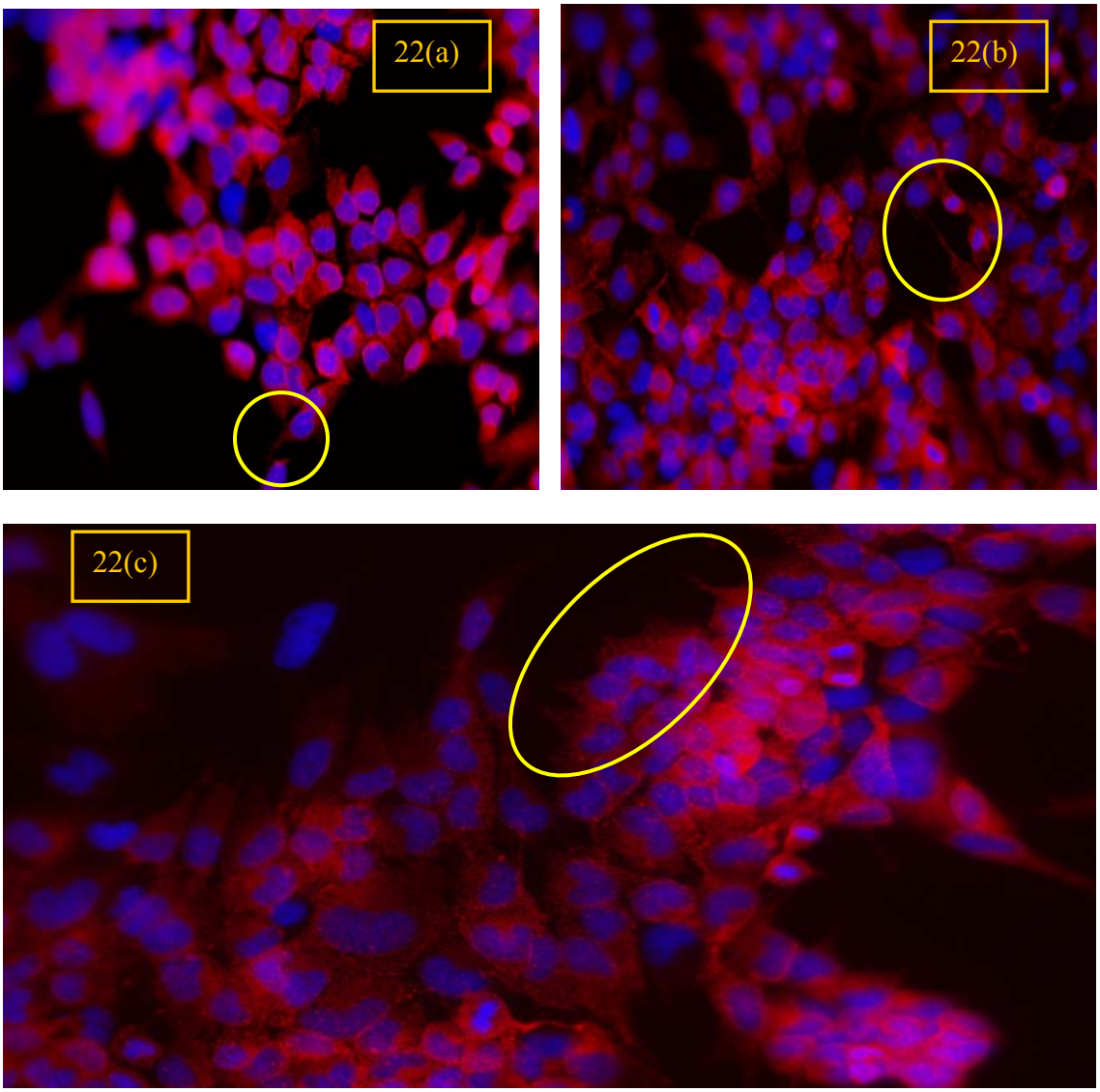

Figure 22: (a) Image showing overlay of DAPI and AP staining of polished titanium.
(b) Image showing overlay of DAPI and AP staining of polished and anodized titanium.
(c) Image showing overlay of DAPI and AP staining of polished and NaOH treated titanium.

Image of polished titanium surface in figure 22(a) shows nucleus and other regions of cell. Filapodia as shown in the insert of figure 22(a) is the organelle with which cells adhere to the surface of titanium. Cells were found covering the surface of titanium in monolayer and did not cover the surface completely leaving some areas untouched. This observation is consistent with results from phase-II and phase-III. Osteoblasts spread uniformly on the surface of anodized titanium. It was found that filapodia are a little longer and more numerous on polished and anodized titanium than on polished titanium as shown in the insert of figure 22(b). The specific nature of forming cell grouping was found consistent with polished and NaOH treated titanium and these groups were evenly distributed across the surface. Cells formed multiple layers in such colonies. The insert in the figure 22(c) show the filapodia of osteoblast on polished and NaOH treated titanium. They were found attached to the surface firmly through these filapodia which act as anchoring agents.

4.2 Discussion

Titanium was found to be the “best” biomaterial which has attracted many researchers to explore its capability in osteointegration. Its performance in cell attachment and proliferation was evaluated by numerous scientists with different surface modification treatments (such as physical, chemical and combinations thereof) and with different adhesive protein coatings (such as fibronectin, vitronectin, collagen and some peptides). Previous study of Mata et al.[6] with UMR 106-01 osteoblast cells has shown that cells

were able to adhere to culture surface using defined textures of varying depth on standard plastic substrate.

The current study extends Mata et al. research work with UMR 106-01 osteoblasts to different surface treated titanium specimens in terms of cell attachment and proliferation; simulating a situation where cells interact only with surface texture. The following plots illustrate the consolidated results of all the phases of this thesis.

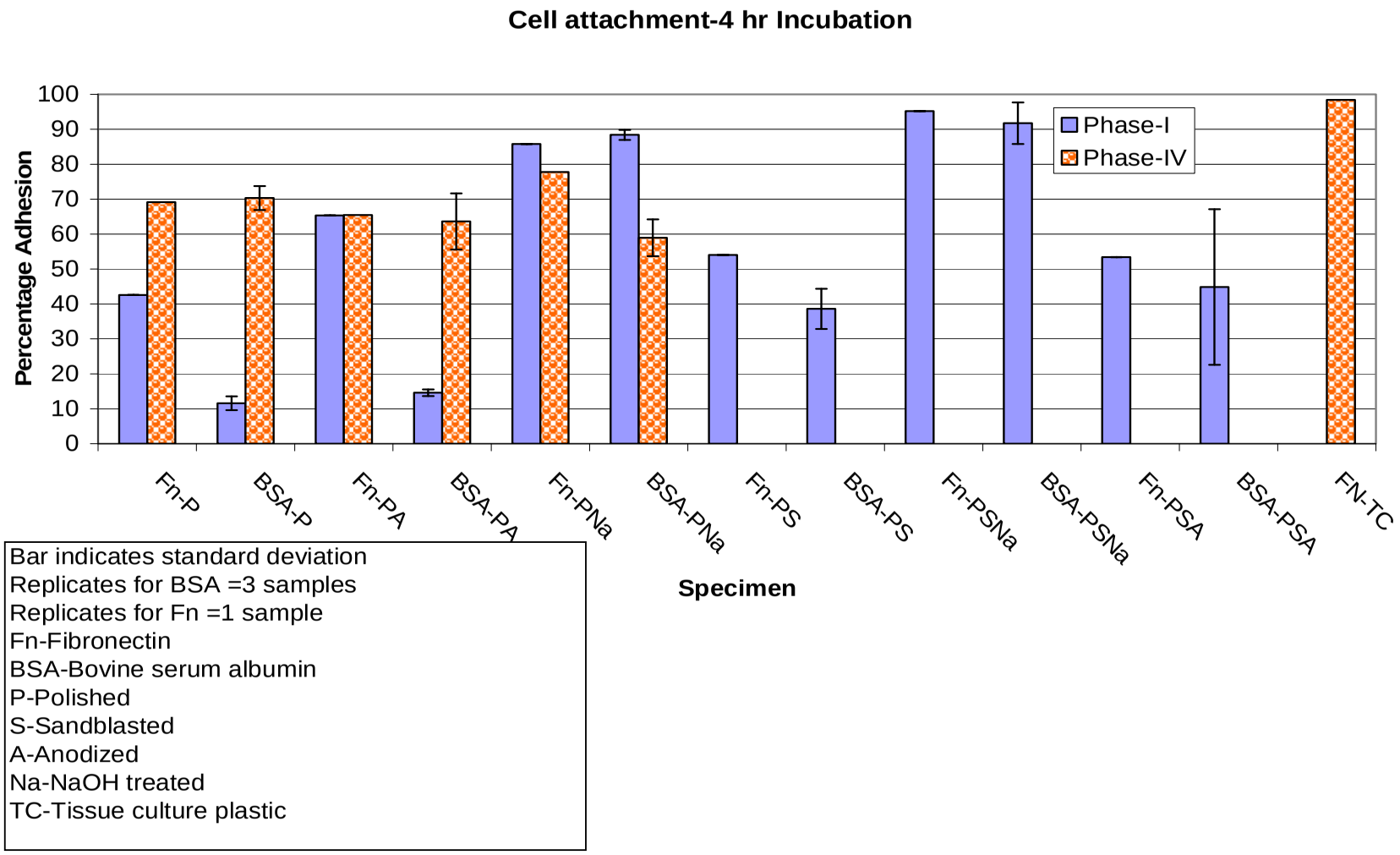

Figure 23. Osteoblast cell attachment results for all the phases – 4 hr incubation.

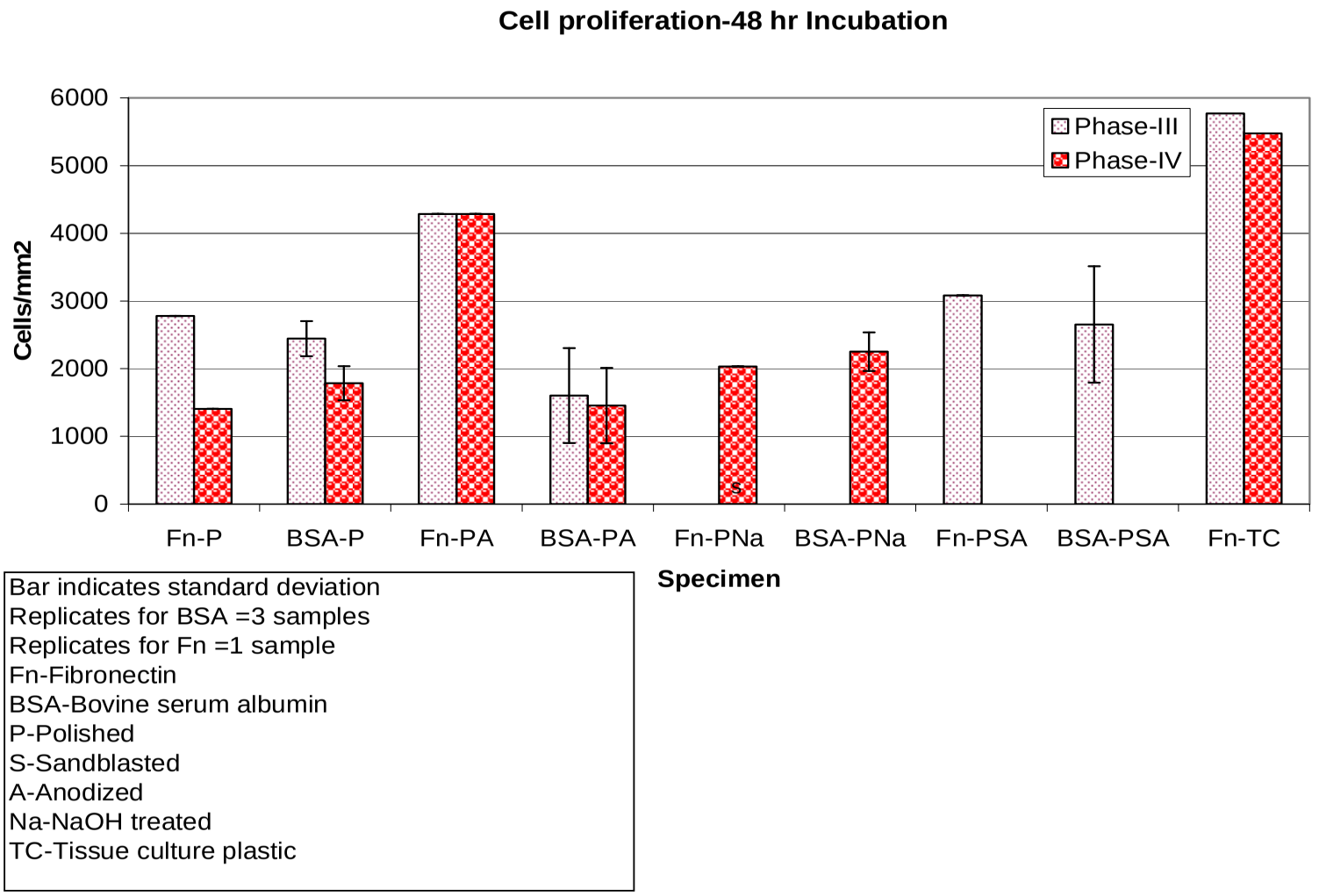

Figure 24. Osteoblast cell proliferation for all the phases – 48 hrs incubation.

From figure 23 and 24 it was found obvious that some differences existed between phase-I, phase-III and phase-IV. They are cell inoculation density, titanium surface modification procedure, employment of different persons to produce surface modifications on titanium and different BSA batches. The BSA lot used in phase-I, II and IV were tested for its effectiveness and compatibility, whereas the lot used in phase-III did not undergo any such tests.

Observing the results obtained for BSA coated polished titanium surface as shown in figure 23, a huge difference was found between phase-I and phase-IV. BSA coated polished and anodized titanium surface showed in figure 23, exhibited large difference between phase-I and phase-IV. It was believed that titanium might have not been properly anodized in phase-I. The physical appearance of titanium samples in phase-I and phase-IV after anodization was found different and also no statistical difference was found between polished titanium and polished and anodized titanium in phase-I. Consistent results were found for cell proliferation results between phase-III and phase-IV, shown in figure 24.

A decrease in percentage adhesion of osteoblast cells was noticed between phase-I and phase-IV for BSA coated polished and NaOH treated titanium. The reason for such inconsistency between phase-I and phase-IV is not known. Osteoblast cells were found bonding with the titanium surface firmly as an anchoring agent.

Polished titanium surface was produced using #1200 silicon carbide grit paper which will produce a surface roughness value ranging in nano-meters. Anodization in 0.5% wt/wt HF resulted in nano-tubes with an average diameter ranging between 50-60 nm (figure 8). Treating with 5M NaOH solution resulted in micro scale texture formation. SEM images in the figures 8 and 9 suggest that performing anodization or NaOH treatment on such fine polished surface would not alter the surface roughness to a great extent Such treatment leaves polished surface with nano-and microtexture on the surface. Though no statistical difference existed between polished titanium, polished and anodized titanium, and polished and NaOH treated titanium in phase-IV, the cell morphology suggests interesting results. From the fluorescence images of phase-IV, polished and anodized titanium surface exhibit elongated morphology compared to polished surface. Cells on polished titanium were found to be spreading flat on the surface and they did not have many extensions. NaOH treated titanium showed similar elongated morphology (similar to the anodized surface) as well as has noticeable anchoring which help cells to keep tightly bonded with the surface.

Extensive contradictory literature exists on the relationship between osteoblast cell attachment and proliferation and surface roughness. Some authors such as Buser et al.[19], Schneider et al.[20] etc found that surface roughness could improve cell adhesion while some authors such as Zhao et al.[15], Batailon et al.[16], Lee et al.[17] etc found that smooth or polished surfaces improves cell attachment.

Zhao et al.[21] work with MG63 cells explains the effect of surface micro-structure and surface energy on cell number. Cells were cultured on tissue culture plastic, smooth pretreated titanium surface with surface roughness (Ra) of 0.2 μm, acid etched titanium surface with Ra of 0.83 μm, and sandblasted and acid etched titanium surface with Ra of 3-4 μm. After 6 days in culture, cell numbers were calculated and found that smooth pretreated titanium and acid etched titanium specimens exhibited cell numbers same as those on tissue culture plastic. Cell number on sandblasted and anodized titanium has shown a value 44% lower than smooth pretreated surface. Zinger et al.[22] found that micro scale roughness improves MG63 cell attachment, spreading and, differentiation. At the same time submicron scale roughness contributes to the local factor production. Zhao et al. and Zinger et al. results show very good agreement with the current work where the anodized titanium and NaOH treated titanium surfaces are believed to have the sub-micron structure.

Ketul et al.[23] revealed that 40% more marrow stromal cells were present on anodized titanium surface compared to flat titanium (Ra for the flat surface not provided) after 7 days in culture. Anodized surface had nano tubes with an average diameter of 80 nm and 400 nm in depth. Polystyrene surface was setup as control surface and as expected highest number of cells were found on it. Cells were calcein stained and fluorescence imaged. Clusters of cells were found on nano tube anodized titanium surface. When allowed in culture for 3 weeks cells mineralized and produced calcium and phosphorous whose concentration was 50% higher for nano tubular anodized titanium surface than flat titanium surface. Results of Ketul et al. work is very much relevant and supports the

current research study though some differences in experiment procedure exists such as BSA coating titanium samples, serum free medium for initial cell attachment (4 hr incubation) and 2 days in serum medium culture.

4.2.1 Relationship Between Cell Adhesion and Specific Growth Rate

Figure 25 illustrate the relationship between initial cell attachment and specific growth rate for phase-IV. No direct relationship was found between cell attachment and growth rates for phase-IV but there exists a connection between protein coating, percentage adhesion and specific growth rate. Though tissue culture plastic performed highest cell attachment, the specific growth rates were equivalent with BSA coated polished and NaOH treated, and polished and anodized titanium. FN coated polished and anodized titanium and polished and NaOH treated titanium exhibited better growth rates than their counter parts which are coated with BSA protein.

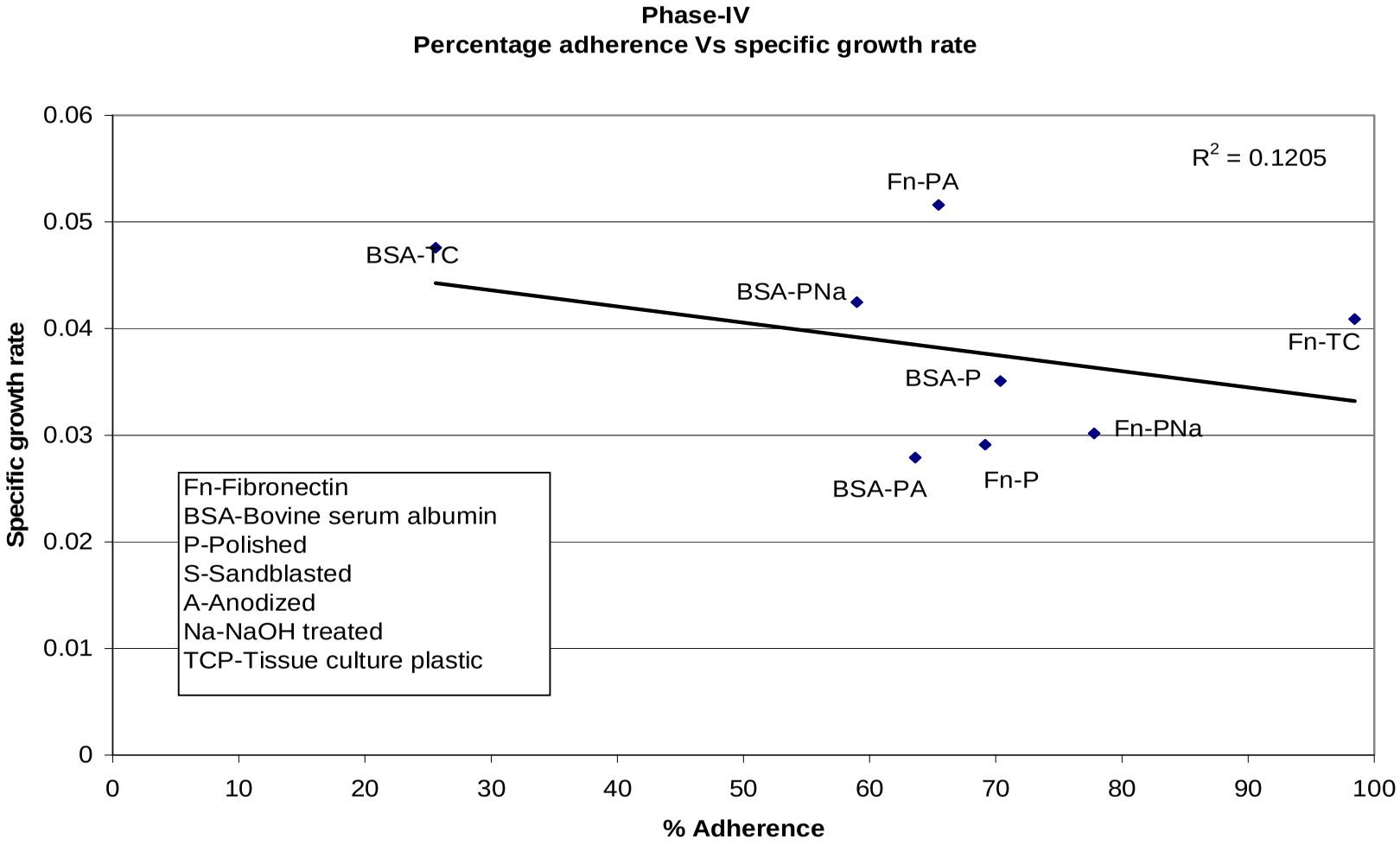

Figure 25. Relationship between specific growth rate and percentage adhesion in phase-IV.

Figure 26 depicts interesting correlation plot facts about the effects of proteins on osteoblast cell attachment and growth on titanium surface. Cells exhibited similar specific growth rates on both Fn-TC and BSA-TC. Polished titanium has shown similar cell attachment values for different protein coatings. Anodized BSA and Fn coated samples has large difference in their growth rates but little difference in their cell adhesion values. NaOH treated samples has shown appreciable difference between both BSA and FN coated samples in terms of cell attachment and growth rate. It can be concluded that there exists a relationship between surface texture, protein coating, cell adhesion and specific growth rate.

Figure 27 illustrates that an inverse relationship exists between growth rate and attachment among the BSA coated samples. Interestingly it was found that polished titanium has the highest cell adhesion but nearly the smallest growth rate. NaOH treated titanium shows the highest growth rate but lowest cell adhesion than polished titanium and anodized titanium. BSA coated tissue culture plastic exhibited least cell attachment but highest specific growth rate.

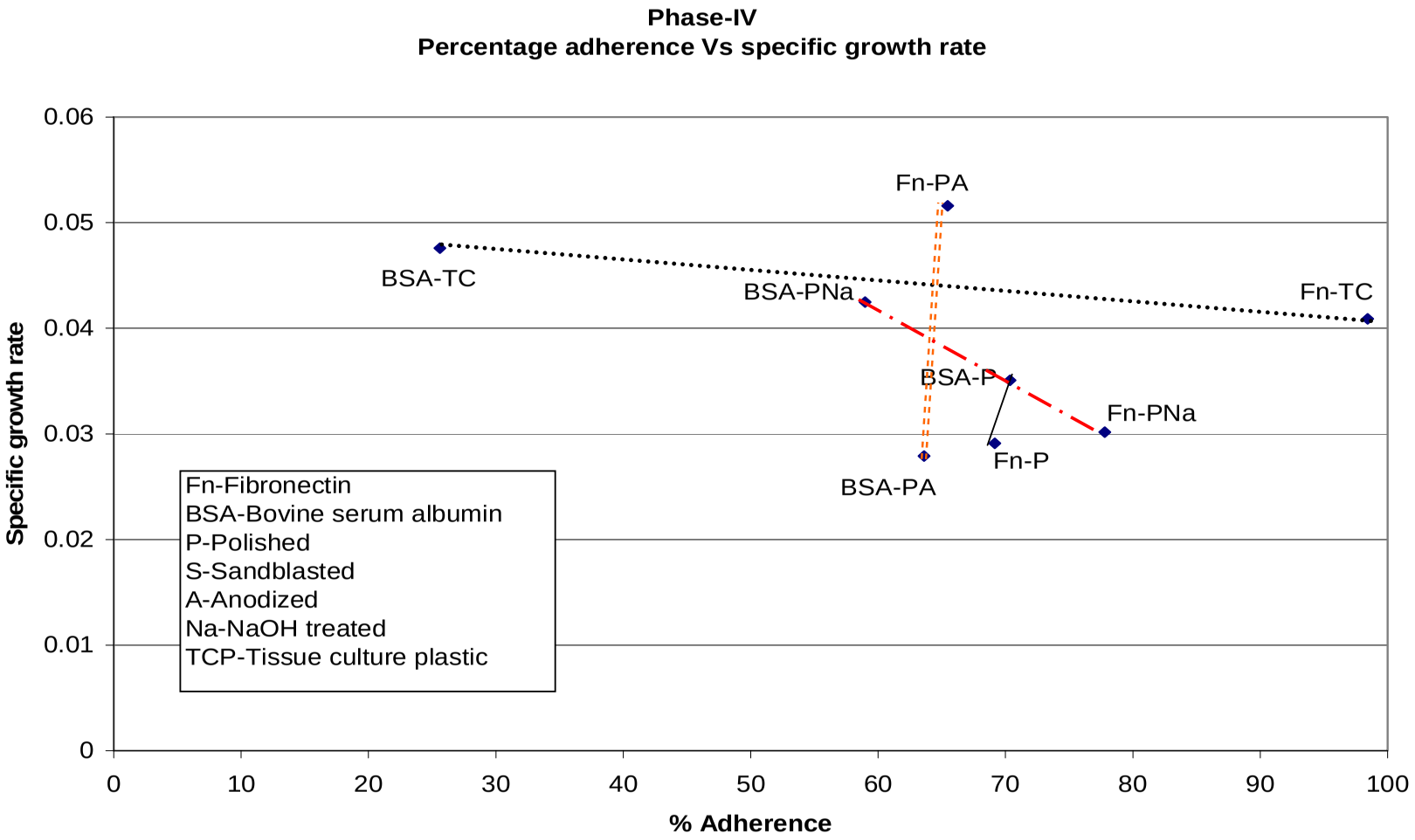

Figure 26. Effect of protein coating on percentage adhesion and specific growth rate in phase-IV.

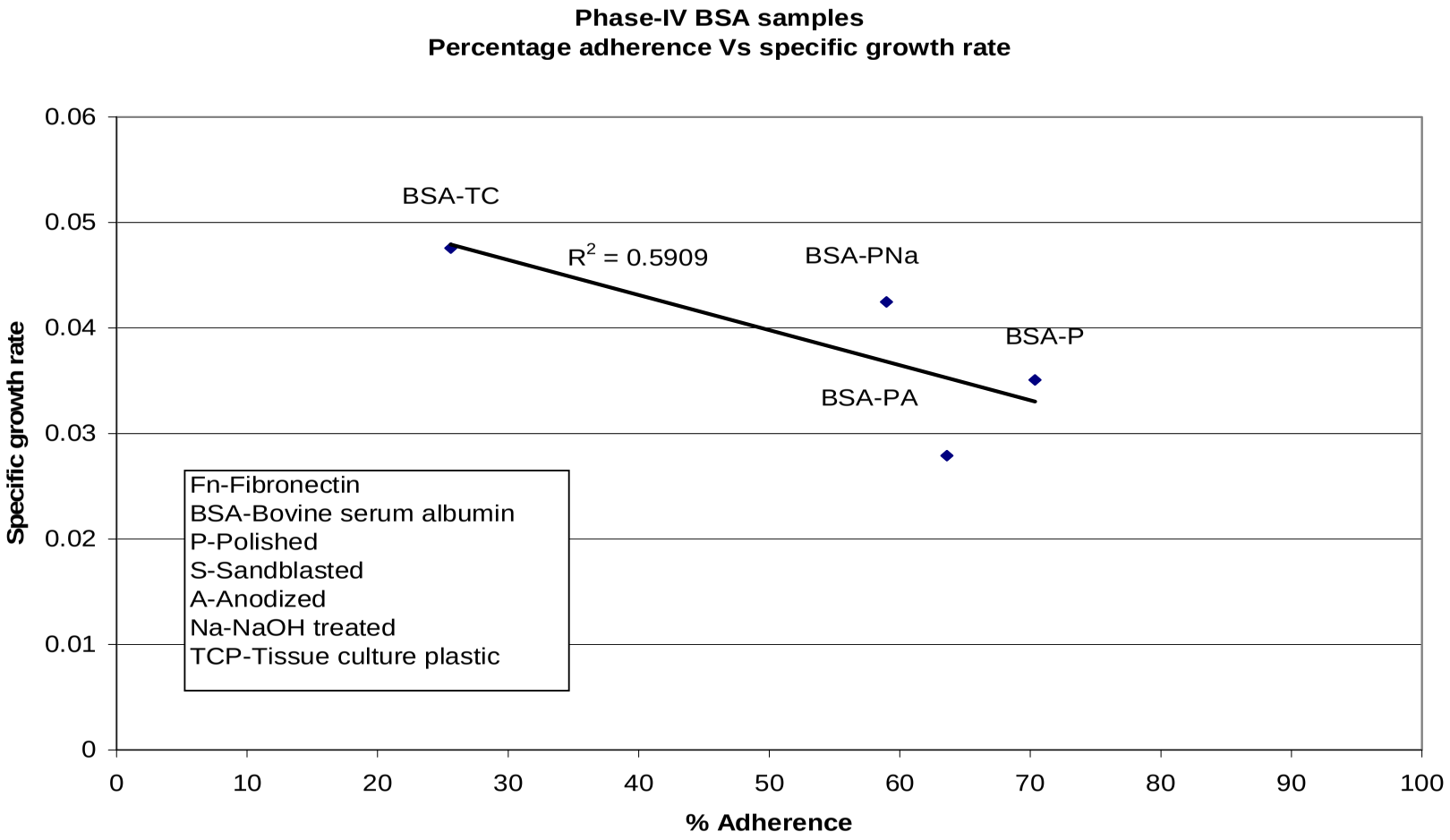

Figure 27. Graph showing growth rate and percentage adhesion specific to BSA coated samples in phase-IV.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this study, titanium alloy (Ti-6Al-4V) was treated with different surface modifications such polishing, sandblasting, anodizing and alkali (NaOH) treatment. Effect of treated titanium alloy surfaces on UMR 106-01 osteoblast cells was investigated. “Exciting” results were found and are summarized below:

- All the surface modification procedures adopted in this study were found to be non-toxic to cells.
- Polished titanium surface exhibited appreciable initial cell attachment and good cell proliferation. Fluorescence images revealed that UMR cells on polished surface were flat and have short filapodia relative to other treated titanium.
- Sandblasting improves cell attachment when compared with polished titanium. But it does not show any additional effect other than polished titanium on NaOH treated samples.

- Anodized titanium surface exhibits relatively lower cell attachment (50-60%) but cell numbers after 48 hrs in culture were found to be equivalent with NaOH treated titanium. Fluorescence images revealed that cells were found to be spreading uniformly and their long filapodia suggests their bonding with the surface
- NaOH treated titanium show the highest cell attachment in the study (other than the positive control of tissue culture plastic), though cell numbers were found to be on par with others after 48 hrs incubation period. It can be inferred from fluorescence imaging that cells were bonding with the surface using their filapodia as an anchoring agent. This feature of NaOH treated titanium separates it from the rest of the surface-modified titanium materials in developing better biocompatible implant.

The special feature of cells anchoring with the surface of NaOH treated titanium, may address the issue of implant loosening in orthopedic surgeries. From the 4 hr cell attachment study and 48 hr proliferation study, it can be concluded that NaOH treated titanium provides support in initial hours of implantation and as well as bolster proliferation and at the same time helps the bone tissue to have a tight bonding with the surface.

5.2 Recommendations

The following are the recommendations for further studies in order to know in and out of titanium implants:

1) Enough care to be taken to completely wash out ethanol from titanium while sterilizing. Any leftover traces of ethanol may effect cell survival. Other titanium sterilization techniques such as gamma and UV radiation methods can be incorporated.
2) Longer cell culture experiments (2-3 weeks) are to be performed in order to study formation of extra cellular matrix and mineralization.
3) Several other cell lines exist for implant in vitro studies such as, intentionally immortalized cell lines, non-transformed clonal cell lines, and primary cultures. These experiments done should be repeated with other cell lines to determine any variations in the performance of the implant materials.

REFERENCES

1. D.M.Brunett, P.Tengvall, M.Textor, P.Thomsen. Titanium in Medicine; Springer: New York, c2001, p 1-1019.

2. Information source: Raymond Choi, Sarah E Campbell. Dental Economics. http://www.dentaleconomics.com/display_article/275090/55/none/none/Focus/The-growing-popularity-of-mini-dental-implants.

3. Information source: Nanotech wire. http://nanotechwire.com/news.asp?nid=1718

4. U.Meyer, A.Buchter, H.P.Wiesmann, U.Joos, D.B.Jones. Basic reactions of osteoblasts on structured material surfaces. European Cells and Materials. Vol.9, 2005, p 39-49.

5. Yunzhi Yang, Renee Cavin, Joo L Ong. Protein adsorption on titanium surfaces and their effect on osteoblast attachment. Journal of Biomedical Materials Research Part A. Volume 67A, Issue 1, 2003, p 344 – 349.

6. Alvaro Mata, Xiaowei Su, Aaron J. Fleischman Shuvo Roy, Bruce A. Banks, Sharon K. Miller, and Ronald J. Midura. Osteoblast Attachment to a textured surface in the absence of exogenous adhesion proteins. IEEE Transactions on Nanobioscience. Vol. 2, no. 4, 2003, p 287-294.

7. H. Zreiqat, F. Ahu Akin, C. R. Howlett, B. Markovic, D. Haynes, S. Lateef, L. Hanley. Differentiation of human bone-derived cells grown on GRGDSP-peptide bound titanium surfaces. Journal of Biomedical Materials Research part A. Vol 64A, Issue 1, 2002, p 105-113.

8. Bren et al. Effect of surface characteristics of metallic biomaterials on interaction with osteoblast cells. Proceedings of the 7th World Biomaterials Congress, 2004, p 17-21.

9. Keller JC, Schneider GB, Standford CM, Kellog Bradley. Effects of implant microtopography on osteoblast cell attachment. Implant Dentistry. Vol 12, 2003, p 175-181.

10. Keller JC, Schneider GB, Standford CM, Perinpanayagam.H, Clegg.M, Zaharias.R, Seabold.D. Implant surface roughness affects osteoblast gene expression. J.Dent. Res.Vol 82(5), 2003, p 372-376.

11. Degasne, M. F. Basle, V. Demais, G. Hure, M. Lesourd, B. Grolleau, L. Mercier, D. Chappard. Effects of Roughness, Fibronectin and Vitronectin on Attachment, Spreading, and Proliferation of Human Osteoblast-Like Cells (Saos-2) on Titanium Surfaces. Calcif Tissue Int. Vol 64, 1999, p 499–507.

12. Klinger, D. Steinberg, D. Kohavi, M. N. Sela. Mechanism of adsorption of human albumin to titanium in vitro. Journal of Biomedical Materials Research, Vol 36, Issue 3, 1998, p 387-392.

13. Xuanyong Liu, Paul K. Chu, Chuanxian Ding. Surface modification of titanium, titanium alloys, and related materials for biomedical applications. Material Science and Engineering R. Vol 47, 2004, p 49-121.

14. Andriana Soares Santiago, Euler Araújo dos Santos, Márcia Soares Sader, Marcelo Felippe Santiago, Gloria de Almeida Soares. Response of osteoblastic cells to titanium submitted to three different surface treatments. Braz Oral Res,. Vol 19(3), 2005, p 203-8.

15. Ge Zhao, Olivier Zinger, Zvi Schwartz, Marco Wieland, Dieter Landolt, Barbara D. Boyan. Osteoblast-like cells are sensitive to submicron-scale surface structure. Clin. Oral Impl. Res. Vol 17, 2006, p 258–264.

16. P. Linez-Bataillon, F. Monchau, M. Bigerelle, H.F. Hildebrand. In vitro MC3T3 osteoblast adhesion with respect to surface roughness of Ti6Al4V substrates. Biomolecular Engineering. Vol 19, 2002, p 133-141.

17. T.M.Lee, R.S.Tsai, E. Chang, C.Y.Yang, M.R.Yang. The cell attachment and morphology of neonatal rat calvarial osteoblast on the surface of Ti-6Al-4Vand

plasma-sprayed HA coating: Effect of surface roughness and serum contents. Journal of Materials Science: Materials in Medicine. Vol 13, 2002, p 341-350.

18. Information source: CellDeath.de, Andreas Gewies. http://www.celldeath.de/apometh/dapi.html

19. D. Buser, N. Broggini, M. Wieland, R.K. Schenk, A.J. Denzer, D.L. Cochran, B. Hoffmann, A. Lussi, S.G. Steinemann. Enhanced bone apposition to a chemically modified SLA titanium surface. J Dent Res. Vol 83(7), 2004, p 529-533.

20. G.B. Schneider, H. Perinpanayagam, M. Clegg, R. Zaharias, D. Seabold, J. Keller,C. Stanford. Implant surface roughness affects osteoblast gene expression. J Dent Res. Vol 82(5); 2003, p 372-376.

21. G. Zhao, A.L. Raines, M. Wieland, Z. Schwartz, B.D. Boyan. Requirement for both micron- and submicron scale structure for synergistic responses of osteoblasts to substrate surface energy and topography. Biomaterials. Vol 28, 2007, p 2821–2829.

22. Zinger O, Zhao G, Schwartz Z, Simpson J, Wieland M, Landolt D, et al. Differential regulation of osteoblasts by substrate microstructural features. Biomaterials. Vol 26, 2005, p 1837–47.

23. Ketul C. Popat, Lara Leoni, Craig A. Grimes, Tejal A. Desai. Influence of engineered titania nanotubular surfaces on bone cells. Biomaterials. Vol 28, 2007, p 3188–3197.

24. Setti S. Rengachary.M.D. Bone morphogenetic proteins: basic concepts. Neurosurg Focus. Vol 13(6), December 2002, p 1-6.

APPENDICES

APPENDIX-A

A. Cleaning and sterilization of titanium metal disc (Revised):

1) Take 100 ml of acetone-ethanol in 1:1 ratio in a 500 ml conical flask.
2) Soak titanium samples in the flask for 1 hr under fume hood.
3) Wash with milliQ water.
4) Take 2 ml of laboratory cleaning agent ie., RBS-35 detergent in a 500 ml conical flask and make up to 100 ml with tap water at 50^0C.
5) Take one set at a time from six different sets of titanium specimens.
6) Drop the titanium discs carefully in to the cleaning solution such that textured surface is facing upside.
7) Connect one end of the hose tube to the flask using rubber stopper and the other end to house vacuum with inline moisture absorbing filter.
8) Allow the titanium specimens to soak under vacuum for about 3-4 hours at room temperature.
9) Swirl/tap the contents of the flask intermittently in order to remove the trapped gas molecules from the specimens.
10) Wash the samples with milliQ water extensively (about 10 washes) in a beaker.
11) Sterilize the samples by soaking them in 70% ethanol overnight.
12) When ready for use, remove the samples from ethanol solution under laminar air flow hood and transfer them to silicone well.
13) Wash the Ti samples with PBS 3-4 times and then continue for further processing.

B. Titanium discs:

1) Micro textured surface on the titanium specimens are created in different types. They are: a) polished, b) polished & anodized, c) polished & NaOH treated, d) sandblasted & cleaned, e) sandblasted & NaOH treated and f) polished sandblasted & anodized.
2) Each specimen is about 25.5 mm in diameter and 4 mm in thickness.
3) Specimens are textured only on one side of disc.

C. Cell culture:

Cell attachment (4 hr incubation study):

1) Take 6 well tissue culture plates with silicone rubber well in it and place titanium discs in it with micro texture upside.
2) Wash the discs with 1 ml of 1X PBS 4 times to get rid off any ethanol traces.
3) Coat the surface with fibronectin (positive control) and BSA (test group) as per procedure mentioned in APPENDIX-B. Setup test group in triplicate.

4) Trypsinize and split the cells from tissue culture (T75) flask and count them in hemocytometer. Run DNA assay on the remaining cells for accuracy in cell number.
5) Cell density: 500 cells/mm^2
6) Inoculate 0.26 * 10^6 cells per well (approximate area 5.09 cm^2 per well) in 1 ml of serum free media of 0.5% BSA and incubate for about 4 hrs at 37^0C in a humid environment of 5% CO_2.
7) After 4 hrs collect the media suspension in a 50 ml centrifuge tube and wash the well with 1ml PBS recover and pool the initial non-adherent cell suspension. Repeat the washings for about 5 times.
8) Centrifuge the non-adherent cells and aspirate the supernatant, re-suspend the cell pellet in 0.53 ml of formamide/ 1% SDS lysis buffer.
9) Cells adhering to the surface of the titanium disc are lysed by adding 0.53 ml of formamide/ 1% SDS lysis buffer per well.
10) Place lid, seal with parafilm. Heat both the adherent and non-adherent cells for 2 hrs at 60 ^{0}C.
11) Allow the plate to cool down.
12) Sonicate lysates and store at 4 ^{0}C until ready to measure DNA and protein assays.

Cell proliferation (48 hr incubation study):

Repeat steps of 4hr incubation study from 1 through 5

6) After 4 hours aspirate the 0.5% BSA media and add 1ml of 10% FBS media.
7) Allow the cells in culture for about 48 hrs in incubator.
8) Aspirate the media and wash the well with 1 ml of HBSS. Repeat washings for 3 times.
9) Lyse the cells with 0.53 ml of formamide/ 1% SDS lysis buffer per well.
10) Place the lid, seal with paraffin. Heat for 2 hrs at 60 ^{o}C.
11) Allow the plate to cool down.
12) Sonicate lysates and store at 4 ^{o}C until ready to measure DNA assay.

Fluorescent imaging:

1) Setup one titanium sample each from four different types of microtextured surfaces to 4 hr incubation and 48 hr incubation. Procedure as above.
2) Procedure for nucleus and alkaline phosphatase (ALP) staining as per procedure mentioned in APPENDIX-C

SEM cell fixation protocol:

1) Fix in 2.5% glutaraldehyde, 4% paraformaldehyde solution for 30 minutes.
2) Rinse with PBS saline for 30 minutes. Repeat this rinsing twice.
3) Wash with milliQ water for 5 minutes. Repeat this step twice.
4) Dehydrate with:
    a. 50% ethanol- 15 minutes
    b. 70% ethanol- 15 minutes
    c. 80% ethanol- 15 minutes
    d. 90% ethanol- 15 minutes
    e. 95% ethanol- 15 minutes
    f. 100% ethanol- 15 minutes
    g. 100% ethanol- 15 minutes
5) Rinse with ethanol and hexamethyl disilazane reagent (HMDS) in 1:1 ratio for 15 minutes
6) Critical point drying with HMDS for 15 minutes.
7) Allow the specimen to dry and gold sputter the surface for SEM imaging.

APPENDIX-B

Preparation of fibronectin 2 μg / ml:

1) Take the stock fibronectin i.e., 5 mg and dilute to 1 mg/ml in 5ml of Phosphate buffered saline without Ca & Mg (PBS).
2) Add 100 μL of 1 mg/ml of fibronectin to 50 ml of PBS. This would make up to 50 ml of 2 μg / ml.

Fibronectin and BSA coating:

1) Add 943 μL per well of 2 μg / ml fibronectin solution
2) Incubate for about 2 hrs at 37^0C
3) Wash with 1.32 ml of PBS each time for about 10 washes
4) Add 1ml of 0.5% medium to each well, incubate overnight at 37 ^{0}C in culture incubator before use (**post blocking with BSA**)
5) To prepare BSA coating on plastic/ titanium surface, add 943 μL of 0.5 % BSA medium and wash the surface 3 times.
6) Add 1 ml 0.5% BSA medium and allow the samples to stay overnight in incubator.

APPENDIX-C

Objective: To verify osteoblast phenotype characteristics by alkaline phosphatase activity using vector red from vector laboratories and to view cell nucleus using DAPI staining.

a) Fixing of cells

Procedure:

1) Collect supernatant media from silicone rubber wells and store at $4^{o}C$.
2) Wash cells with 1ml of cold Hanks BBS (phenol red free).
3) Fix cells with 1ml of 4% paraformaldehyde in PBS.
4) Refrigerate plates for 2-72 hours wrapped in paraffin film.

b) Alkaline phosphatase staining

1) Carefully wash the silicone well with 1ml of PBS with a residence time of 5-10 mins. Repeat washing for 5 times to remove excess fixative from the titanium samples.
2) Prepare the vector red substrate working solution just before use in a test tube. Take 5 ml of 100 mM Tris-HCl, pH 8.2-8.5 buffer and add 2 drops of reagent 1 from vector red assay kit and mix well. Add 2 drops of reagent 2 and mix well. Add 2 drops of reagent 3 and mix well.
3) Precaution to be taken that, steps 2 and 3 are done in dark to get good staining.
4) Drop in sufficient amount of substrate solution such that the surface of titanium is enough covered.
5) Incubate titanium specimens with substrate solution at room temperature until suitable staining develops, about 20-30 minutes.
6) Remove the vector red substrate and discard in to waste container.
7) Wash with 1 ml of Tris-HCl buffer with a residence time of 5 minutes.
8) Treat with 0.05% of Triton 1X 100 solution and leave for 5 minutes undisturbed
9) Wash with 1ml of PBS for about 2 minutes.

c) Counter staining with DAPI

1) Thaw the DAPI stock solution of concentration 1 mg/ml. The solution can only be thawed once, no reuse of thawed stock solution.
2) Prepare a 5 µg/ml solution of DAPI in PBS from the 1mg/ml stock solution. Keep the solution in a controlled location to prevent from light and temperature.

3) Drop the DAPI solution in to the silicone wells and cover the plate with foil for 10-15 minutes at room temperature. Protect plate from light as DAPI dye is photosensitive.
4) Remove the DAPI solution and discard the waste in to waste container.
5) Wash the titanium sample with 1 ml PBS for 5 times with a residence time of 10 minutes each. Discard the waste in to the waste container.
6) Add the vecta shield without DAPI to the titanium samples and place a cover slip over the specimen.
7) Then mount the slide on to the fluorescence microscope and adjust the filter systems accordingly to view the cells on Ti specimen.
8) Handle all the above steps using gloves as little is known about the toxicity and carcinogenic characteristics of substrate components.
9) Take images for documentation.