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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Controlled Nanostructures for Enhanced Biological Responses and Release of
Incorporated Biomolecules

A dissertation submitted in partial satisfaction of the
requirements for the degree
Doctor of Philosophy

in

Materials Science and Engineering

by

Karla Sue Brammer

Committee in charge:

Professor Sungho Jin, Chair
Professor Jennifer Cha
Professor Vlado Lubarda
Professor Eduardo Macagno
Professor Gabriel Silva

2011

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The Dissertation of Karla Sue Brammer is approved, and it is acceptable
in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2011

DEDICATION

To my family and fiancé (Jeff, Kay, Carrie, Kurt, and Hesham)

EPIGRAPH

*"We will go to the moon. We will go to the moon and do other things, not
because they are easy but because they are hard."*

- John F. Kennedy, Jr.

"All it takes is all you got."

- Marc Davis

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PUBLICATIONS AND PATENTS

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ABSTRACT OF THE DISSERTATION

Controlled Nanostructures for Enhanced Biological Responses and Release of
Incorporated Biomolecules

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University of California, San Diego, 2011

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In terms of biomaterial development and implant technology, the cellular response can be affected by topographical circumstances. In the field of *in vitro* cell biology, there is a growing body of data that shows how cells respond positively to substrate topography, particularly in the nanoscale regime. The role of a substrate is more than merely providing mechanical support; it acts as an intelligent surface providing physical, chemical and topographical signals, which

guide and control cellular behavior. Fabricating nanostructures on surfaces provides features that are on the same scale as cellular features and biological entities. Influencing cell behavior using substrate nanotopography is an attractive strategy for regenerative medicine applications and advanced tissue engineering. New fabrication technologies and new nanotechnologies have provided biomaterial scientists with enormous possibilities when designing customized tissue culture supports and scaffolds with controlled nanoscale topography. The goal is to effectively design scaffolds by choosing the appropriate combination of biomaterials, surface chemistry, geometric design, and physical properties to be able to (a.) tailor towards applications as challenging and complex as stem cell differentiation and to be able to (b.) release incorporated biomolecules for therapeutically enhancing cellular environments. This work has emphasis on the observed effects of nanostructured surfaces on various cell behaviors, specifically morphological modulation, enhanced functionality, and guided stem cell differentiation into lineage specific cell types using nanotubes and nanopillars. Additional discussion will focus on sustained, improved bioavailability of drugs incorporated in surface nanostructured “motherships” for long term drug release. Lastly, new scaffold topographic designs and surface structure variations are considered with unique approaches to further i) control cell behavior for tissue engineering therapies and implant designs and ii) tailor release of biomolecules for therapeutic applications.

CHAPTER 1

1. Introduction: Cell-Surface Interactions

In recent years, nanoscale sciences and engineering have provided new avenues for engineering materials down to molecular scale precision. The resultant materials have been demonstrated to have enhanced properties and applicability; and these materials are expected to be enabling technologies in the successful development and application of nanomedicine (1). Research efforts have soared as more and more knowledge is poured into precise control and manipulation of nanomaterial properties for many innovative and exciting applications. Nanotechnology has found a natural partner in biomedicine, where the biological entities and processes involved, such as mammalian cells (1-100 μm), proteins and enzymes (1-10 nm), genetic coding and cellular functioning on the molecular level rely on and are surrounded by size scales similar to or smaller than the materials concerned. The development of nanostructures, such as those discussed in this work, allows unprecedented access to and intimate interaction with the fundamental subunits of biology.

1.1 Nanostructures of and Around the Cell

1.1.1 Cellular Nanofeatures and Functions

The basic structural, functional and biological unit of the human body is the cell. The human body contains ~50-75 trillion cells operating in harmony (2). There are a variety of cell types, each one specialized for a specific role with distinct features and functions dictated by the surrounding tissue. There are

kidney cells, nerve cells, skin cells, bone cells, the list goes on...with complex characteristics and unique roles to be carried out diligently to form the working human body. The cell is anything but simple.

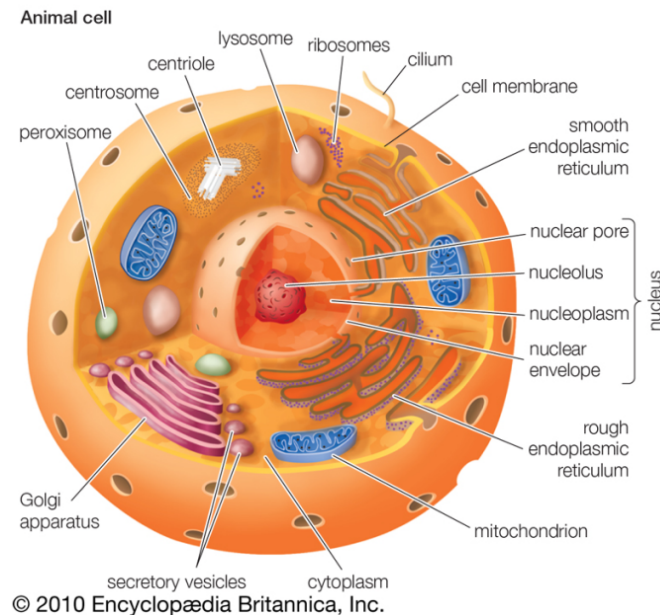


Figure 1.1-1. Schematic illustration of a typical cell (3). The cell organelles within the cell body have distinct geometries and are labelled in the figure accordingly.

Cells are typically 1-100 μm in size, and in general are spherical in nature. However, depending on the specific type of cell, the cell shape can take on several forms such as tubular, elongated, polygonal, oval, triangular, etc. The cell shape will dictate the function of the cell and the cell is most likely shaped and formed by external and internal stimuli (this can include tension, mechanical stress, or pressure from interacting with the surrounding environment as well as biomolecules and signalling from within, respectively). Cells, although micrometer in dimension, are very reactive and have sensitive features on their interior and exterior that are nanometer in dimension. The functioning structures

inside the cell are called organelles, little organs, with interesting nano-geometries. Each organelle adapts and carries out its own vital function. **Fig.1.1-1** shows the intricate features and complex components of a typical cell.

This work will emphasize three main nanoscale components that have been described in full in the text book reference (4) :

- *The cell membrane* ---the perimeter and protector of the cell that regulates the entrance and exit of the substances, thereby maintaining internal balance. It is the gate keeper, the border between outside and in.
- *The cell cytoskeleton*---makes up the internal framework which gives each cell its distinctive shape and high level of organization. It is the infrastructure of the cell.
- *The cell nucleus*--- this is referred to as the “brain” and programming center of the cell. The function of the nucleus is to control all activities carried on in the cell.

At the perimeter of the cell is the *cell membrane*, also called the plasma membrane. A schematic of the cell membrane is illustrated in **Fig.1.1-2**. The cell membrane is a semipermeable phospholipid flexible bilayer with a variety of biological molecules and essential proteins embedded within that are involved in a large array of cellular processes. The essential proteins called *integrins* (integral proteins, globular proteins shown in **Fig. 1.1-2**) that span through the membrane are critical as cell receptors which inform the cell of the environment and evoke a response. Integrins perform outside-in signaling as well as operate in

inside-out mode. The cell membrane is the intermediate anchor point for the extracellular environment or extracellular matrix (ECM) on the outside of the cell and the intracellular *cytoskeleton* on the inside of the cell. The transmembrane integrin proteins transduce information from the external environmental ECM to the inside of the cell, allowing rapid and flexible responses to changes in the environment.

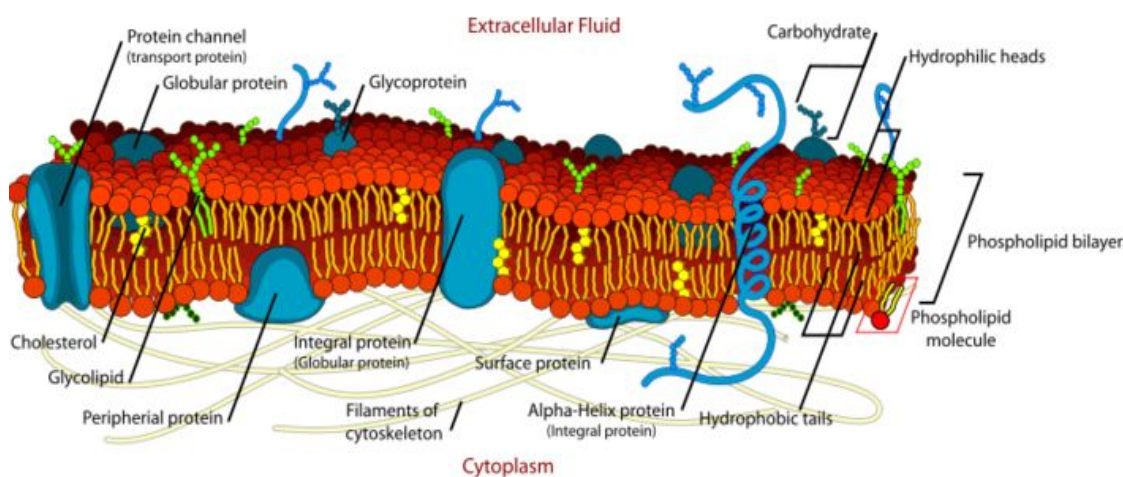


Figure 1.1-2. Public domain, courtesy Mariana Ruiz. Schematic Illustration of the cell membrane.

The *cell cytoskeleton* is present in the interior area of the cell cytoplasm. The cytoskeleton is considered the nano-infrastructure of the cell, which is anchored to the inside wall of the cell membrane. It maintains the strength and shape of the cell. The cytoskeleton allows the cell to adapt, reorganize and change shape. The cytoskeleton is dynamic scaffolding that provides “tracks” in the form of protein filaments for structure and to transport of organelles, signaling molecules, vesicles, even physical forces such as stress can be transmitted along the filaments of the cytoskeleton. The cytoskeleton is made up of three types of

filaments 1) micro-filaments, f-actin (8-10nm in diameter) 2) intermediate filaments (8-10nm in diameter) and 3) microtubules (25nm in diameter).

The cytoskeleton also functions to give support to and protection for the *cell nucleus*, the largest of the cell organelles. The intermediate filament components of the cytoskeleton secure the nucleus. The cell nucleus houses all the genetic programming for the cell by regulating gene expression. The nucleus controls all activity of the cell.

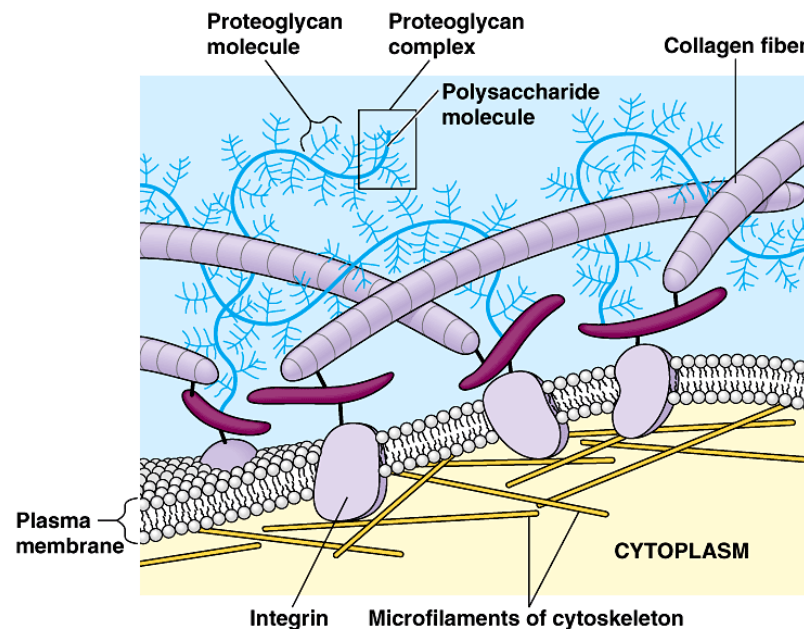
The underlying connection and focus of this report, between the cell *membrane*, the cell *cytoskeleton*, and the cell *nucleus* follows that:

- i. The cell membrane will be influenced from the external surrounding environment.
- ii. This causes the transmembrane cell sensors or integrins to relay a message to the internal cytoskeleton.
- iii. The cytoskeleton will rearrange its components and pass along a message in the form of signaling molecules, or physical stress or strain forces, inducing morphologic shape changes to the cell and relays an input to the nucleus.
- iv. The nucleus will cause direct changes in the initiation of new or altered cell functions and activities.

1.1.2 Influence of Extracellular Nanostructures

The extracellular environment is comprised of several factors that stimulate and interact with the cell and initiates a response. The key stimulants are i. physical stimulation (i.e. flow of biologic fluid, pressure, stretch), ii. soluble

factors (i.e. biochemicals, ionic salts, hormones, growth factors), iii. intercellular contract between neighbor cells, and iv. biomimetic substrates or the underlying ECM. This report focuses on the latter. The normal cell environment involves a complex network of ECM with intricate features that are nanoscale in dimension. In many instances the capacity of a cell to regulate its functions such as to proliferate, differentiate, and to express specialized operations intimately depends on the presence and maintenance of an intact ECM (5). **Fig.1.1-3** illustrates the cross-section of the cell membrane and complex nature of the extracellular matrix outside the cell. The integrins are embedded in the cell membrane, directly connecting the ECM molecules (collagen fibers and biomolecules) and cytoskeleton components (actin microfilaments).



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Figure 1.1-3. Cross-section of the cell membrane revealing the intricate structure of the extracellular matrix on the outside of the cell and the direct connection to the cytoskeleton (actin microfilaments) on the inside of the cell(6).

In 1986, Chester coined the definition of a biomaterial as a “non-living material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body” (7). Cells in our body are predisposed to interact nanostructured surfaces in the form of the ECM for optimal functioning (8). Therefore, in order to truly interface with biological systems, fabricating nanofeatures upon biomaterial surfaces provides features that are on the same scale as the bio-environmental features of the natural ECM that will allow intimate interact with cells. Observing what happens on biomaterials with different surface nanostructures can help to understand and uncover pathways that promote the desired cellular response, via surface engineering approaches, to aid in tissue regeneration and beyond. Furthermore, this work is designed to reflect and contribute to the definition of nanomedicine (1): “The monitoring, repair, construction and control of human biological systems at the molecular level, using engineered nanodevices and *nanostructures*.”

1.2 Engineering Nanostructures for Cell Interactions

Cell-substrate interactions play a crucial role in biology. Understanding these interactions helps explain cell behavior *in vitro* and *in vivo*, allowing us to use the knowledge gained to control and manipulate cell behavior. A fundamental principle of manipulating these interactions is the aspect of improving the design and integration of medical devices and medical implants inside the body. Tissue and organ formation results from a complex coordination of cell-substrate adhesion, proliferation, execution of cell functioning, migration, differentiation, and so on. Interaction of cells with the ECM via cell

transmembrane *integrins* is at the center of these events (9-11), because the integrins are so crucial in relaying messages from the outside-in. Experimenting with artificial biomaterial surfaces and gaining insight on the influence on cell behaviour will contribute greatly to regenerative medicine and tissue engineering schemes.

1.2.1 The Role of the Surface

A number of surface properties have been revealed to affect cell attachment and subsequent behavior, including surface composition, surface charge(12), surface energy(13-14), surface oxidation (15), solidity(16), curvature(17), and surface features and shapes (18-19). The initial event that occurs at the surface when a material is put *in vitro* or *in vivo* culture conditions is the adsorption of proteins (occurs in less than 1 second) which are present in cell culture media and natural biological fluids. The proteins adsorbed will make up the cell's "perceived" ECM. The surface characteristics directly impact the adhesion of these proteins and the configuration of the proteins. There are several material factors that affect how the proteins will adhere thus impacting how the surface is perceived by the cell. Some material factors include and are not limited to surface chemistry, surface energy/tension/wettability, surface roughness, crystallinity, surface charge, size of the features (nano vs. micro), geometry of the features, ion release, and other mechanical properties such as elasticity. The cellular events, post protein adsorption, that occur at the surface are as follows (**Fig. 1.2-1**):

- a. Initial contact of the cell with a bed of adsorbed proteins dictated by surface characteristics.
- b. Formation of bonds between cell surface receptors (integrins) and the protein functional groups (ligands).
- c. Cytoskeletal reorganization with progressive spreading of the cell on the substrate for increased attachment strength and subsequent response.

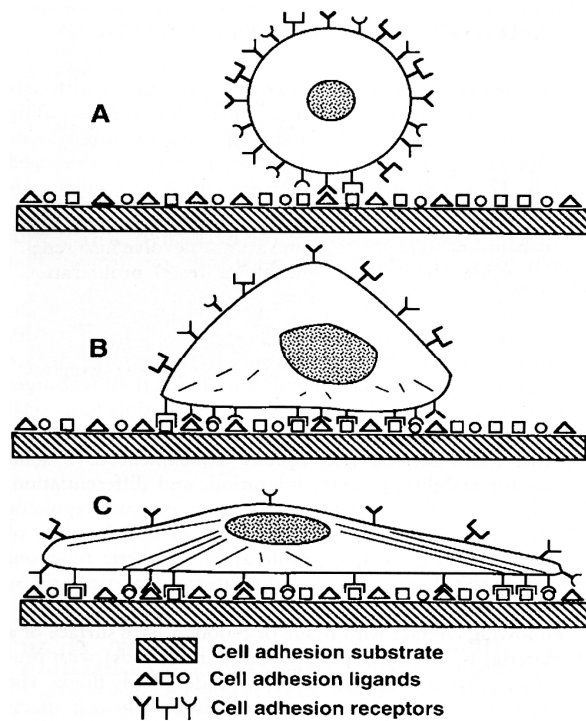


Figure 1.2-1. Cellular events at the surface(20).

In studying biomaterials, it is beneficial to understand the protein adsorption characteristics which are ultimately dependent on the surface features of the biomaterial. Transmembrane cell receptors (integrins) interacting with the protein layer are governed by molecular events at the nanometer

scale. Focal adhesions, the sites of cell attachment to the underlying substrate, play a pivotal role in all subsequent cell actions in response to nanotopography. These dynamic adhesions are subject to complex regulation involving integrin binding to ECM components, and the reinforcement of the adhesion plaque by recruitment of additional proteins (21). The term ECM is referred throughout this work in two ways 1) as the nanostructure surface where cells interact, attach, and function on and 2) the extracellular molecules/proteins/minerals etc. that are secreted by the cells adding to the unique nanostructured environment and allowing for proper functioning, both contribute to the extracellular environment.

In addition to their adhesive functions, integrins mediate bidirectional signalling between the cell and the ECM, activating both direct *mechanotransductive signalling* and indirect molecular cascades that regulate transcription factor activity, gene and protein expression, and ultimately growth and differentiation, as will be discussed in the following sections.

For non-leaching materials, the body will “read” the material through its surface. This brings much attention to the surface of biomaterials, especially because surfaces are uniquely reactive, much different from the bulk, readily contaminate (especially in fluid, more so in biofluids), and in terms of the size of the features, in particular nanostructures possess a higher surface area to volume ratio than microstructures.

1.2.2 Scaffold Engineering

Tissue engineering has been defined as “understanding the principles of tissue growth, and applying this to produce functional replacement tissue for

clinical use" (22). Tissue engineering utilizes living cells in combination with engineered extracellular matrices or scaffolds to evaluate the physiological response of tissues. The scaffold is designed to recapitulate the *in vivo* milieu and influence cells based on simulating different micro/nanoenvironments. Many researchers are interested in investigating and understanding which way a cell reacts to different kinds of scaffolds, also known as substrates, which are the underlying biological surfaces at which cells will attach and grow.

The ability of the substrate to influence cell orientation, migration and cytoskeletal organization was first noted by Harrison in 1911 (23). He grew cells on a spider web and observed that the cells oriented themselves to follow and grow along the length of the web fibers. This phenomenon was named physical guidance. Later in 1964, it was first proposed that cells react to the topography of their scaffold environment, where the surface shape and physical features themselves resulted in a cell response (24). Since then, numerous studies have shown that many cell types react strongly to the topography of the scaffold of which they are grown.

A major determinate in cell behavior is cell spreading and the morphology it assumes. It has been shown that cell spreading controls growth (25-26), gene expression, protein synthesis, and differentiation functioning (27-30). Because it has also been revealed that the surface topography directly regulates how the cell adheres, the cell shape and the extent of spreading (31-32), it can be hypothesized that it is possible to directly control cell behavior by means of the surface. Although the importance of topographic cues may vary

for different cell types, its relevance is unquestionable. Emerging literature presents many interesting findings on the effects of topography (33), more so on the effects of topography with nanometer sized features. In general, nanosized features have been proven to improve cell behavior over micrometer features (34-35). It has been found that nanotopography stimulates many different cell types in many different ways:

- Influences protein organization
- Enhances cell adhesion
- Alters cell morphology/shape
- Affects proliferation
- Initiates intracellular signaling
- Provides contact/physical guidance
- Mediates cell differentiation and cell functioning

Incorporating topographical consideration into the design of scaffold surface environments is becoming increasingly important in light of these studies. The role of the substrate guides and controls cell behaviour at the cell-surface interface.

There is a vast parametric space within which the size, geometry, and pattern of the topographical features should be investigated. The potential gain of these studies would likely be enormous both in fundamental understanding of the central processes in cell biology and in application of a new approach to advance biomaterials and implant design.

1.2.3 General Types of Nanotopography

In general, the topographic influence of the surface on cell behavior is quite distinguishable. The fact that in *in vivo* circumstances the extracellular matrix (ECM) or substratum with which cells interact includes topography at the nanoscale brings up the need to investigate cell-substrate interactions at the submicron level. With advances in nanofabrication technologies the promise of and the unknown information about topographical effects in manipulating the cell-substrate interaction is now being uncovered.

New fabrication technologies and new nanotechnologies have provided biomaterial scientists with enormous possibilities when designing customized tissue culture supports and scaffolds with controlled nanoscale topography. The main challenge in designing nanotopography of a scaffold is to choose the appropriate combination of material composition, size, geometry, structure, and properties (i.e. stiffness, elasticity, roughness). A few general designs of surface topography and desired tissue interactions are:

1. *Lithographic surfaces*— provides patterns of precise topographies (grooves, islands, pillars, holes) using clean room techniques such as photolithography and reactive ion etching. Early studies included surfaces with grooves or ridge features with distinct heights, widths and pitch for controlling cell adhesion, morphology, and orientation (36). The groove structure was first used to provide “contact guidance” or physical guidance. The effects on cells are based on the notion that ridges have sharper angles that aid in orienting cells better. It was determined that

cells adhere better when aligned with a surface texture or line pattern of the ridges. One of the first applications was sought for wound healing. While photolithography is limited by diffraction limitations and often features remain in the micron range, electron beam lithography can be used to produce nanoscale patterns. Some other methods such as laser ablation, x-ray lithography and imprint lithography may also be used, but few cell culture studies have been conducted on substrates produced by these techniques (37).

2. *Porous, roughened and textured surfaces*—this design is based on increased surface area which promotes the adhesion of cells through integrin and focal adhesion contacts and improves tissue ingrowth, resulting in a mechanically stable device. These surfaces have been found to have potential in mechanical interlocking and reducing the interfacial motion. It has been hypothesized that introducing pores to the surface could be a strategy for improving tissue attachment and *in vivo* implant integration success, especially orthopedic (bone) implants. It has also been shown that textured surfaces play a much broader role in influencing cell behavior and functioning including differentiation states of cells and protein synthesis (38). Chemical etching (removing material at the surface) or physical roughening such as grinding or blasting of the surface are some examples of methods used to create altered surface textures.

3. *Fiber-meshed surfaces*—can generate gradients of pore networks, and more closely imitates extracellular matrix protein fibers, such as collagen. Electrospinning is a promising and versatile technique for generating loosely connected 3D porous mats with high porosity and high surface area (39). The fibers can be randomly or aligning on the surface. There are many options for polymeric material types which include bioresorbable/biodegradable and natural (PLGA) or synthetic types.

Examples of some typical types of nanotopographies studied for controlling cell behaviour is shown in **Fig. 1.2-2 (40)**.

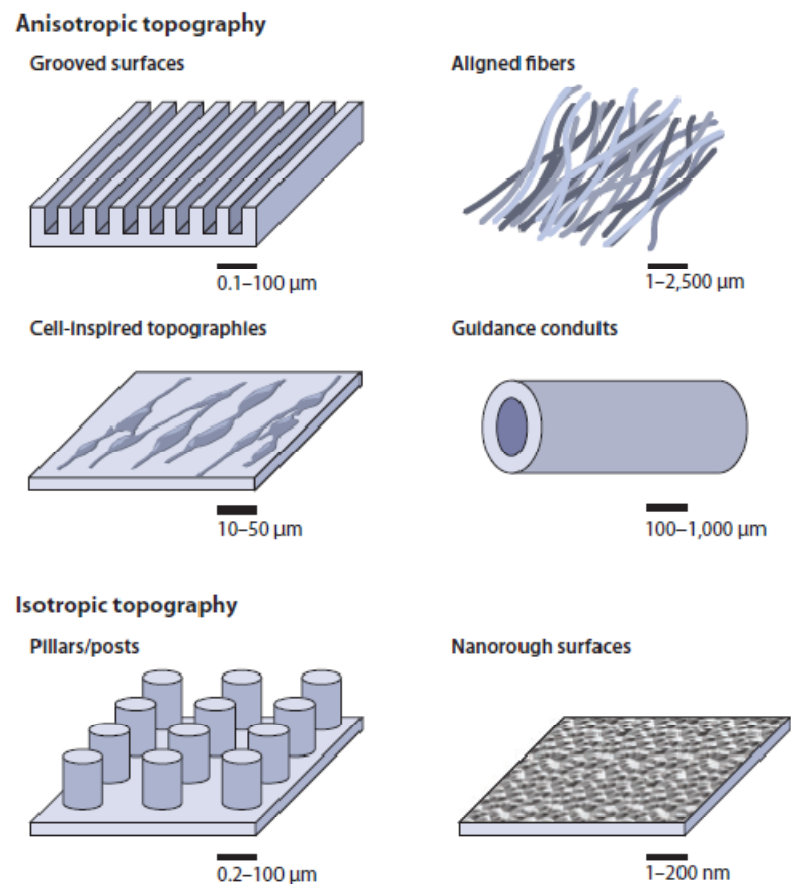


Figure. 1.2-2. General nanotopography designs for influencing tissue interactions (40).

The availability of nanofabrication techniques with uniform and well defined surface morphologies has facilitated a systematic investigation into the role of substratum morphology on cell physiology. However, this particular area of research is still in its infancy, more studies are needed. It is still not completely possible to predict what types of nanoscale features would be the most effective in generating the desired cellular response.

1.3 Scope of the Dissertation

Recent studies suggest that substrate topography may impact cell physiology in many previously unknown ways including, cell adhesion, morphology, function, and even differentiation. The effects on cell behaviour seem to be mediated via modulation of cell integrin and focal adhesion and spreading governed by underlying surface characteristics. Subtle differences in substrate characteristics could translate into significant differences in cell behaviour. The surface characteristics are an important parameter which can be used to optimize substrata for *in vivo* implant technologies and *in vitro* cell culture schemes. The data reviewed in this work makes three important points.

First, in the use of textured systems such as nanotubular shaped titanium dioxide (TiO₂), there lies the opportunity for improving orthopaedic implant performances by optimizing the surface oxide morphology on titanium (Ti) implants. Elucidation of cell behaviour with respect to cell growth, function and differentiation should help the design of better implants. For example, by optimizing geometry of nanotube pores we can increase function of bone cells for enhanced bone forming ability and allow directed differentiation of bone

marrow stem cells into mature bone cells for rapid integration *in vivo*. Design of the surface texture will be motivated from the understanding that substrate topography modulates cell shape upon initial adhesion, which in turn sets the stage and affects long term cell behaviour. The TiO₂ nanostructure is not limited to orthopaedic applications; it also has great opportunities to advance vascular stent, osteo-chondral, and anti-inflammatory technologies.

Secondly, for systems that contains textures that compare different chemistries or surface properties (including surface wettability, feature size, and feature density), there lies the opportunity to investigate novel topographies for understanding mechanisms and controls therein for modulating stem cell differentiation for industrial cell culture. Surface engineering approaches that alter the physical and material chemical composition of the surface topography could be used as an effective tool and as an alternative to soluble biochemical means (such as growth factors or hormones) of directing stem cell interactions and subsequent differentiation. This aims to highlight the importance in the physical and chemical composition aspects of material carriers for stem cell based tissue engineering schemes.

Thirdly, for systems that contain textures that have the capacity to store and release biomolecules for controlled drug delivery, there lies the opportunity to bring a localized therapeutic aspect to implant surfaces and biomedical devices. For instances, ceramic porous surfaces of alumina have the capacity to allow local release of antibiotics for orthopaedic implant technologies. Nanowire carriers made of silicon provide not only provide drug loading and release

capabilities but also provides anti-biofouling and bio-erosion properties for advances in slow release on the surface of biomedical devices. Loading nanostructures with therapeutic agents can provide a more reliable antibiotic protection at the surface of an implant or biosensor site and has a combinatory affect for enhanced integration into the body.

1.3.1 Objectives and Motivation

The overall objectives of this research is to i) systematically elucidate the effects of surface nanostructures on *in vitro* cell behaviour and to explain these results via biochemical or biophysical mechanisms working toward the goal of optimized implant surfaces and ii) reveal the potential of surface nanostructures as therapeutic carriers and to characterize and tailor the release kinetics for controlled drug delivery working toward the goal of improved biomedical device applications. To achieve these goals, the specific aims are:

- a. To prepare surface nanostructures with defined features and material characteristics.
- b. To characterize the nanostructures and material properties using suitable surface techniques.
- c. To apply suitable assays for measuring cellular response to different surface nanostructures.
- d. To explain the difference in cellular response to different surface nanostructures.
- e. To design substrates with desired nanotopography and achieve better control of cell physiology.

- f. To utilize the surface nanostructures as potential therapeutic carries.
- g. To describe the biomolecule release kinetics from surface nanostructures.

This study involves the important and exciting direction of research in nanomedicine that will help gain better understanding of the interaction between cells and nanostructures. This will facilitate the creation of next generation biomaterials with well-defined nanotopographies that can elicit the desired cellular and tissue response as well as provide therapeutic benefits.

CHAPTER 2

2. TiO₂ Ceramic Nanotubes

Advances in biomaterial surface structure and design have improved tissue engineering. This chapter is a report on titanium dioxide (TiO₂, or titania) nanotube surface structuring for optimization of titanium (Ti) implants utilizing nanotechnology. The TiO₂ nanotube surface structure formation, mechanism, bio-characteristics, and emerging role in tissue engineering and regenerative medicine are reviewed. The main focus will be on the unique 3-D tube-shaped nanostructure of TiO₂ and its effects on creating profound impacts on cell behavior. Specifically, we will discuss how the adhesion, proliferation, and phenotypic functionality are enhanced on surfaces with TiO₂ nanotube surface structuring. TiO₂ surface nano-configurations are advantageous in regulating many positive cell and tissue responses for various tissue engineering and regenerative medicine applications, for orthopaedic, osteo-chondral, vascular implant applications and beyond.

2.1 Vertically Aligned TiO₂ Nanotubes by Anodization

It is well known that titanium (Ti) and its alloys have been used as implantable biomaterials because they have high-quality mechanical properties, a native oxide layer that is resistant to corrosion, are biocompatible and bioactively react with native human tissue. In recent studies, vertically aligned TiO₂ nanostructures fabricated by electrochemical anodization have

become apparent as primary candidates for direct control of many types of cell behavior for progressing tissue engineering.

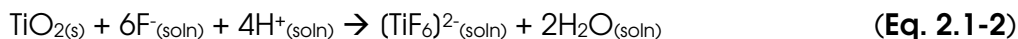
2.1.1 Electrochemical Anodization

TiO₂ nanotubes prepared by anodization in fluorine-ion containing electrolytes are described first in this chapter. In general, the mechanism of TiO₂ nanotube formation in fluorine-ion based electrolytes is said to occur as a result of three simultaneous processes: the field assisted oxidation of Ti metal to form titanium dioxide, the field assisted dissolution of Ti metal ions in the electrolyte, and the chemical dissolution of Ti and TiO₂ due to etching by fluoride ions, which is enhanced by the presence of H⁺ ions (41). TiO₂ nanotubes are not formed on the pure Ti surface but on the thin TiO₂ oxide layer naturally present on the Ti surface. Therefore, the mechanism of TiO₂ nanotubes formation is related to oxidation and dissolution kinetics.

Metallic Ti naturally contains a stable, passivation surface layer of TiO₂ that tends to inhibit further chemical reactions to occur on the Ti surface. When the passivation layer is damaged the layer is generally restored quickly. This restoration reaction occurs when the TiO₂ layer is in contact with air or water. This reaction produces titanium oxide as well as hydrogen gas, according to the following reaction (42):



When the Ti surface is in contact with the aqueous fluorine containing electrolyte solution, the TiO₂ layer forms rapidly. The detailed dissolution mechanism of TiO₂ with regard to the formation of TiO₂ nanotubes in fluorine containing solutions has been proposed to be sufficiently described by the following reaction (43):



In this process, an intermediate layer (TiF₆²⁻) is formed predominantly at the surface of Ti in the fluorine-containing solution. It is known that the TiO₂ nanotube pore formation is based on both electrical field assisted dissolution of TiO₂ layer and the chemical dissolution by the fluorine-containing electrolyte. Both dissolution reactions occur at the same time and play a critical role in understanding the formation of TiO₂ nanotubes at the Ti surface. Briefly, when the Ti surface is first etched by the electrolyte solution, very small pits (sub-nanometer scale) are formed on the Ti surface and are rapidly restored to a TiO₂ layer. With continuous corrosion of the Ti surface by HF ions, the pits become nano scale pores. As reaction time goes by, nano scale pores become the main body of nanotubes, and the small pits which are still constantly formed at the latter stage of the processing become the interspaces between nanotubes. Another proposed mechanistic model further explains that prior to pit formation, there is an occurrence of microcracks in the TiO₂ layer, which further develop and guide the formation of the pits (43). The final fabricated structure is shown in

Fig 2.1-1 with a schematic illustration of the electrochemical anodization apparatus and the TiO_2 nanotube arrays presented by scanning electron microscopy images (SEM) and cross-sectional a transmission electron microscopy (TEM) image.

Furthermore, based on the mechanism of nanotube formation, it is inherent that the nano-tubular structure formation depends on both the intensity of applied voltage and the concentration of fluorine ions in solution. It is well-known that by increasing the applied voltage, larger diameter nanotubes can be formed. This aspect of diameter manipulation using applied voltage will be further emphasized and the effects on cell function and fate is also discussed.

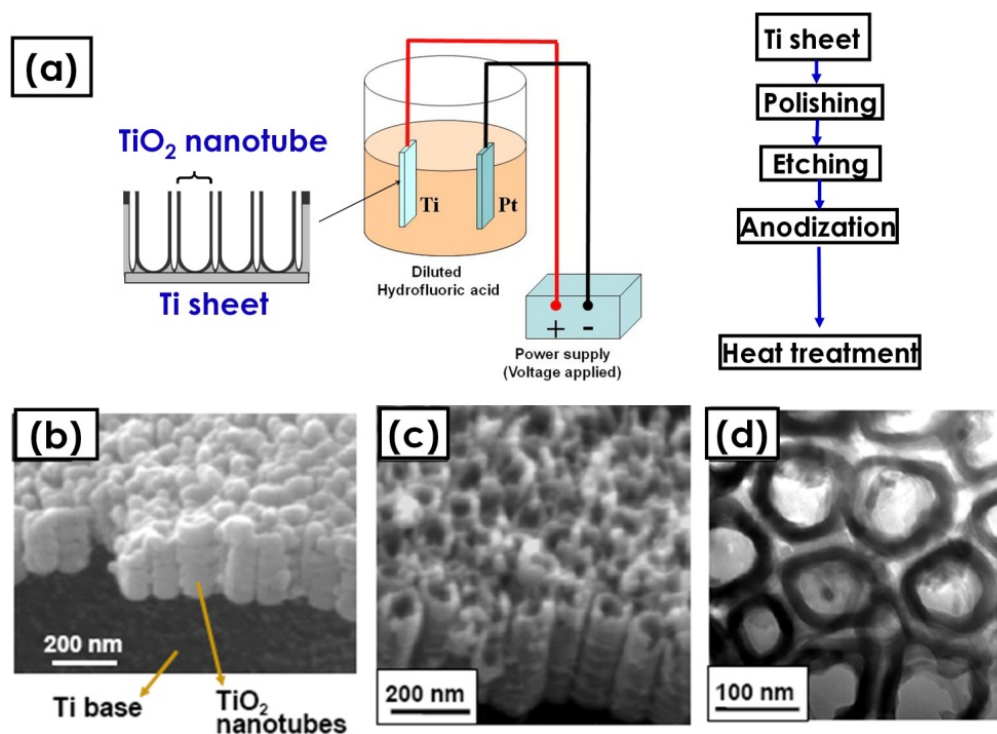


Figure 2.1-1. TiO_2 nanotube surface structuring. **(a)** Schematic illustration of electrochemical anodization and experimental flow chart to form TiO_2 nanotube arrays. **(b)** Scanning electron microscopy (SEM) image showing vertically aligned TiO_2 nanotube arrays on a Ti sheet. **(c)** Higher magnification SEM image. **(d)** Cross-sectional transmission electron microscopy (TEM) image.

2.2 Orthopaedic Implant Considerations

The most common and successful biomaterial being used for bone implants is titanium, which does not elicit an inflammatory response *in vivo*. The bone bonding generally occurs without the common connective tissue layer that forms from the body's immune response (foreign body reaction), between the implant metal and the underlying bone surface (44-46). The majority of joint implants fail because they become loose at the biomaterial/bone interface, suggesting that poor osseointegration contributes to the failure (47). It is therefore logical that attempts are made in the tissue engineering field to replicate the porous structure of bone for implants for tissue ingrowth and mechanical interlocking. However, porous structures at present lack the mechanical strength needed for load bearing. There are a variety of modified titanium implants on the market that focus on incorporating rough structures and chemical coatings to improve osseointegration for bone implants, but the implant materials are far from being optimal.

While a thin TiO_2 passivation layer on the Ti surface can impart improved bioactivity and better chemical bonding to the bone (48), other techniques have been developed to further enhance the bioactivity of a pure Ti surface, such as direct coating of bioactive materials like hydroxyapatite and calcium phosphate (49-51). However, even though these surface modified layers have good bioactivity and high surface area, they tend to delaminate at the interface between the implant and the bone due to the relatively large, micrometer-regime thickness of the coated layer on Ti (52), presumably due to

the stress accumulation commonly seen in a thick coating of foreign material. In order to overcome this problem, some plasma spray Ca-Si based ceramic coatings have been developed but still have roughness and layer thickness in the micrometer range. For the purposes of this study, the focus is on nanoscale thickness surface coatings. Therefore, developing an implant bioactive surface layer having high surface area for enhanced bonding yet thin enough, in the nanometer range per se, to minimize delamination would be desirable.

Recent reports indicate that modifying Ti surfaces with TiO_2 nanotubes for orthopedic applications significantly enhances the mineral formation (53), adhesion of osteoblasts *in vitro* (54), and strongly adherent bone growth *in vivo* (55), showing better bone bonding characteristics than conventional micro-roughened Ti surfaces by sandblasting. One physical advantage of the TiO_2 nanotube surface system is that it is composed of and created directly from the native underlying Ti constituent, unlike the foreign ceramic and spray coatings on Ti or Ti alloyed surfaces mentioned previously. As well, the nanotube layer is at most ~300nm tall (for the purposes of this work) which in the scheme of things is a much thinner layer and this nanometer length scale eliminates the tendency of delamination prevalent in thick micrometer layers.

2.2.1 Improved Bone-Forming Functionality on Diameter-Controlled TiO_2 Nanotube Surface

The Jin lab was the first to demonstrate that TiO_2 nanotubes, **Fig.2.1-1** can significantly accelerate osteoblast (bone cell) adhesion and proliferation at the biomaterial/tissue interface and enhance bone mineral formation. The TiO_2

nanotubes are formed as vertically aligned configuration, with an average diameter of ~100 nm, a height of ~300 nm, and a wall thickness of ~10 nm. According to published research (56), nanotube arrays on titanium surfaces induced proliferation of osteoblasts by as much as 300 – 400% compared to non-modified titanium surfaces. In other research groups studying nanoporous materials, major accomplishments have been made in the generation of geometrically defined surfaces with the fabrication of Al and Si nanostructured surfaces. There is rapidly increasing evidence that the lateral spacing of features on the nanoscale can impact and change cell behaviour (57-59). Therefore, in order to optimize the lateral spacing of the TiO₂ nanotube system, by changing the geometry of the nanotubes, four different pore sizes (30, 50 70, and 100nm in diameter) were created for examination of bone cell response. In this section, cell behaviour of osteoblasts cultured on various inner pore diameters of vertically aligned TiO₂ nanotubes was investigated. The nano-size effect on osteoblast cell adhesion, morphology and osteogenic functionality for biomaterial implant optimization was explored. Here it is reported that a unique variation in cell behavior is obtained even within such a narrow range of nanotube dimension.

2.2.2 Experimental

TiO₂ Nanotubes Fabrication

TiO₂ nanotube surfaces were created using a two-electrode setup anodization process as described previously (53-54). Briefly, a cp-Ti sheet 0.25 mm thick, (Alfa-Aesar, 99.5% metals basis, USA) was used for this process. The

nanotubes were prepared in a 1:7 volumetric ratio of acetic acid ($\geq 99.99\%$ purity, Sigma-Aldrich) to 0.5 w/v% hydrofluoric acid in water (EM Science, 48 w/v %, USA) at 5, 10, 15 and 20 V for 30 minutes. A platinum electrode (Alfa-Aesar, 99.9%, USA) served as the cathode. The samples were then washed with deionized water, dried at 80°C , and heat-treated at 500°C for 2 hours in order to crystallize the as-fabricated amorphous structured TiO_2 nanotubes into anatase structure. The anatase phase was confirmed by Raman spectrometry using an argon laser (Horiba, model iHR320, Imaging Spectrometer, at wavelength 514.5nm). The samples ($1.27 \times 1.27 \text{ cm}^2$) used for all experiments were sterilized by autoclaving before usage. A flat Ti sheet cut into identical sized pieces was used as a control after being chemically cleaned by acetone and iso-propanol for 10 minutes in an ultrasonic cleaner, dried, and autoclaved.

Atomic Force Microscopy

The atomic force microscope (AFM) was used to characterize the roughness of the samples. The AFM apparatus was a Veeco scanning probe multi-mode microscope with a nanoscope IV controller. The average roughness (R_a) was measured for all experimental samples (Ti and 30-100nm TiO_2 nanotube surfaces) in tapping mode using Micromasch tapping cantilever tips (NSC15/NoAl) over a $1.0 \mu\text{m}^2$ scan area.

Contact Angle Measurement

The measurement of contact angle for the 30-100nm TiO_2 nanotube surfaces was carried out by a video contact angle measurement system Model No. VSA 2500 XE (by AST Products, Inc.). A small amount of D.I. water droplet (~

3mg) was placed on the nanotube surfaces to measure static contact angle. The measurement of contact angle is a simple method for analyzing the surface energy and the hydrophilic nature of the surface.

Osteoblast Cell Culture

For these studies, MC3T3-E1 mouse osteoblast (CRL-2593, sub-clone 4, ATCC, USA) were used. Each 1 mL of cells was mixed with 10 mL of alpha minimum essential medium (α -MEM; Invitrogen, USA) in the presence of 10% v/v fetal bovine serum (FBS; Invitrogen, USA) and 1% v/v penicillin-streptomycin (PS; Invitrogen, USA). The cell suspension was plated in a cell culture dish and incubated under 37°C, 5% v/v CO₂ environment. When the concentration of the MC3T3-E1 osteoblastic cells reached $\sim 3 \times 10^5$ cells/mL, they were seeded onto the experimental substrate of interest (TiO₂ or Ti) which were placed on a 12-well polystyrene plate, and stored in a CO₂ incubator until the assays were performed at selective incubation periods to observe cell morphology and count viable attached cells as a function of incubation time. The concentration of the cells seeded onto the specimen substrate was 1×10^4 cells/well.

Cell Counting and Viability Test

In order to count adhered osteoblasts on experimental specimens, a coulter particle counter (Model Z-1, Beckman Coulter Inc., USA) was utilized. Fluorescein diacetate (FDA; Sigma, USA) staining was conducted to measure cell elongation. FDA stains the cytoplasm of viable cells for microscopic examination. At 2 and 24 hours after plating, the cells on the substrates were washed with phosphate buffered saline (1X PBS) solution (Invitrogen, USA) and incubated for

approximately 30 seconds with FDA stock (5 mg dissolved in 1 mL acetone) dissolved in PBS (10 μ L/10mL), and washed once more. The samples were then inverted onto cover-slips, mounted, visualized and photographed using a green filter by a fluorescence microscope. (DM IRB, Leica Co., USA).

SEM for Substrate and Cell Morphological Examination

After 2 and 24 hours of culture, the cells on the substrates were washed with PBS and fixed with 2.5 w/v% glutaraldehyde (Sigma, USA) in PBS for 1 hour. After fixation, they were washed three times with PBS for 15 minutes each wash. Then the cells were dehydrated in a graded series of ethanol (50, 70, 90, and 100% v/v) for 30 minutes each and left in 100% ethanol until they were dried by critical point dryer (EMS 850, Electron Microscopy Science Co., USA). Next, the dried samples were sputter-coated with metal for SEM (scanning electron microscopy) examination. The morphology of the TiO₂ nanotubes as well as that of the adhered cells were observed using SEM (XL30, FEI Co., USA).

Immunofluorescence of Cytoskeletal Actin

After 24 hours of culture, the cells were fixed in 4% paraformaldehyde in 1X PBS for 15 minutes at room temperature. Once fixed, the cells were washed twice with 1X wash buffer (1 X PBS containing 0.05% Tween-20). To permeabilize the cells 0.1% Triton X-100 in 1X PBS solution was added for 10 minutes. The cells were washed twice with wash buffer. Then the samples were incubated for 1 hour at room temperature in blocking solution 1% BSA/1X PBS. After incubation, cells were washed three times for 5 minutes each wash with 1X wash buffer. TRITC-conjugated phalloidin (1:1,000 Chemicom International) in 1X PBS was

added and incubated for 1 hour at room temperature. The cells were washed three times with 1X wash buffer for 5 minutes each wash. In order to stain the nucleus, DAPI (1:1000, Chemicon) in 1X PBS was added and incubated for 5 minutes at room temperature. Again the cells were washed three times with 1X wash buffer for 5 minutes each wash. The samples were then inverted onto glass slides with a dab of Fluormount-G (Southern Biotech), visualized and photographed using a red (actin) and blue (DAPI) filter by a fluorescence LEICA DM IRB microscope.

Data Analysis for Cell and Nuclear Elongation/Area

To quantify the differences in cell morphology observed in the SEM results for 2 and 24 hours, the major and minor axes were measured by use of the scale bar and the major/minor ratio was used as the elongation ratio. For nuclear morphology, boundaries of the DAPI stained nuclei were outlined by using Image J software, a public domain image processing and analysis program developed by the NIH. The nucleus major and minor axes and spreading area were measured by Image J. The major and minor axes results were displayed for the purpose of this study as a ratio for elongation.

MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) Assay

To estimate the density of viable cells, an MTT assay was employed. After the selected incubation periods, the samples were washed by PBS and transferred to a new 12-well polystyrene culture plate with 1 mL of media. 100 μ L of MTT dye agent (Sigma, USA) was added to each well. After 2 hours of incubation in 5% CO₂ incubator, 1 mL of iso-propanol solution from the

manufacturer was added to each well and the polystyrene plate was shaken for to dissolve all the MTT crystals. The absorbance of each solution was measured at the wavelength of 570 nm with the subtraction of the 650 nm background by UV-VIS spectrophotometer (Biomate™ 3, Thermo Electron Co., USA).

Alkaline Phosphatase (ALP) Activity Test

After the selected incubation periods, the samples were washed by PBS and transferred to a new 12-well polystyrene culture plate. 500 μ L of 0.1 w/v % triton X-100 (Sigma, USA) was added to each well to study the lysis of cells. After incubating in 5% CO₂ incubator for 2 hours, the solutions were transferred (2 mL) to a micro centrifuge tube and frozen at -80°C for 2 hours. After 3 times freezing-thawing cycles to homogenize the solutions, aliquots of the solutions were used for measuring total protein content (Bradford protein assay kit, Bio-Rad Laboratories, USA) and 350 μ L of the solutions were used for ALP activity test. 350 μ L of ALP substrate solution (ELPN-500, Bio-Assay Systems, USA) was added to each solution and the solutions were mixed at room temperature for 30 minutes. After 30 minutes, 350 μ L of 1M sodium hydroxide (NaOH; Fisher Scientific Co., USA) was added to stop the reaction. The absorbance of each solution was measured at the wavelength of 405 nm by UV-VIS spectrophotometer.

Statistical Analysis and Error Bars on Graphs

Sigma Plot software (2001) which specializes in scientific data analysis and presentation was utilized for demonstrating statistical significance for the assays. The graphs show the average $\pm$ standard error bars associated with the sample size or N values shown in a box in the upper portion of each graph. When

relevant, Student t-tests or one-way ANOVAs were performed using the pairwise multiple comparison procedure. P values used to show significance are described in the figure legends, respectively.

2.2.3 Results and Discussion

Fig. 2.2-1 (a) shows the SEM micrographs of 30, 50, 70, and 100 nm TiO₂ nanotubes. The self-assembled layers were generated by anodizing Ti sheets. The images show highly ordered, vertically aligned nanotubes with four different pore sizes between 30-100nm, created by controlling potentials ranging from 5 to 20 V. The nanotubes differ in diameter and height proportionally with a diameter to height ratio of 1:3. The nanotube surface on the anodized Ti substrate has a well defined and structurally sturdy morphology. In this study as well as subsequent sections utilizing vertically aligned TiO₂ nanotubes, the maximum diameter size was limited to 100 nm in order to unify the experimental conditions and composition of electrolyte solution. Flat substrates of Ti with a native TiO₂ oxide layer having analogous chemical composition of the TiO₂ nanotube surface were used as control surfaces for the purposes of this study.

Previously, it was demonstrated that osteoblasts proliferate at a faster rate and have up-regulated levels of alkaline phosphatase activity on heat-treated anatase phase TiO₂ nanotubes over as anodized, amorphous TiO₂ nanotubes and controls of flat Ti with a native (amorphous) oxide layer (54). The anatase phase plays an important role in proliferation and cell morphology (54, 60). Since this phase phenomenon has already been shown, heat-treated anatase nanotubes were chosen in all *in vitro* studies and it was decided to vary

only the dimensions of the nanotubes, not crystal structure. Flat substrates of Ti with a native TiO_2 oxide layer, although an amorphous oxide layer, is used only as a base substrate for comparison. The primary focus of this work is to observe the cellular behavior based on the changes in nanotube dimension and to elicit the effects of nanotopography on osteoblasts.

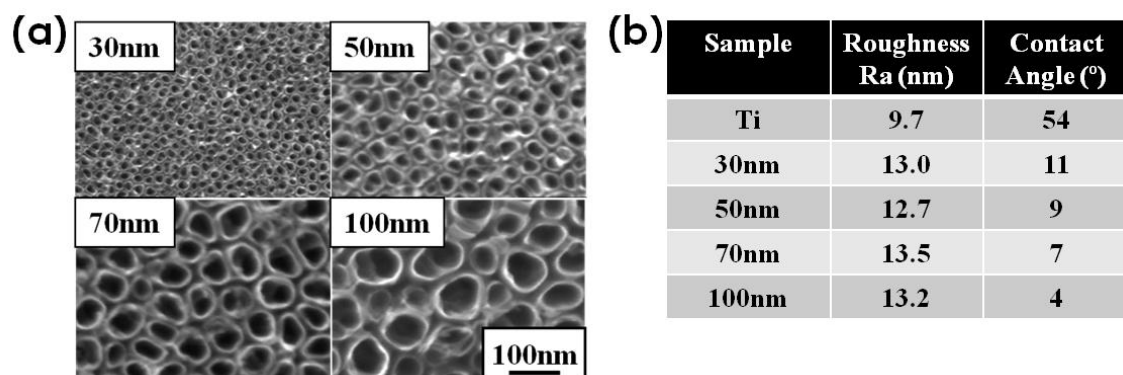


Figure 2.2-1. Physical characterization of different size nanotube surfaces. **(a)** SEM micrographs of self-aligned TiO_2 nanotubes with different diameters. The images show highly ordered nanotubes with four different pore sizes between 30-100nm. **(b)** Table with average roughness (Ra) and surface contact angle measurements for Ti and 30-100nm TiO_2 nanotube surfaces.

In terms of current biologically active implants, enhanced surface roughness is one of the important factors in providing the proper cues for a positive osteoblast response to implanted materials. However, much of the research related to the effect of macro and micro-roughness on cellular responses and bone formation are inconclusive due to the non-uniformity of macro and micro-roughness stemming from crude fabrication methods like polishing, sand blasting, chemical etching and so on. An important aspect of our nanotube system shown in the SEM images is that the nano-topography can

feature a more defined, reproducible and reliable roughness than micro and macro-topography for enhanced bone cell function *in vivo*. Although, the heights of the nanotube walls increase proportionally to the increasing diameter, there is no evidence of changes in surface roughness between the different sized nanotubes based on atomic force microscopy (AFM) data (**Fig.2.2-1 (b)**). As expected, the nanotube surfaces have a slightly higher roughness over flat Ti, but between the nanotubes, there appears to be no difference. However, it can be assumed that the surface area on the nano-scale may be affected based on the various sizes and the surface area probably increases proportionally with increasing nanotube size. It is expected that the surface area to be 3 times higher on the 100nm diameter nanotubes compared to the 30nm diameter nanotubes, respectively.

Although it has not been directly measured, it is also interesting to note that there are inter connecting spaces in-between the nanotube walls (~10nm observed by TEM for 100nm diameter nanotubes (54) and shown in **Fig. 2.1-1**) which, even after cell adhesion or confluency, can possibly allow for continued fluid flow of culture media and increased exchange spaces for gas, nutrients, and cell signaling molecules for an overall enhanced cell environment. It should be mentioned that fluid flow occurs in the human body naturally in the interstitial spaces around bone cells due to circulation and repetitive loading and unloading of bones during activity (61). TiO₂ nanotubes may provide increased channeling for proper fluid exchange for improving bone remodeling signaling molecules and functioning characteristics. It appears in **Fig.2.2-1 (a)** that the

spaces and gaps in between the nanotubes increase with increasing diameter, therefore 100nm diameter nanotubes may be most advantageous for allowing fluid and nutrient flow to occur.

Fig.2.2-2 indicates the SEM pictures of adhered osteoblasts on flat Ti and 30, 50, 70, and 100 nm TiO₂ nanotubes after 24 hours of incubation time. The shapes of the osteoblast cultured on flat Ti and on various sizes of TiO₂ nanotubes were noticeably different. The osteoblast on flat Ti indicated a more round shape in appearance without cellular extension and filopodia propagation (**Fig.2.2-2** and **Fig. 2.2-3**), but those cultures on TiO₂ nanotubes became increasingly more elongated with increased inner pore dimension and showed a number of filopodia extension at leading edges (regardless of the size of nanotubes) (See yellow arrows at **Figs. 2.3-2** and **2.3-3**). It can be speculated that the interplay between the cell and the nanotubes allows for enhanced dynamic propagation and an overall increase in osteoblast activation indicated by the filopodia.

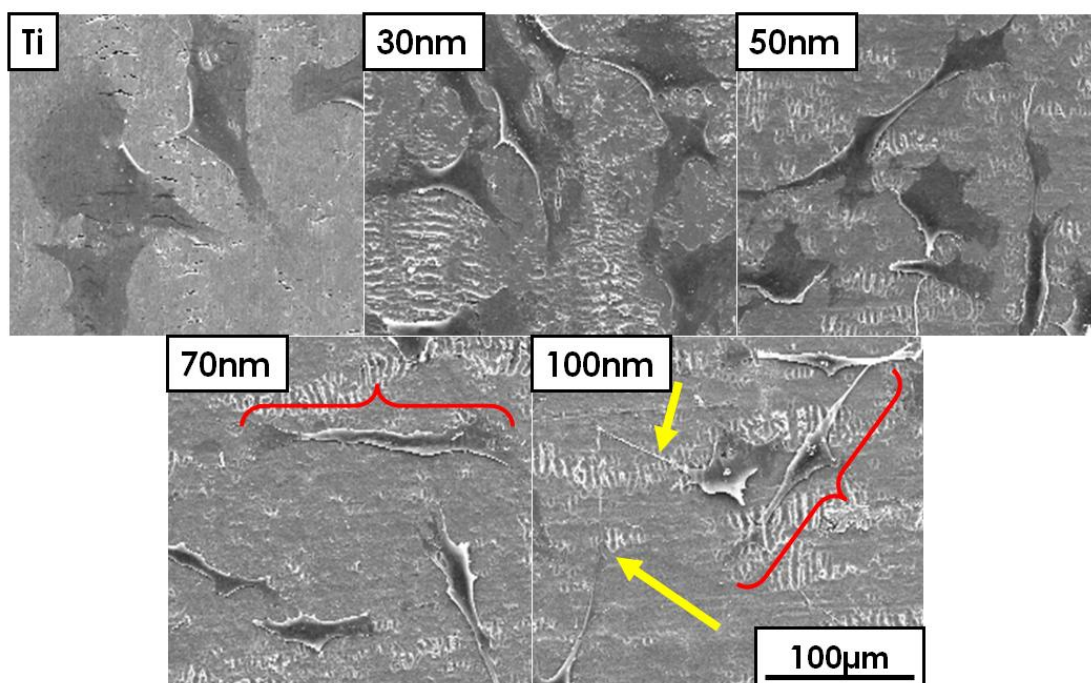


Figure 2.2-2. SEM micrographs of osteoblasts (which appear dark) on flat Ti and 30, 50, 70, 100nm diameter TiO₂ nanotube surfaces after 24 hours of culture incubation. Arrows indicate strikingly long cellular extensions probing across the substrate on the 100nm nanotubes. Red brackets show increased cellular elongation on the larger ~70-100nm diameter nanotubes. Flat and more rounded shaped cells (dark colored) are shown on Ti and 30-50nm TiO₂ nanotube surfaces.

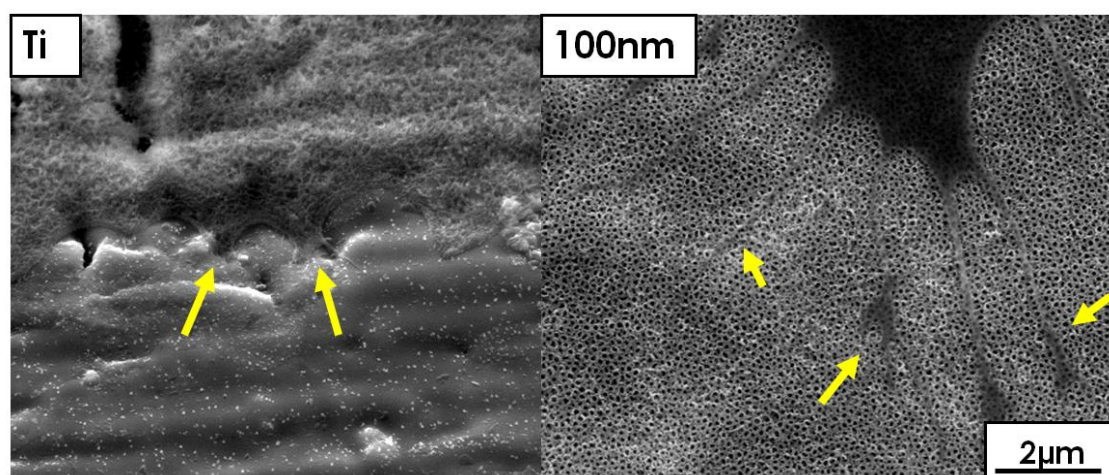


Figure 2.2-3. Higher magnification SEM micrographs of osteoblasts on flat Ti vs. 100nm TiO₂ nanotube surfaces. A much more pronounced protrusion of filopodia with significantly longer configuration is seen on the TiO₂ nanotube surface.

Immunofluorescent images of cytoskeletal actin (red) for cells cultured on the different surfaces are displayed in **Fig. 2.2-4** with corresponding DAPI nuclear staining (blue). The same elongation trend after 24 hours of incubation in the SEM images is present in the actin images, where elongation increased with increasing nanotube diameter.

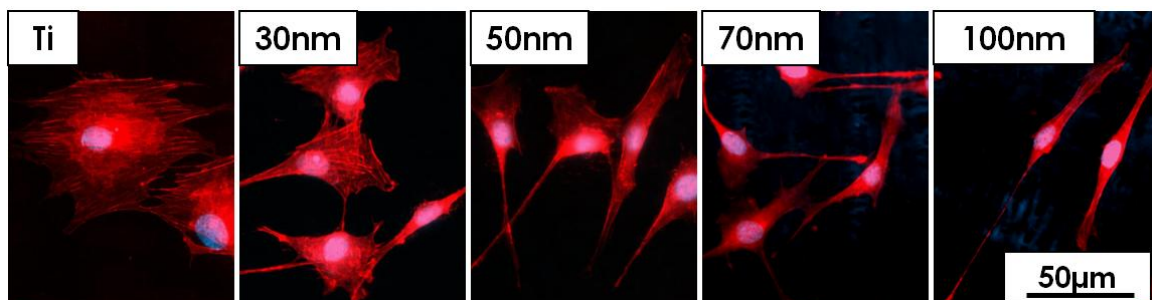


Figure 2.2-4. Immunofluorescent images of cytoskeletal actin (red) and nuclei staining DAPI (blue) for osteoblasts on Ti and 30, 50, 70, 100nm diameter TiO₂ nanotube surfaces after 24 hours of culture incubation. The images seem to suggest that there is a trend of increasing cellular elongation with increasing size of nanotube diameter.

The results of the number of adhered cells as a function of incubation time revealed that there was a significant difference between flat Ti and all sizes of TiO₂ nanotubes as seen in **Fig. 2.2-5 (a)**. However, there was no difference among all TiO₂ nanotubes except 30 nm nanotubes in the initial two days of incubation, but by day 7 all the nanotubes showed that they were statistically the same probably due to confluency on the surfaces. Therefore, all the nanotubes triggered a greater cell adhesion over Ti substrate, with 30nm TiO₂ nanotubes having the highest degree of cell adhesion in the first two days. It is reasonable that TiO₂ nanotubes promoted greater cell adhesion partly due to the nano topography and partly due to the significantly enhanced hydrophilic

surface characteristics of the TiO₂ nanotubes showing a contact angles less than 11° (**Fig. 2.2-1 (b)**), while Ti is more hydrophobic in nature with contact angle of ~54° (62). From the quantitative results of cell elongation (See **Fig. 2.2-5 (b)**), the aspect ratio of length to width increased significantly as the inner pore of TiO₂ nanotubes increased, and 100 nm TiO₂ nanotubes showed the longest cells among all experimental conditions, with the average cell elongation ratio as much as 10 – 15.

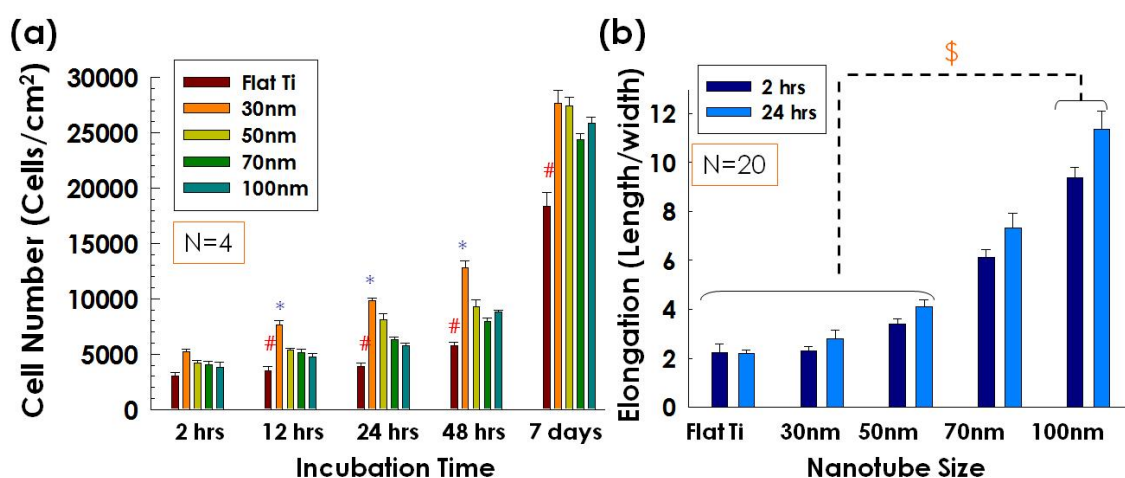


Figure 2.2-5. (a) Cell number vs. incubation time and **(b)** elongation vs. nanotube size. The bar graphs show the average $\pm$ standard error bars. P values after performing t-tests reaching statistical significance $P < 0.05$ are marked on the graphs: # denotes significance between flat Ti and all four different sized TiO₂ nanotube surfaces, * indicates significant difference between 30nm TiO₂ nanotubes and the larger size (50-100nm) nanotubes, \$ denotes significance between 100nm TiO₂ nanotubes and flat Ti, 30 and 50nm TiO₂ nanotube substrates.

Interestingly, the cell nuclei also exhibit a somewhat similar trend of increased elongation with increasing nanotube diameter, with the 100nm TiO₂ nanotubes showing the most significantly elongated nuclear shape (by ~20-25%) as demonstrated in **Fig.2.2-6 (a)**. It can be hypothesized that the nucleus

organelle elongation on the TiO₂ nanotube surfaces is in part due to the gross elongated cytoskeletal morphology of the cell. It has been reported that cell shape maintained by the cytoskeletal assembly may also facilitate nuclear shape distortion which may promote DNA synthesis by releasing mechanical restraints to DNA unfolding, changing nucleocytoplasmic transport rates, or alternating the distribution and function of DNA regulatory proteins that are associated with the nuclear protein matrix (63). A change in the nuclear structure has an effect on the 3-dimensional internal organization (64). It appears that the osteoblasts are adapting to the nanotube substrate nanotopography by organizing both external and internal shapes. **Fig.2.2-6 (b)** shows that there is also a difference in nuclear spreading area illustrating a decreasing area with increasing nanotube diameter. However, further analysis with a greater number of cells may be needed to confirm the nuclear shape/area analysis in this study and its implications on cell function.

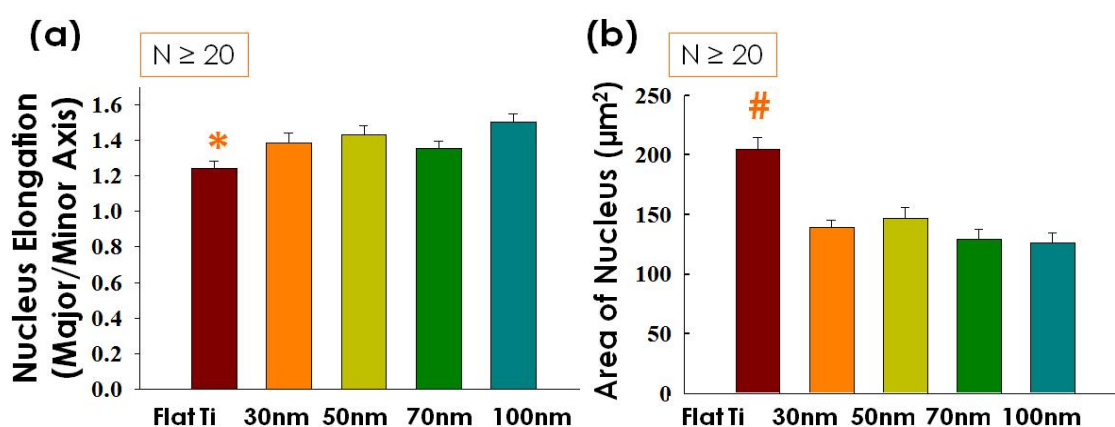


Figure 2.2-6. (a) Nuclei shape and elongation (b) Area of Nuclei. The bar graphs show the average $\pm$ standard error bars. P values after performing t-tests reaching statistical significance $P < 0.05$ are marked on the graphs: * indicates significant difference between flat Ti and 50nm and 100nm TiO₂ nanotube substrates, # denotes significance between flat Ti and all four different sized TiO₂ nanotube surfaces.

When comparing the effect of different diameter of TiO₂ nanotubes, it was found that there were distinct size regimes for controlling precisely the cell behaviors of initial adhesion and growth vs. elongation of osteoblasts. The number of adhered cells on the smallest 30 nm diameter nanotubes was notably higher than all other sizes of nanotubes, but the cells started to be more elongated on nanotube diameters above 70 nm. It has been recently observed that this phenomenon of adhesion vs. elongation is determined by initial adsorption of proteins, namely fibronectin and albumin, from the serum in the culture media (65). Osteoblasts cultured on 30 nm TiO₂ nanotubes easily adhered to the nanotube surface because of the large number and thorough distribution of protein nanoparticles already covering the whole surface of the nanotubes shown in **Fig. 2.2-7**. However, proteins on 100 nm TiO₂ nanotubes can only adhered sparsely at the top wall surface owing to the presence of large empty nanotube pore spaces (**Fig. 2.2-7**). It is probable that osteoblasts must expand their filopodia further to find a protein deposited surface, extending across larger areas and thus eventually forming an exceedingly elongated shape. Thus altering the density of extracellular matrix (ECM) attachment sites affects the shape of adhered cells. It has been reported that the focal attachments made by the cells with their substrate determine cell shape which, when transduced via the cytoskeleton to the nucleus, result in expression of specific phenotypes (66). Cells respond to the amount and area of ECM that is available for binding (67). In fact, cells do not see a naked material, *in vivo* or in *in vitro* culture. At all times, the material is conditioned by the components of the fluid in which the

material is immersed, whether it is serum, saliva, cervical fluid or cell culture media (34). As the cell begins to adhere and spread on the nanotubes, there will be a dissimilar protein density and extra cellular configuration based on the nanotube diameter.

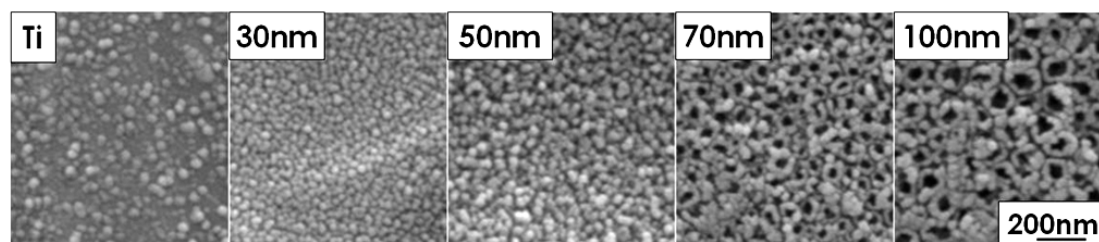


Figure 2.2-7. SEM micrographs of flat Ti and 30, 50, 70, 100nm diameter TiO₂ nanotube surfaces after 2 hours of culture showing protein adsorption from media.

To confirm the cell adhesion assay and to estimate the density of viable cells, an MTT assay was employed. From the results of the MTT assay (See **Fig. 2.2-8 (a)**), there were significant differences between flat Ti and all TiO₂ nanotubes at 24 and 48 hours of incubation time. As shown in **Fig. 2.2-8 (a)**, the trend of the MTT results comparing the flat Ti (with native TiO₂ surface) vs. TiO₂ nanotubes was the same as that revealed on the graph with the number of adhered cells as a function of incubation time, **Fig. 2.2-8 (a)**. The two results were in agreement.

To explore the osteogenic functionality of the osteoblasts and the effect of different nanosize TiO₂ nanotubes, alkaline phosphatase activity was measured. **Fig. 2.2-8 (b)** indicates that the alkaline phosphatase activity (ALP) of osteoblasts, an *in vitro* marker of bone formation, increased proportionally with increasing size of the TiO₂ nanotube diameters. All TiO₂ nanotubes elicited a significant up-regulation of alkaline phosphatase activity over flat Ti. Even though

an explanation for the effect of TiO₂ nanotube size on the ALP of osteoblasts has not been determined, it can be speculated that there may be a correlation between initial protein adsorption, cell and nuclei elongation and ALP activity of osteoblasts because the results of the elongation and ALP activity tests show similar trends, where 100 nm TiO₂ nanotubes induced much increased elongation and the highest ALP functionality.

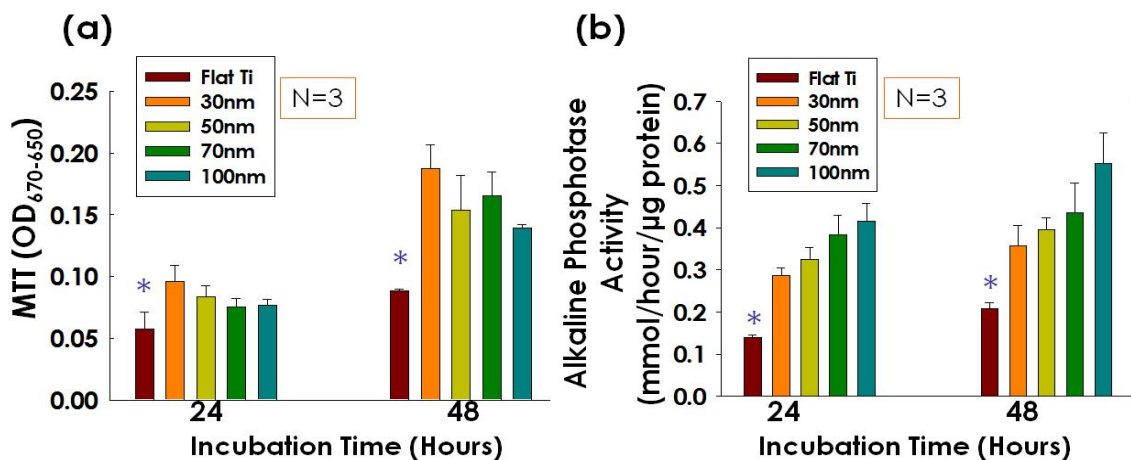


Figure 2.2-8. (a) MTT assay data showing the optical density (OD) of reaction product of the MTT working solution and **(b)** alkaline phosphatase activity of osteoblast cells cultured on flat Ti, and various sized TiO₂ nanotubes 24 and 48 h of incubation. The bar graphs show the average $\pm$ standard error bars. P values after performing t-tests reaching statistical significance $P < 0.05$ are marked on the graphs: * denotes significance between flat Ti and all four different sized TiO₂ nanotube surfaces.

Further studies about this correlation are currently being conducted. The results of ALP would indicate that 100nm TiO₂ nanotubes having the most increased biochemical activity of osteoblast cells could promote the greatest integration of material into surrounding bone. While the current-state-of-the-art self assembly process of TiO₂ anodization does not easily allow fabrication of TiO₂ nanotubes with the diameter larger than ~100nm with the electrolyte used in this

study, it would be interesting to study the effect of even larger diameter TiO₂ nanotubes, possibly using a modified chemical process, on osteoblast adhesion and bone growth.

These results are in contrast to Park et al.'s data (68-69) reporting that the optimum length scale for cell vitality and differentiation is on smaller diameter (~15 nm) TiO₂ nanotubes and declined significantly with increasing pore size showing dramatically reduced cellular activity and a high extent of cell death near ~100 nm diameter nanotubes. This difference in the cell behavior as compared to our data might be caused by the substantially different nature of the TiO₂ nanotubes (as-anodized, amorphous phase TiO₂ nanotubes used in Park et al, vs. heat-treated and crystallized, anatase phase TiO₂ nanotubes in our case). Our heat treatment also allows for evaporating toxins, such as fluorine, from the electrolyte solution remaining on the surface which may induce toxicity to the cells especially if more ions are accumulated in larger (~100nm) pore sized diameters. Further studies on the effect of TiO₂ nanotube dimensions, surface chemistry, and crystal structure on cell growth behavior of different cell types would be valuable for understanding of the nature of cell behavior on nanotube substrates.

In summary, various diameters of TiO₂ nanotube surfaces were prepared by anodization, and their osteogenic functionalities and induced cellular responses were investigated. The experimental data indicated that a substantially improved alkaline phosphatase activity of osteoblasts was obtained, together with increased elongation of the cells and nuclei, when

cultured on larger diameter TiO₂ nanotubes. In this report, a trend was revealed showing that increasing nanotube diameters led to increased elongation/stretching of cell bodies and increased levels of alkaline phosphatase, bone-forming ability. Especially, large 100nm diameter nanotubes have the greatest potential for bone implant material because of the much induced 11:1 (length:width) osteoblast elongation and highest up-regulated level of alkaline phosphatase activity over smaller (30-70nm) diameter nanotubes. Such a trend can be utilized for improvement and control of the bone forming functionality for advanced orthopedic implant technologies.

2.3 Additional Tissue Engineering using TiO₂ Nanotubes

2.3.1 Osteo-chondral Applications: Nanotube Surface Triggers Increased Chondrocyte Extracellular Matrix Production

With an ever increasing elderly population with osteo-arthritic disease and an estimated 1 million or more total joint arthroplasties performed annually in the United States, cartilage defects remain a major concern in orthopaedics (70). Current treatments for cartilage repair are less than satisfactory, and rarely restore the necessary function nor return the tissue to its native normal state (71). Nonetheless, emerging tissue engineering and regenerative medicine techniques hold a great promise in the efforts to generate functional cartilage tissue substitutes. Artificial cartilage prepared from cultured chondrocytes offers promise as a treatment for cartilage defects (72), but connecting this artificial soft tissue to bone in the attempts to restore the defected cartilage is difficult. One strategy employed in this section is to develop a dually functional substrate

that supports the growth and attachment of cartilage tissue on one extremity and encourages osseointegration, a direct structural and functional connection to living bone, on the other. This substrate should be an engineered interface between artificial cartilage and native bone (73).

Advances in scaffold design and cell culture have improved cartilage tissue engineering (74). In the attempts to replace cartilage, most research involves the combination of *in vitro* expansion of chondrocytes with synthetic or natural polymer scaffolds. Constructs have been comprised of materials such as polyglycolic acid (75), polylactic acid (76-79), various co-polymers (80-81), polyethylene glycol (PEG) hydrogels (82), as well as other extracellular matrix protein combinations (83-84). Although the response of chondrocytes to the polymer substrates provided valuable information to advance the repair of chondral lesions, there are still complications to overcome and most are lacking the dual functionality and suitable mechanical properties needed to fully integrate with native bone tissue. Some attempts of sewing and press fitting (85-86) have been made to integrate artificial cartilage and host tissue, but for large defects and long term applications these methods deem unsuccessful. It seems necessary that various other types of integrative cartilage/bone biocompatible constructs, biomaterial designs, and methods need to be evaluated to successfully incorporate cartilage with bone in the attempts to repair cartilage defects.

In recent studies, Ti has emerged as a candidate material in cartilage tissue formation as well. It has been demonstrated that a micrometer porous

substrate of Ti-6Al-4V provided conditions that favored cartilage tissue formation by influencing cell attachment, spreading and the amount and composition of cartilaginous tissue that forms (87-89). Not only porosity, but also surface geometry and topography have been found to have positive effects on the behavior of chondrocytes (88).

Nanoscale topographic effects have been illustrated in nanostructured poly-lactic-co-glycolic acid (PLGA)/nanophase titania (TiO_2) composites, which have elicited an enhanced chondrocyte response compared to surfaces with a conventional or micrometer topography (90). In this portion of the report, it is hypothesized that nanotopographical cues, from porous nanotubular structured substrates made of TiO_2 , already being an osseointegrating biomaterial (54-55), may also be a candidate for providing an alternative way to positively influence cartilage formation and the cellular behavior of cartilage chondrocytes.

2.3.1.1 Experimental

TiO₂ Nanotube Fabrication

Vertically aligned TiO_2 nanotube surfaces on Ti foils were created using an anodization process similar to the method described previously (56, 91) and described in detail in section 2.2.2 of this report.

Chondrocyte Cell Culture

Primary bovine cartilage chondrocyte (BCC) cells were obtained at passage number 0, originally isolated from bovine cartilage. The cell culture medium was composed of DMEM (Dulbecco's Modified Eagle's Medium) high glucose (4.5g/L) (Invitrogen) and supplemented with 10% FBS (fetal bovine

serum) (Omega Scientific), 10mM HEPES (Sigma), 0.1mM non essential amino acids (Sigma), 0.4mM proline (Sigma), 50mg/L Vitamin C (Sigma), and 1X Penicillin/Streptomycin (Sigma). The cells were cultured in a humidified 95% air/5% CO₂ incubator at 37°C. All experiments were conducted with BCC cultures at passage number 0 and never passed to avoid any de-differentiation or loss of phenotype. After thawing from liquid nitrogen the cells were immediately seeded onto substrates for experiments. For assays lasting ≥ 5 days, media was changed on every third day. Substrates were placed in Nunc 12-well culture dishes for all cell culture experiments with 1ml of media. For a baseline control the polystyrene well surface, optimized for tissue culture, was used as a base substrate (Nunc catalogue no. 150628) in the experimental assays.

Scanning Electron Microscopy (SEM) for Cell Morphological Examination

After 5 days of culture, the cells on the experimental substrates (Ti and 30, 50, 70, 100nm diameter TiO₂ nanotubes) were fixed and methods were followed as described earlier in Section 2.2.2.

Cell Viability

To verify the living status of the cells, a fluorescein diacetate (FDA) (Sigma) staining was conducted after 5 days of culture, methods are described in full in Section 2.2.2.

Data Analysis for Cell Shape

In order to quantify the differences in cell morphology observed in the FDA image results, the numbers of round cells vs. flattened, fibroblastic cells were counted. For each substrate (polystyrene, Ti, and 30, 50, 70, 100nm diameter TiO₂

nanotubes) at least 1300 cells from three fields were used for the analysis. The percentages from each field were averaged and the final results were graphed with standard error bars.

Glycosaminoglycan (GAG) Functional Assay

The glycosaminoglycan (GAG) assay was performed by analyzing the culture medium in contact with the Ti, TiO₂ nanotubes, and polystyrene control plates after 5 days of incubation. The substrates were individually placed in wells of a 12-well polystyrene dish (Nunc). After allowing the cells to attach for 24 hours, the Ti and TiO₂ samples were removed from the dish and placed in a fresh 12-well for isolation during the next 5 days of incubation in complete media. The media was collected and analyzed using a dimethylmethylene blue (DMMB) assay similar to Farndale et al. (92). Briefly, the color reagent was prepared by dissolving 4mg DMMB (Sigma) in 250ml water containing 0.76g glycine (EMD Chemicals Inc.), 0.593 g NaCl (Sigma) and 23.75ml HCl, to give a solution at pH 3. For the color reaction with the collected media, 100μL of each sample was pipetted into a spectrophotometer disposable Plastibrand® UV-Cuvette followed by the addition of 1ml of color reagent. The absorbance was immediately read at 525nm using a spectrophotometer (Biomate_3, Thermo Electron, Madison, WI). The assay was calibrated by use of reagent blanks and standards containing up to 2.5μg chondroitin sulfate sodium salt from bovine cartilage (Sigma). The results were expressed per cell to normalize the data and the polystyrene plate was used as a baseline condition to measure the levels of secretory GAG products from the flat Ti and nanostructured TiO₂ culture

conditions. The final results collected from five samples of each substrate were averaged and graphed with standard error bars. The average cell density at the time of assay were approximately 6.7×10^4 /cm² for polystyrene, 8.1×10^4 /cm² for flat Ti, and 7.8×10^4 , 8.2×10^4 , 6.5×10^4 , 6.3×10^4 /cm² for 30, 50, 70, 100 TiO₂ nanotube substrates. The polystyrene substrate had approximately twice the sample area of the Ti and TiO₂ nanotube substrates.

Real-time Polymerase Chain Reaction (PCR)

In order to test if there was any loss of phenotype at longer culture times of two weeks, total RNA of the cells on the polystyrene, Ti and TiO₂ nanotube substrates were extracted with Trizol (Sigma), and reverse-transcribed into cDNA by qScript™ cDNA Synthesis Kit (Quanta BioSciences). Real-time PCR was performed by Taqman® Gene Expression Assays (Applied Biosystems), and the information of Taqman® PCR primer is as follows; GAPDH (Bt03210913_g1), Aggrecan (Bt03212186_m1) and Collagen Type II (Bt03251861_m1). Real-time PCR were carried out using Taqman® Fast Universal PCR Master Mix and 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). cDNA samples (1 µL for total volume of 20 µL) were analyzed for gene of interest and for house-keeping gene GAPDH. The comparison test of cycle-threshold point was used to quantify the gene expression level of four samples of each experimental group. In this study, all levels of expression were normalized by the level of expression of the polystyrene culture dish.

2.3.1.2 Results and Discussion

The dimensions of the nanotubes were varied in order to determine if the size of the nanotube diameters would play a role in the chondrocyte behaviour. The nanotube substrates used in this work are analogous to Section 2.2 used to optimize osteoblast behavior. The SEM micrographs shown in **Fig.2.2-1** are the same nanotube surfaces used in this section of the report. Because pore size has been shown to influence chondrocyte behaviour (88), the relative pore size distribution is shown in the **Table 2.3 -1** with the estimated pore size and standard deviation for each nanotube dimension.

Table 2.3-1. The average pore size estimated from the SEM images for the different nanotube diameters and the standard deviation respectively.

Nanotube Diameter (nm)	30	50	70	100
Estimated Inner Pore Size From SEM images (nm)	27.02	46.37	69.55	99.61
Standard Deviation (nm)	4.43	2.76	7.87	7.32

Fig.2.3-1 shows comparative SEM images of bovine cartilage chondrocytes (BCCs) cultured for 5 days on flat Ti vs. different diameter (30, 50, 70, 100nm) TiO₂ nanotube surfaces. The chondrocytes on the flat Ti substrate show signs of possible flattening and spreading on the surface denoting a more fibroblastic appearance. On the nanotube surfaces however, a much more characteristic chondrocyte morphology was observed. Round, spherical shaped cells are present (yellow dotted lines) on the nanotube surfaces. Prominent extracellular matrix (ECM) fibrils were secreted by and extend from the cells on the nanotube surfaces and project over the surfaces surrounding the cells in a

compact matrix. It should be noted, that chondrocytes are highly active cells that maintain the ECM of cartilage. This type of ECM section seen in the SEM images is a positive sign for showing active cells on the nanotube surfaces. Higher magnification SEM observations of BCCs in **Fig.2.3-2** clearly reveal the striking difference in the secretion of ECM fibrils between the flat Ti vs. TiO₂ nanotube surfaces. As shown clearly in the right side image, the cells have secreted very dense, fibril-type ECM material on the nanotubular surfaces which was lacking on the flat Ti.

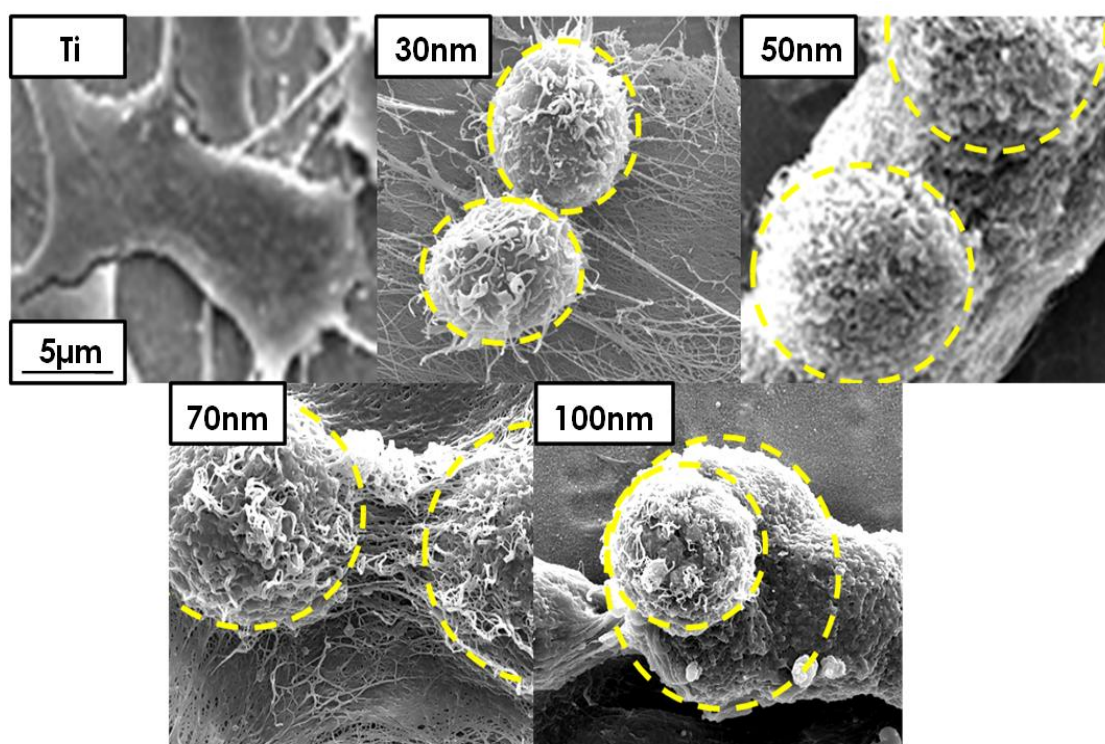


Figure 2.3-1. SEM micrographs of bovine cartilage chondrocytes (BCCs) on flat Ti and 30, 50, 70, 100nm diameter TiO₂ nanotube surfaces after 5 days of culture. The yellow dashed lines show the round characteristic shape of the chondrocytes on the nanotube surfaces, which is lacking on the Ti substrate

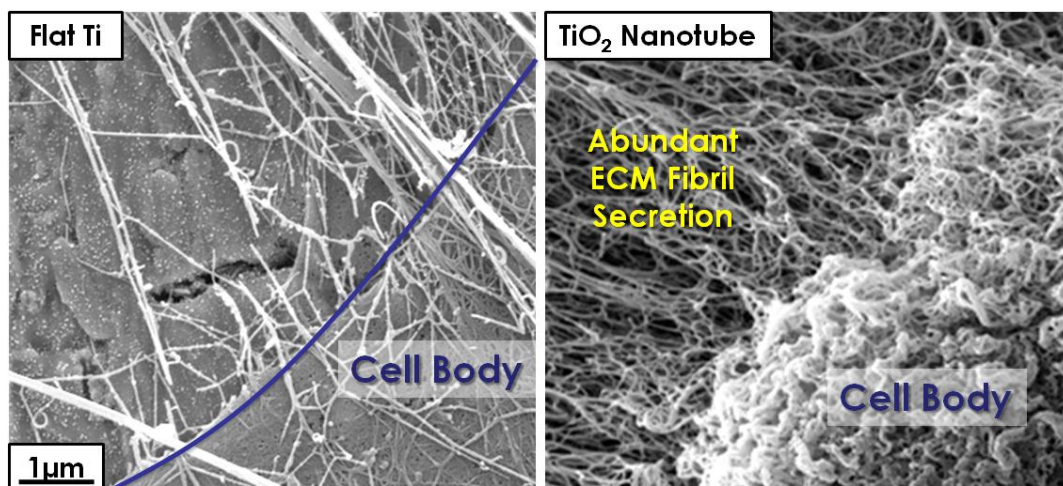


Figure 2.3-2. Higher magnification SEM observations of BCCs reveal a striking difference in the production of extracellular matrix (ECM) fibrils between the flat Ti without a nanostructure vs. TiO₂ nanotube surfaces. Fibrils are abundant and extending from all areas of the chondrocyte cell creating a dense network of ECM on the nanotube substrates.

In the SEM micrographs (**Figs. 2.3-1 and 2.3-2**), the nanotubes substrates appear to have the advantage of inducing a positive response from the chondrocytes because the cells enhance the ECM fibril secretion and organization compared to the flat Ti surface. The flat Ti most likely lacks surface structuring cues for signaling ECM fibril production and organization. One possibility is that ECM protein formation into dense fibrils on the surface may be “nano-inspired” to form on the nanotube structure because of the precise dimensions or fine scale cues of the top surface (tip of the vertical wall) of TiO₂ nanotubes having a physically confined geometry which could aid in fibril formation. It was demonstrated previously that the nanotubes produced bio-active nanostructured formations of sodium titanate nanofibers directly on the top of TiO₂ nanotube walls when the nanotubes were exposed to NaOH solution

(91). ECM proteins once secreted, in this study, may also self-assemble according to the top-wall surface geometric nanocues.

Another advantage to the nanotube system is that the nanotubular structure can possibly be utilized as nano-depots to store and entrap extra biomolecules and nutrients while the fluid spaces in-between the nanotube walls allow for the exchange of gas, nutrients, proteins and cell signaling molecules for an overall enhanced cell environment, as mention above. It appears that the spacing or volume in-between the nanotubes increase with increasing diameter (**Fig.2.2-1**). Larger diameter (50-100nm) nanotube interspacing quite possibly increases the ECM production because of the increased storage capacity and ability for flow. While the exact mechanism for stimulating cellular ECM fibril and molecule production on the nanotube surface is unknown, recent studies have also revealed with SEM images that a nanotube surface stimulates increased extra cellular matrix production in both bone and endothelial cell cultures (93-94), which agree with our SEM findings (**Figs. 2.3-1 and 2.3-2**).

In order to verify the living status of chondrocytes grown on the substrates, BCCs on control polystyrene (a Nunc 12-well tissue culture dish, used only as a base substrate for comparison because it is optimized for *in vitro* cell culture but well known that it is ill sufficient for *in vivo* implantation), flat Ti, and 30, 50, 70, 100nm TiO₂ nanotube substrates were incubated with the viability marker fluorescein diacetate (FDA), a cytoplasmic fluorescent dye. The FDA (green fluorescence) images are illustrated in **Fig.2.3-3**, practically all the cells have stained green and are alive. Similar to the SEM images at 5 days, the shape of

the BCCs in **Fig.2.3-3** on flat Ti elicit more of a fibroblast shape with increased spreading, but the BCCs on the nanotube substrates are portraying a much more characteristic round chondrocyte shape.

The morphological analysis based on the FDA observations shown in **Fig. 2.3-4 (a)** may imply that the nanotubes induce a more spherical chondrocyte cell shape. Fibroblastic shaped cells were observed on the flat controls. The percentage of round shaped cells was significantly lower for BCCs on the polystyrene, Ti, and the smallest diameter (30nm) nanotube substrates compared to the larger diameter 50, 70, and 100nm TiO₂ nanotube surfaces respectively. It was also determined that all diameter nanotube surfaces were significantly higher than flat Ti which probably indicates that the cell shape was influenced by the presence of the nanostructure itself. The highest percentage of round cells was observed on the larger 70nm pore size sample reaching approximately 80%.

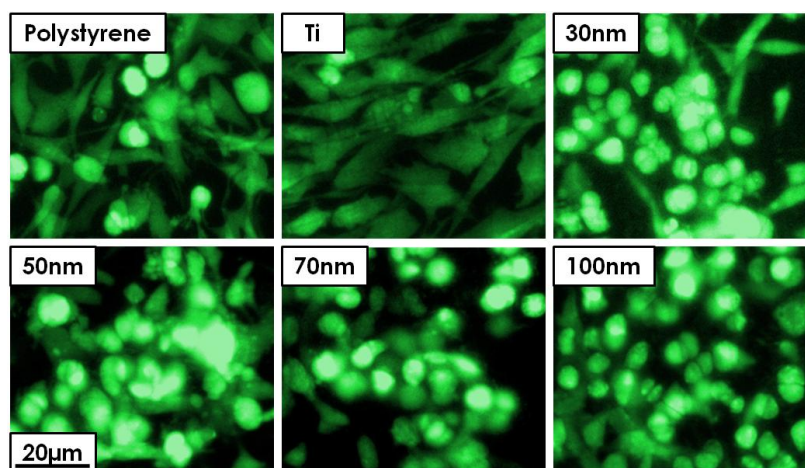


Figure 2.3-3. Viability of BCCs using FDA live cytoplasmic staining. Cells were cultured for 5 days on control polystyrene culture dishes, flat Ti and 30, 50, 70, 100nm diameter TiO₂ nanotube surfaces. More round shaped cells are observed on the nanotube surfaces, more cell spreading and fibroblastic shaped cells are seen on flat controls.

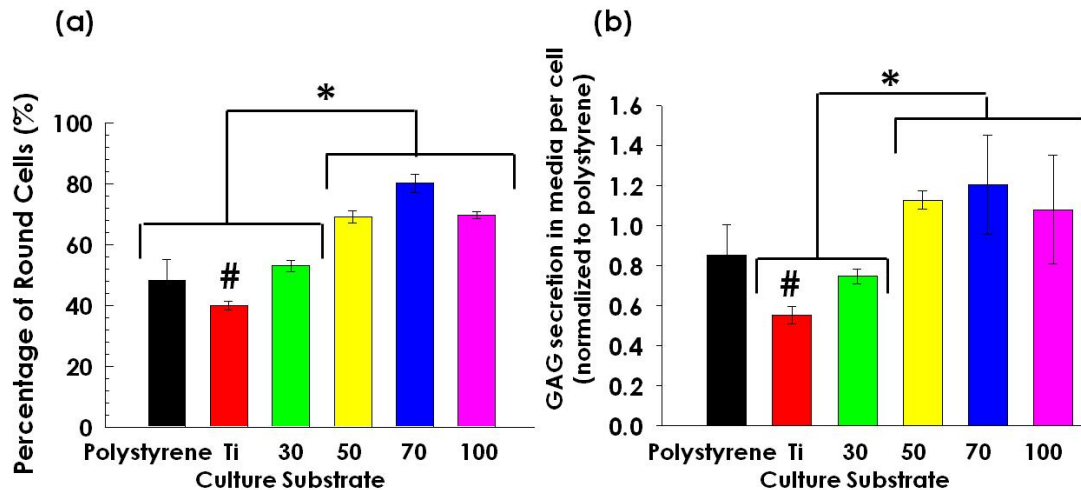


Figure 2.3-4. (a) BCC cell shape analysis. **(b)** Glycosaminoglycan (GAG) secretion in the media. The bar graphs show the average $\pm$ standard error bars. P values after performing one-way ANOVAs reaching statistical significance $P < 0.05$ are marked on the graphs: * denotes significance between the bracketed groups, # indicates significance between flat Ti and all (30-100nm) TiO_2 nanotube surfaces.

An additional aspect of the nanotube geometry is that it could potentially promote the preservation of the characteristic chondrocyte spherical morphology (**Fig. 2.3-4 (a)**), particularly on large (50-100nm) diameter nanotubes, which were shown to statistically enhance the maintenance of round shaped cells. It has been proposed before that retention of the chondrocyte spherical morphology, with less cells undergoing fibroblastic type flattening, was retained on porous surfaces over flat due to the differences in the ability of the chondrocytes to adhere to the substratum (95-96). The reasoning was that cells adhered strongly on the flat surface so spreading occurred, but less strongly on porous surfaces allowing them to remain round. Because there are fewer lateral points for cell integrin contact on large diameter nanotubes compared to smaller diameter nanotubes (68), with the points available only at

the top of the nanotube walls and confined by the large pore/hole in the middle of the nanotube, cells may have a hard time initially anchoring (say in the first few hours of incubation). In response they might secrete their own ECM proteins and fibrils for fixing themselves to the surface while remaining spherical due to the lack of surface contact. Quite the opposite was observed with chondrocytes on the flat Ti, which seemingly spread easily along the surface deficient in ECM fibril production probably because contact obstructing cues and the nanopore gaps are not present so it is easy to achieve full cell body contact and strongly adhere with the surface as also proposed by Sawtell et al. (97). However, as the nanopores get larger and cell contact points become few and far between on nanotube surfaces, chondrocytes have a harder time adhering and spreading, but it may not necessarily have an effect on the number of cells that adhere but how they adhere through means of secreting more ECM. The schematic in **Fig. 2.3-5** describes the difference in cell morphology and spreading determined by the size of the nanotube and lateral spacing of the attachment points confined by the diameter of the nanotube.

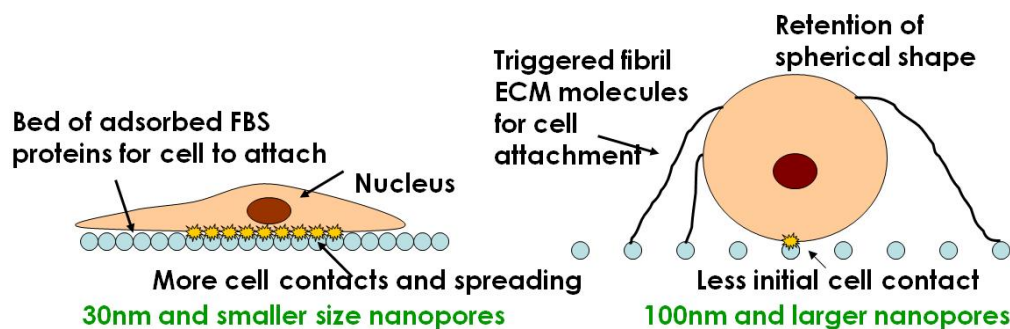


Figure 2.3-5. Chondrocyte cell adhesion and spreading schematic determined by the size of the nanotube diameter and focal attachment sites.

It was formerly assumed that focal contacts should be a specific length in order to promote adhesion and that the maximum overall contact of the cell with its substrate was most favourable (98). Yet, more recent studies suggest that cell-flattening or spreading is not always compatible with differing cell types, particularly in the case of chondrogenesis (99-102). Thus, the type of focal adhesion and its geometry can influence the shape the cell assumes, ultimately influencing the phenotypic expression. It has been reported that the dedifferentiation of chondrocytes in culture is usually associated with changes in cell morphology, from a rounded to a spread one (103). The results reported here suggest that creating pores by fabricating nanotubes on Ti surfaces provides a more favorable environment for the retention of the rounded morphology and the prevention of chondrocyte spreading, reducing the risk of a loss of phenotype.

Because chondrocytes are very dynamic cells that produce and maintain the cartilaginous matrix, which consists mainly of collagen and proteoglycans, it is important to test the biochemical ECM production on the different experimental surfaces. Therefore, to further evaluate the response of BCCs for this comparative report, the glycosaminoglycan (GAG) secretion in the media and PCR relative transcription levels of aggrecan and collagen type II elicited by the different surface conditions was also studied.

In **Fig. 2.3-4 (b)** the GAG amount secreted in the media per cell in contact with the different substrates was evaluated relative to control polystyrene culture dishes. Clearly, the 50-100nm diameter nanotubular surfaces

had noticeably increased GAG secretion compared to flat Ti and the smallest 30nm TiO₂ nanotube surfaces. The highest value, ~100% increased GAG secretion in the media compared to flat Ti, was observed in the 70nm diameter media. Nonetheless, all diameter nanotube surfaces elicited values for GAG secretion that were significantly higher than flat Ti, another observation of the effect of the nanostructure itself.

There also seems to be a correlation in the increased GAG secretion in the media and the reduction of fibroblastic shaped cells. Specifically, TiO₂ nanotubes with diameters in the range of 50-100nm had significantly higher levels of both round shaped cells and GAG secretion over flat Ti and small 30nm TiO₂ nanotube surfaces. Both data sets peaked at ~70nm diameter nanotubes with the highest retention of spherical shaped cells and GAG secretion in the media, possibly reaching a threshold point in nanotube diameter. When comparing porous vs. flat substratum, it was previously demonstrated that GAG release to the media was greater on porous surfaces (95), in fact all the porous nanotube surfaces (30-100nm) were significantly higher than flat Ti, without pores, in our studies as well. It was explained that there was a loss of chondrocytic phenotype by cells on the flat surface, as suggested by the greater proportion of cells demonstrating a fibroblastic morphology, which is in agreement with our findings. Although this correlation cannot be fully determined by the scope of this report, it would be interesting to further elucidate this observation.

In order to ensure no loss of phenotype on the experimental surfaces, PCR analysis was conducted for two chondrogenic markers, namely aggrecan and

collagen type II. The results for quantitative PCR analysis of aggrecan and collagen type II are illustrated in **Fig. 2.3-6**. For both chondrogenic markers, the nanotubes do not appear to have any loss of phenotype and have similar levels of expression as the tissue culture dish. When compared to flat Ti, all diameter nanotube surfaces have remarkably higher relative levels of aggrecan expression. And, the nanotube surfaces with pore size ~70nm had significantly higher values of collagen type II over flat Ti. It seems that the graphs show 70nm TiO₂ nanotubes had the most favorable effects on up-regulating the levels of both aggrecan and collagen type II over the other experimental samples.

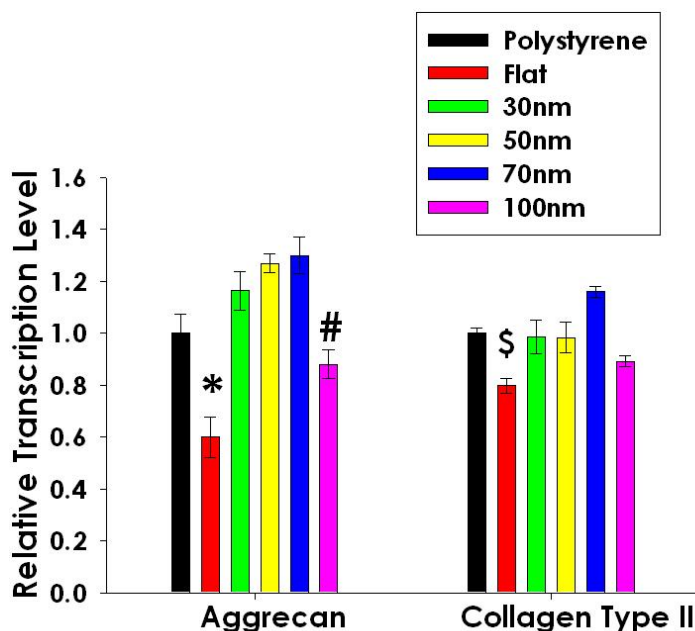


Figure 2.3-6. Quantitative PCR analysis for Aggrecan and Collagen Type II. The graph shows the average $\pm$ standard error bars. P values after performing one-way ANOVAs reaching statistical significance $P < 0.05$ are marked on the graphs: * denotes significance between flat Ti and all five other experimental surfaces, # denotes significance between 100nm TiO₂ nanotubes and the other 30-70nm TiO₂ nanotubes, \$ indicates significance between 70nm TiO₂ nanotubes and the flat Ti surface.

PCR analysis was conducted to ensure that the surfaces did not contribute to the undesired loss of phenotype. The secretion and structure of the ECM in chondrocyte culture is composed of two basic macromolecules that are essential for the structural and functional integrity of cartilage, namely aggrecan and collagen type II (104). Not only is the chondrocyte morphology, ECM fibril production, and GAG secretion more favorable on the nanotube substrates, there is also an up-regulation in the chondrocyte relative transcription levels for both aggrecan and collagen type II, showing significantly higher levels over flat Ti (**Fig. 2.3-6**). With similarity to the other data sets (**Fig. 2.3-4 (a) and (b)**), the peak transcription level is shown on the 70nm diameter nanotube surface for both aggrecan and collagen type II, again possibly revealing a threshold point in the nanotube dimensions. Unexpectedly, the transcription levels drop off with larger 100nm diameter nanotube surfaces.

Thus, it was demonstrated that even within a small range of dimensions (30-100nm), cell behavior can be influenced differently on nanoporous substratum by changing the size of the pores. It has been suggested that the differences in cell responses can be explained by the presence of different curvature in the pores providing optimum compression and tension of cell mechanoreceptors (105-106). Along the same lines, Dalby et al showed cells cultured on nanofeatures of slightly different symmetry and with varying degrees of disorder induces changes in adhesion formation which impacts on cytoskeletal tension, affects indirect mechanotransductive pathways, and impose morphological changes as well as gene expression in cells (107). It was

also reported that the basement cell membrane specifically in contact with a nano-structured surface will suffer tensile and relaxation mechanical forces that will rearrange its components and/or open ion channels that will trigger cell behavior (108). This may help explain the slight difference in cellular responses by the different nanotube dimensions as it is reported here. More on this aspect of mechanotransduction will be discussed in Chapter 3. In summarizing the chondrocyte cell results, a correlation may be drawn from all the assays that, the spherical round shape and ECM production (GAG secretion and PCR levels) of the chondrocytes was enhanced on porous TiO₂ nanotube surfaces, reaching a phenotypic chondrocyte functionality/nanotube diameter threshold at approximately ~70nm.

Nonetheless, as Ti is a well accepted orthopaedic implant material, the results obtained are very encouraging and suggest that the use of nanotube structures could up-regulate production of extracellular matrix components of chondrocytes. In addition, the TiO₂ nanotube surface exhibits significantly augmented, mechanically and chemically strong osseointegration with existing bones with a minimal chance of bone loosening evidenced by *in vitro* data (54), and our preliminary *in vivo* animal data indicating a strong new bone integration on the nanotube surface with reduced soft tissue trapping (109). Therefore, a Ti implant with all surfaces covered with the nanotubes can be potentially designed and utilized, for some specific types of articular cartilage injuries, to serve with dual function of accelerated osseointegration to the existing articular bone surface at the bone-facing contact interface while the exposed nanotube

surface can be applied for cartilage tissue regeneration by providing positive surface nanostructuring effects on chondrocyte extra cellular matrix production. A schematic representation of the prospective goal of an osteo-chondral TiO_2 nanotube dual surface is shown in **Fig 2.3-7**.

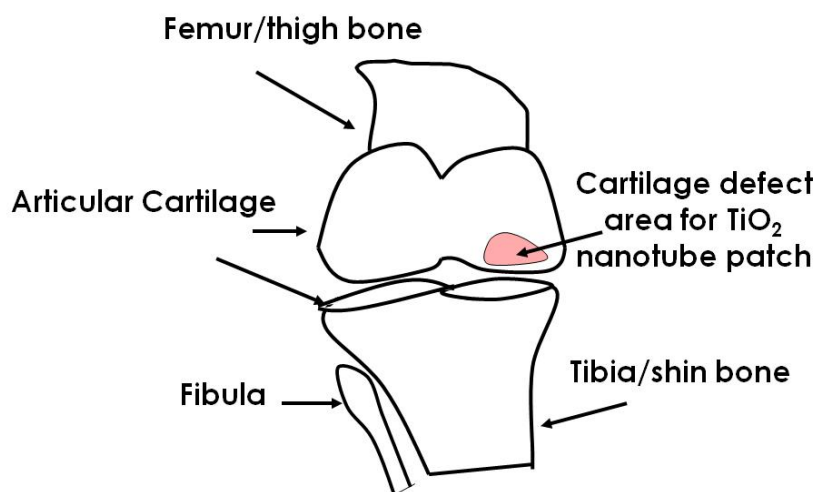


Figure 2.3-7. Schematic illustration of the osteo-chondral area of the knee where a dual side TiO_2 nanotube patch would facilitate cartilage repair and bone growth simultaneously.

2.3.2 Vascular Stent Applications: Enhanced Cellular Mobility Guided by TiO_2 Nanotubes

According to a recent report by the American Heart Association approximately one million coronary stent procedures are performed every year in the United States (110). Ideally, following the deployment of the stent the inner lining of the artery should grow over the stent struts. However, once the stent is implanted into the artery, the time course of arterial healing varies from patient to patient and all patients are at a risk for formation of a blood clot, known as thrombus, inside the vessel at the stented site. Late stent thrombosis may occur

months or even years after implantation and has become a complex clinical problem due to a lack of endothelialization, or coverage of endothelial cells over the inner stent wall, where the stent fails to be fully integrated in the vessel (111-112). The delayed endothelialization of the intra-coronary stent is believed to be the major factor for the risk of late stent thrombosis because the exposed stent surface acts as a nucleation site for thrombosis to arise (113).

Once inserted, the stent is in direct contact with the endothelium and endothelial cell recruitment, migration and coverage are critical for arterial healing and complete endothelialization (114). In order to stimulate a positive reaction upon stent deployment and injury to the vessel wall, there is a need for an increase in the tissue/material interaction to stimulate faster wound healing. The main criteria for stent surfaces are primarily that they should not facilitate platelet aggregation and they should demonstrate thromboresitivity (115). In the material selection of a vascular stent, the material should have surface properties that enable full integration of the stent as a part of the vessel and facilitate structurally sound endothelialization. The surface property of a material affects the rate of cellular migration which is a major requirement for the success of stent implantation and ultimately the rate of endothelialization. Previous studies by Sprague et al. demonstrated that grooved surfaces double the migration rate of endothelial cells over polished and smooth controls (116). The aim of this study however is to examine a different type of surface topography, nanotubular surfaces, based on the cellular response of primary bovine aortic endothelial cells (BAECs).

Even though it has been reported that endothelial cells function better on periodic or patterned nanostructures opposed to random nanostructures (8), the concept of nanostructured surfaces has not extensively been looked at for potential stent surfaces. Additional studies have shown how nano cues may affect endothelial sensing, spreading, and attaching (117), indicating that a nanostructured stent surface may be a promising approach to faster endothelialization. With this hypothesis, the use of a nanotubular surface has been explored in this section for the endothelialization with BAECs *in vitro*. The nanotubular surfaces have been evaluated based on cellular migration, adhesion, spreading, morphology, and functional properties compared to flat controls for potentially improving stent endothelialization.

2.3.2.1 Experimental

TiO₂ Nanotube Fabrication

Vertically aligned TiO₂ nanotube surfaces on Ti foils were created using an anodization process similar to the method described previously by Oh et al (56, 91) and described in detail in Section 2.2.2 of this report. The cell assays were preformed with the 100nm diameter nanotubes unless otherwise noted in the methods.

Endothelial Cell Culture

Primary BAECs (bovine aorta endothelial cells) were obtained at passage number 8, originally isolated from bovine aorta with collagenase. The cell culture medium was DMEM (Dulbecco's Modified Eagle's Medium) high glucose and high L-glutamine (Invitrogen) and supplemented with 10% FBS (fetal bovine

serum) (Omega Scientific), 200mM L-glutamine, 100 units/ml penicillin, 100ug/ml streptomycin, and 100 mM sodium pyruvate (Invitrogen). The cells were cultured in a humidified 95% air/5% CO₂ incubator at 37°C. All experiments were conducted with BAEC cultures between passages 9-12.

Cell Viability

To verify viability of cells at various time points, a fluorescein diacetate (FDA) (Sigma) staining was conducted as described in Section 2.2.2. Cells were plated at a density of 1×10^5 cells per ml of complete media.

Scanning Electron Microscopy (SEM) for Cell Morphological Examination

Initially cells were plated on the substrates at a density of 1×10^5 cells per ml complete media. The cells on the experimental substrates were fixed at the desired incubation times and methods were followed as described earlier in Section 2.2.2.

Immunofluorescence of Cytoskeletal Actin

After 24 and 48 hours of culture, the cells initially seeded at a low density of 1×10^4 per ml complete media on the Ti and TiO₂ substrates and control polystyrene 12 well (Nunc) were fixed the methods described in Section 2.2.2 were followed.

Data Analysis for Cell Morphology

To quantify the differences in cell morphology observed in the actin immunofluorescence results for the 24 hour samples, the boundaries of the cells were outlined by using Image J software, a public domain image processing and analysis program developed by the NIH. For each substrate (Ti, TiO₂, and

polystyrene) at least 100 cells from three fields were used for the analysis. The cell area, major and minor axes were measured by Image J. The major and minor axes results were displayed for the purpose of this study as a ratio to describe cell elongation and shape. The final results were graphed and unpaired Student t-Tests were performed on the data in order to calculate p values using Sigma Plot software for demonstrating statistical significance.

Time Dependent Cell Mobility Assay

Cell mobility assay was conducted in a similar manner as reported by Madri and Stenn (118). Initially BAEC cells were plated on to various substrates (polystyrene 12-well culture dish, flat Ti, and 50, 70, and 100nm TiO₂ nanotube surfaces) at a density of $5\text{-}6 \times 10^4$ cells per ml complete media. After growing to confluency, ~24 hours after plating, the samples were rinsed one time with PBS. A sterile cell scraper (Fisher Scientific Cat. No. 08-773-2) was used to scrape off half the cells from the substrates according to a marked middle position on the samples. Following that, the samples were rinsed twice with PBS and the cells that were still remaining on the substrate were put back into culture in complete media, and the cell migration distances into the open area were measured and recorded at each time point of 0, 12, and 24 hours using the viability stain, fluorescein diacetate (FDA) as previously described.

NOx and Endothelin-1 Functional Assays

To further evaluate the response of BAECs for this comparative study, the functional modification by the different surface physiological conditions was also studied. The NOx and Endothelin-1 assay was performed by analyzing the culture

medium in contact with the Ti, TiO₂, and polystyrene control plates after a 24 hour incubation. Prior to the incubation 1×10^5 cells per 1ml medium were seeded onto the substrates. The substrates were individually placed in a 12-well. After allowing the cells to attach for 24 hours, the samples were treated for 4 hours with DMEM supplemented with penicillin and streptomycin only following a procedure similar to that previously described (119). Following treatment, the Ti and TiO₂ samples were removed from the dish and placed in a fresh 12-well for isolation during the next 24 incubation in complete media. The media was collected and analyzed using assay kits following the manufactures' instruction (Cayman, Ann Arbor, MI). Both kits utilized a Thermomax microplate reader (Thermo Fisher Scientific, Waltham, MA). The results were expressed per cell to normalize the data and the polystyrene plate was used as a baseline condition to measure the increased levels of the two secretory products from the flat Ti and nanostructured TiO₂ culture conditions. The average cell counts at the time of assay were approximately 17.0×10^4 for polystyrene, 5.6×10^4 for flat Ti, and 6.1×10^4 for TiO₂ nanotube substrates with the latter two substrates having an area of approximately $\frac{1}{2}$ of that for the polystyrene substrate.

2.3.2.2 Results and Discussion

The endothelium is derived of a layer of flat cells where the individual cells are anchored together to form a continuous monolayer linked together through cell to cell junctions (120). Because the restoration of the endothelium after stent implantation is critical, the Ti and TiO₂ surfaces were compared according to cell viability and monolayer formation. Fluorescein diacetate (FDA) staining, **Fig. 2.3-**

8, revealed that the bare metal, Ti surface may not provide adequate conditions for monolayer formation. Although the cells proliferated over time and fully covered both surfaces, the BAECs on the flat Ti did not appear anchored as indicated by cellular extensions seeming to be detached from the surface. The BAECs on the Ti surface looked aggregated and bunched up, possibly more disease prone. On the other hand, BAECs on the TiO₂ nanotube surface seem to be flatter, less aggregated with fewer detached cellular extensions. Further examination with increased statistics may be needed, but the cells on the TiO₂ surface appear to be evenly spread and formed a more complete monolayer than that on the Ti surface because they were seemingly more stable in the FDA images.

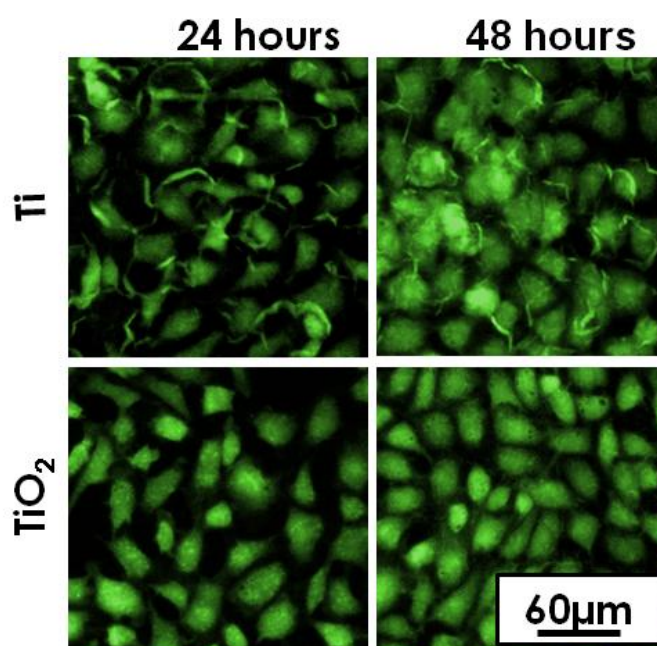


Figure 2.3-8. FDA viability of BAECs on Ti flat and TiO₂ nanotube substrates.

Fig. 2.3-9 show comparative SEM images of endothelial cells cultured (for 2 hours) on flat Ti vs. TiO₂ nanotube surfaces. The flat Ti substrate has a much lower surface area and does not render much topological cues, and hence most likely could not provide for adequate attachment conditions for cellular probing as indicated by **Fig. 2.3-9 (a)** which shows filopodia that are not particularly pronounced. After identical two hours of incubation the BAECs on the nanotubular surfaces, **Fig. 2.3-9 (b)**, show much more pronounced protrusion of filopodia with significantly longer configuration and a high degree of contact (also see the higher magnification insert in **Fig. 2.3-9(b)**). The filopodia are also probing the surface and form more intimate contacts with the nanotubes, sometimes protruding into the nanotube holes. A similar behavior was also observed for osteoblast cells (54, 121). The interplay between the cell and the nanotubes allows for enhanced cellular propagation and an overall increase in cell/substrate interaction. The nanotubes may also facilitate movement because there are more holes, edges, and ledges that act as nano cues.

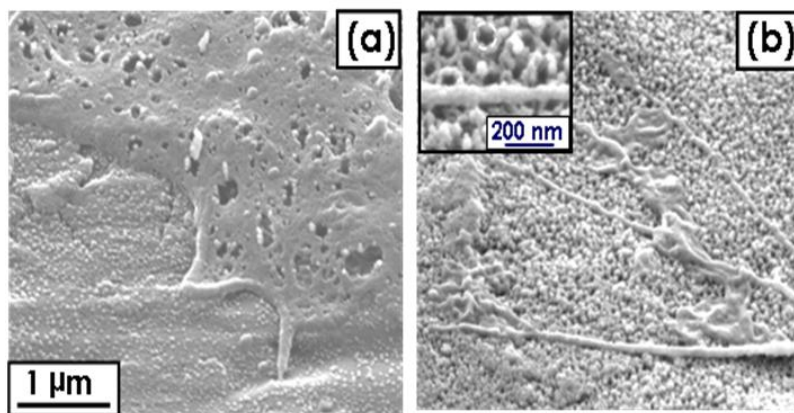


Figure 2.3-9. (a) SEM micrographs of BAECs on Ti, and **(b)** on TiO₂ surfaces after 2 hours of culture. A much more pronounced protrusion of filopodia with significantly longer configuration and a high degree of contact is seen on the TiO₂ nanotube surface.

Clearly, the difference in surface structure has an effect on the response of the BAECs on flat vs. nanotube surfaces. In relation to a cell migration scenario, such as that in wound healing after stent deployment, the nanotube surface has an advantage because the nanotube structure provides a better environment for typical migration processes which involves extension of leading cell edges, adhesion, and pulling forward. The nano features initiate cell probing and contact stimulation for cellular advancement. Because migration of cells onto the stent surfaces is essential for endothelialization and vascular remodeling, the three dimensional environment may give topographical cues that allow for faster cell communication and migration. An additional aspect noteworthy to mention is that the unique geometry of nanotube arrangement with ~ 10 nm spacing between adjacent nanotubes, still allows fluid spaces (as mentioned above) even after the endothelial cells cover the nanotube top surface thus enabling a continued supply of natural blood underneath the attached endothelial cells, as they are normally exposed to blood flow regularly in the artery. It is hypothesized that such a condition may also contribute to a healthier environment for the cells to thrive. It would be interesting to investigate the comparison of the nanotube samples with different diameters for endothelial cell culture so as to elucidate the possible effect of such gap spacing between the nanotubes; this would allow more light to shed on the nature of topography versus chemistry in cell behaviour.

SEM observations of endothelial cells cultured for longer incubation period of 6 hrs, **Fig. 2.3-10**, indicate a striking difference in the formation of extra cellular

matrix between the flat vs. nanotube surfaces. There is a deposition of high density, rough material on the nanotubular surfaces. The nanotubes are well covered with extra cellular matrix (ECM) type material which was also observed in recent studies using the TiO₂ nanotubes for bone cell culture and chondrocytes (122-123). It may be that the nanotubes are actually regulating the cells by facilitating additional ECM deposition, because the ECM distribution and quantity is much greater on the TiO₂ nanotube surface compared to the flat Ti surface. The ECM deposited upon the flat Ti surface appeared to be found in aggregated clumps and appeared sparser in comparison to the nanostructured substrates, however an assay would need to be conducted to be able to quantify this difference. Furthermore, it is speculated that the increase in ECM on the nanotube surface may play a critical role in endothelial homeostasis because it helps form a more natural basal membrane for the cells to be imbedded, a type of "vascular bed" so to speak. The enhanced ECM formation may reduce possible thrombus formation as well because the stent surface is now well coated. The ECM itself serves as a type of substrate for cell adhesion and migration. The composition, density and distribution of ECM exert critical regulatory influences on the cells (124), from both the nanostructure surface and secreted ECM entities. The abundance of ECM on the nanotubes may also trigger faster cell signaling and endothelialization. It is hypothesized that the nanotubes may thus function as a tool for manipulating ECM distribution and ultimately cellular responses.

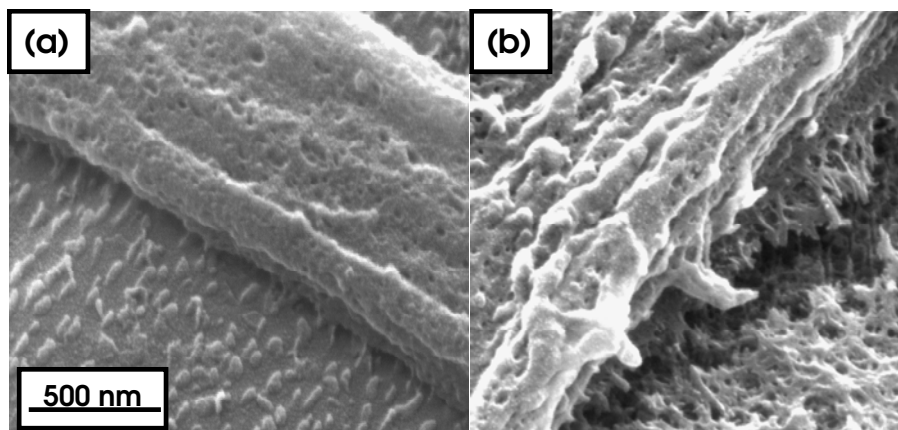


Figure 2.3-10. SEM micrographs after 6 hours of incubation. **(a)** BAECs are deficient in ECM material on the flat Ti controls compared to **(b)** where a coarse ECM material is deposited on and filled in the nanotubes on the TiO₂ surface.

In addition, endothelial cells are known to produce several secretory products which are vital for maintaining vessel physiology including many ECM products such as fibronectin, laminin, collagen (I, II, III, IV, VIII, XVIII), and proteoglycans (125). The nanotubes have the advantage over the flat surface because as the endothelial cells will eventually completely cover the surface there will be a reserve of ECM nutrients for preserving the health of the cells and vessel physiology whereas only a limited supply of ECM would be available on the Ti substrate. The naturally present pores within nanotubes can be utilized as nano-depot to store biomolecule or drugs for controlled slow release (e.g. for drug eluding stents), the rate of which should be controllable with the nanotube diameter and aspect ratio.

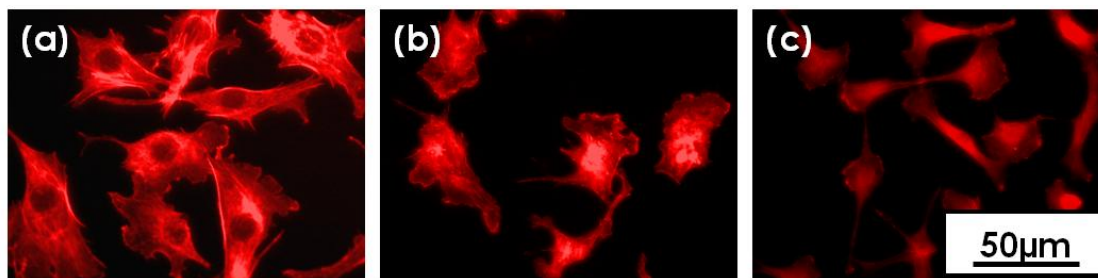


Figure 2.3-11. Immunofluorescent images of cytoskeletal actin for BAECs on polystyrene **(a)**, flat Ti **(b)**, and TiO₂ nanotube **(c)** surfaces after 24 hours of culture. The nanotube surfaces trigger more organized, prominent lamellipodia for increased cell locomotion.

The results for actin showed that the BAEC morphology and cytoskeleton organization depended on the different substrate surface topologies. The images in **Fig. 2.3-11** suggest that the cells on the nanotubes were much more motile because of the more prominent lamellipodia. This implies that the nanotubes were able to provide cues that organized the actin more efficiently for cell locomotion because all the cells have formed advancing fronts. The nanotubes may act as signals for initiating cell migration because the protrusion of prominent lamellipodia is the very first step in the process of cellular movement. The nanotubes possibly even transmit signals between cells more rapidly, speculated by the abundant cell/substrate contacts and ECM cues, as the images show cells crawling toward each other. The nanotubes up regulated cell to cell communication that is not apparent on the Ti and polystyrene controls because cellular interconnects and elongation toward each other were lacking. The actin images show that the nanotube surface has the advantage over the flat surfaces because it allows for more dynamic and coordinated

changes in cytoskeleton organization, locomotion, and cell to cell communication.

The morphological analysis based on the actin observations shown in **Fig. 2.3-12** further imply that the nanotubes are influencing cell area and elongation. The cell spreading area was significantly lower for BAECs on the TiO₂ surfaces compared to the flat controls. As well the minor/major axis ratio was significantly lower on the nanotube surfaces, implying a more elongated, uni-directional, and polarized shape for facilitating migrational traction forces. This type of cell elongated morphology has been revealed in other cell types (including osteoblast cells and mesenchymal stem cells) when cultured on the TiO₂ nanotube surfaces with 100nm diameter pore sizes (65, 121). It has already been demonstrated that the cell shape, rather than the cell spreading area, determines the efficiency of cell migration.

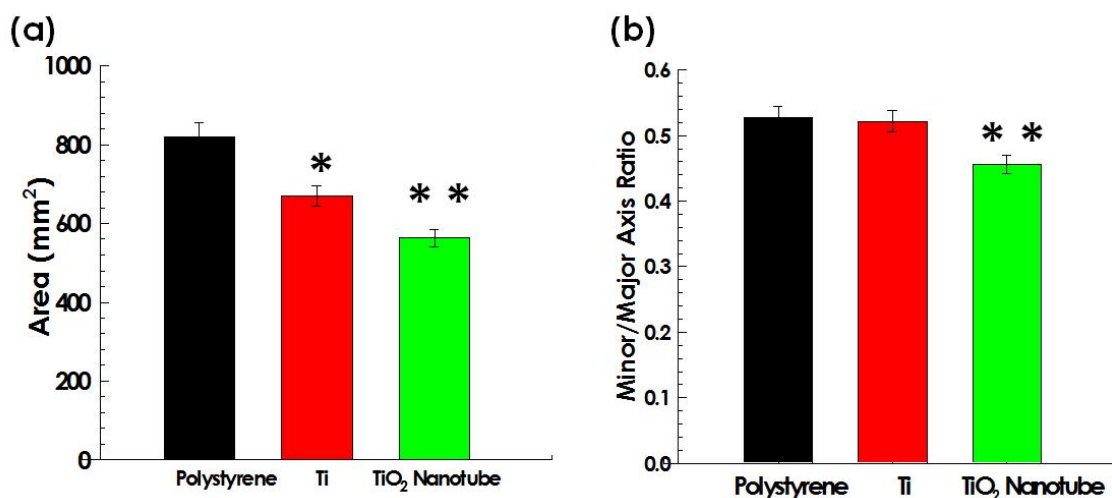


Figure 2.3-12. Analysis of BAEC morphology on polystyrene tissue culture plates, flat Ti, and TiO₂ nanotubes. The spreading area **(a)** and minor/major axis ratio **(b)** are calculated from at least 100 cells from three different fields $\pm$ SEM. * denotes $p < 0.05$ when compared with cells on polystyrene. ** denotes $p < 0.05$ when compared with cells on polystyrene and cells on Ti.

Endothelial cells on 15 μm patterned surfaces had a noticeably lower area and lower shape index (the value 1 for perfectly round and 0 for a straight line), but much higher migrational velocities than cells on larger micro patterned and non-patterned surfaces (124). This suggests that the cells on the nanotubes are probably migrating at faster speeds than on the flat surfaces, and endothelialization rates would also be enhanced by the nanotube surface. To further support this hypothesis, a time dependent study of cell migration was conducted **Fig. 2.3-13**, the result of which clearly suggests faster cell mobility on 100nm TiO_2 nanotube surfaces compared to flat Ti and smaller sized nanotubes of 50-70 nm diameters. It is interesting to note that cells on smaller diameters (30-70) appear less elongated than on the large 100 TiO_2 nanotubes, a general trend was revealed that increasing diameter increased cell elongation (65, 121). The cell elongation has been correlated with diameter size and this study also seems to suggest that increasing diameter size increases endothelial cell mobility, possibly due to the differences in cell morphology determined by the surface nanostructure as well. In vascular repair according to Gottlieb et al.(126) , the nanotubes show qualities that are much more suitable than the flat surfaces because the cells are able to extrude prominent lamellipodia, spread, elongate, and migrate as the actin results in this work suggest.

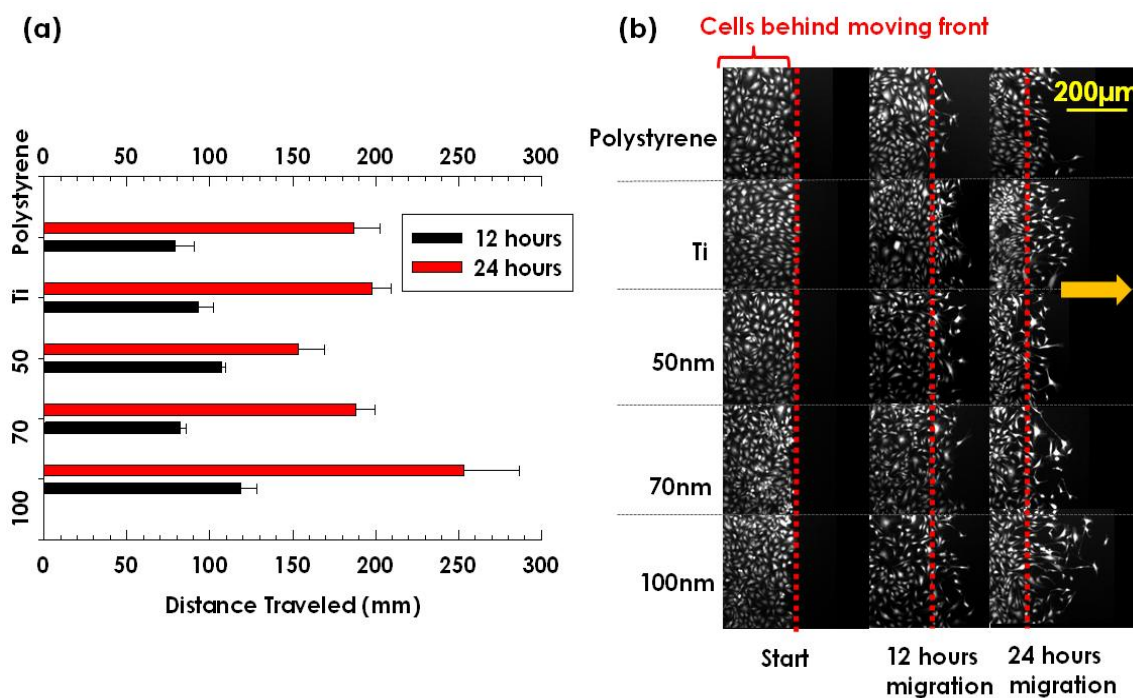


Figure 2.3-13. Time dependent migrational assay for BAECs on polystyrene and Ti flat controls compared to 50, 70, and 100nm diameter TiO_2 nanotube surfaces. **(a)** Represents the quantitative distance traveled by the cells after 12 and 24 hours. **(b)** Microscopic images of FDA stained cells during the migration period. The red dotted line indicates the start line of the cell migration. The cell mobility tests were done individually for each substrate, and the resulting fluorescent optical micrographs were put together into one figure for comparison. The cells on the 100nm diameter TiO_2 nanotube surface have traveled the greatest distance after 24 hours and are much more elongated indicating a migrational morphology with enhanced movement and a higher mobility rate compared to smaller sized nanotube surfaces and flat controls.

Healthy endothelial cells are fundamental for correct maintenance of the vasculature to ensure under normal conditions the appropriate regulation of platelet activation and to avoid blood coagulation (127). In the endothelium, nitric oxide (NOx) is continuously synthesized in order to maintain vascular homeostasis. It is an important vasodilator as it is also called the vascular endothelium relaxation factor, and it inhibits platelet aggregation (114). On the other hand, the release of endothelin-1 counteracts NOx and is a vasoconstrictor that promotes platelet aggregation (119). A stent surface must be able to

condition the cells for proper functioning so that the cells are producing normal biologically active substances, and the surface must provide for a homeostatic environment. For this reason, the levels of NO_x and endothelin-1 produced by BAECs in reaction to the TiO₂ nanotubular and flat Ti surfaces were also investigated. The physical stimuli of the nanotopography of the TiO₂ nanotubes caused a greater increase of the *in vitro* NO_x/Endothelin-1 ratio, see **Table 2.3-1** and Fig. **2.3.13**, compared to flat controls. The TiO₂ surface induced a lower platelet aggregating endothelial state, increasing the vasodilating bioactivity and bioavailability indicative of the augmented NO_x levels per cell in the media. This improves vascular tone and controls blood flow, a central role of endothelial cells *in vivo*. Although, NO_x levels alone produced by the cells increased on both the flat Ti and TiO₂ nanotube surfaces about the same, the peptide levels of endothelin-1 are significantly increased in BAECs on the flat Ti substrate, counteracting the healthy arterial environment. The aim of these experiments was to verify if NO_x and endothelin production by endothelial cells may be affected by the presence of the nanotube structure. The results obtained are encouraging and suggest that the use of the nanotube structure could up-regulate anti-thrombic conditions. These results reveal that a nanostructured surface like TiO₂ nanotubes would provide for better cellular functioning than a bare metal Ti stent.

Table 2.3-1. NOx and Endothelin-1 levels released in the culture media for polystyrene, flat Ti, and TiO₂ nanotube substrates (Average of 4 samples).

	Polystyrene	Flat Ti	TiO ₂ Nanotube
NOx (umol/cell)	2.88E-05	1.06E-04	9.12E-05
Endothelin (pg/cell)	3.51E-03	8.82E-03	6.02E-03
NOx/Endothelin-1 ratio	8.21E-03	1.20E-02	1.51E-02

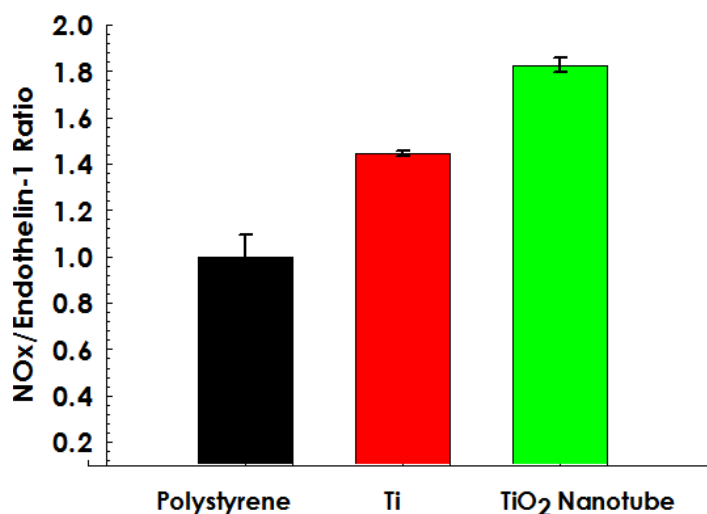


Figure 2.3-14. NOx/Endothelin-1 ratio with respect to the average of the polystyrene $\pm$ standard error.

The beneficial effect of the TiO₂ nanotube structure for enhanced endothelialization, much increased extra cellular matrix formation, and substantially raised level of nitric oxide-endothelin ratio, may possibly be utilized as a safer stent surface with a reduced probability of late thrombosis. Another possible approach of using the nanotube surface may be that a pre-endothelialization *in vitro* with patient's own endothelial cells on the stent surface covered with TiO₂ nanotubes or similar nanostructures can be introduced to ensure complete endothelial coverage prior to stent implantation. The latter

approach would be more applicable for the cases of non-emergency type stent implantation.

An additional aspect of TiO₂ nanotubes on Ti or Ti alloys can be an alternate surface modification replacing the conventional polymer coatings for drug eluting stents. An increased risk of late stent thrombosis associated with polymer carriers on the surface of drug-eluting stents remains one of the challenges in cardiovascular stent technology, which has instigated a renewed interest in the polymer-less, bare metal stent approach. There is an intriguing possibility to utilizing the nanopores and spaces between nanotubes as nanoscale depots to store and slowly release therapeutics.

2.3.3 Anti-Inflammation Applications: Macrophage Inflammatory Response to TiO₂ Nanotube Surfaces

Medical devices, which are commonly used to improve the health of patients, serve to repair joints, reopen blood vessels, and trigger electrical stimuli among numerous applications. Implantation of medical devices is complicated by the creation of trauma caused by the necessary surgery which is needed for the placement of these devices (128). The natural wound healing sequel to this surgical trauma is in turn challenged by the presence of medical devices, leading to an alternate wound healing response around the device generally termed the “foreign body response.” The foreign body response is an inflammatory response to the implanted material, and the intensity of this immediate inflammatory response to implanted devices directly impacts healing

around and tissue integration of the implant, as well as down-the-road functioning of the medical device (128).

Because the body “sees” the surface of the implant, many methods to change the surface properties of biomaterials have been undertaken in hopes of i) improving the desired native cell/tissue adhesion and overall host integration, ii) reducing the unwanted inflammatory response of macrophages and various defense cells, and iii) eliminating the subsequent foreign body response and rejection of the medical device. One method of inquiry towards improving the integration of materials with native tissue, has been to incorporate nanotopography on the surface, which presents a surface with features that are on the same nanoscale as the *in vivo* biological materials such as biomolecules or enzymes, proteins and extracellular matrices (e.g. collagen), cell surface receptors or integrins, etc. that are nanometer in dimension. When incorporated into culture surfaces, nanotopographies can vary greatly, e.g., nanoneedles, nanorods, nanopores or nanospheres, etc.

A primary concern with the use of nanomaterials is the possibility of toxicity. It has been shown that the toxicity of nanoparticles is initiated with the accumulation of free radical oxygen species which overwhelms the cell's or tissue's antioxidant response, resulting in inflammation and eventual cytotoxicity. The toxicity of nanoparticles brings into question the inflammatory response of material surfaces with nanoscale topographic features as well. In terms of immune cell reactions to nanotopography, previous studies on macrophages grown on zinc oxide nanorods exhibited low viability on tall, thin nanorod

surfaces of approximately 50nm diameter (129). On the other hand, titanium dioxide surfaces with nanofeatures slightly larger in diameter (~70nm) and shorter in height have demonstrated much better macrophage adhesion and survival, but exhibited less production of inflammatory cytokines by the macrophages cultured on the nanotopographic surfaces compared to a flat surface of titanium without a nanostructure (130). Glass nanowires have also demonstrated a trend of greater *in vitro* inflammatory response with taller than shorter nanofeatures, although the same study did not detect a significant trend for inflammatory response related to the height for polymer nanofeatures (130). In addition, the culture of macrophages on patterned nanofeatures using several different polymers exhibited no significant differences in the release of inflammatory cytokines(131). Overall, it appears that there is no clear trend regarding macrophage response and inflammation due to nanotopography (for review see ref. (132)). Therefore further studies to elucidate the effect of nanotopography on macrophage response would be extremely valuable for understanding and controlling the inflammatory cell behavior for implantable devices.

Thus far, it has been shown that the TiO₂ surface nano-configuration is advantageous in regulating many positive cell and tissue responses for various applications for tissue engineering and regenerative medicine; therefore it was chosen as the primary substrate of investigation in this research on the macrophage inflammatory cell response. Previously, when comparing the effect of different diameters or inner pore sizes of TiO₂ nanotubes, it was found that

there were distinct size regimes for controlling the behaviors of osteoblast (121), chondrocyte (123), and mesenchymal stem cells (68, 133-134). As well, a study comparing 20nm vs. 200nm pores of aluminum oxide revealed unique differences in the inflammatory response of macrophages (135). Because pore size seems to play an important role in controlled cell behavior, here we investigate the potential influence of differently sized TiO₂ nanotubes on the *in vitro* inflammatory responses of macrophages, and it was found that the geometry of the nanotube may have significant impacts on the inflammatory cell behavior.

While this report will not go in to depth about immune cell response, this section evaluates the response of macrophages to TiO₂ nanotubes with different diameters, with the intention of getting a more complete understanding of the effect of nanotube size on the inflammatory system for biomedical implant purposes. These results show that the nanotube size of the material studied affects the macrophage activation. The different activation states were reflected by morphological changes and secretion levels of TNF, a proinflammatory cytokine. The experimental TiO₂ nanotube surfaces appear similar to previously fabricated surfaces (54, 93, 121, 123, 133-134), and have similar size characteristics and discrete uniform geometries (**Fig. 2.2-1**) as shown in earlier sections. **Fig. 2.2-1** shows highly ordered, vertically aligned nanotube structures with different diameters fabricated by varying the anodization potential.

Fig.2.3-15 illustrates SEM micrographs of macrophages on the Ti and TiO₂ nanotube surfaces after 24 hours of incubation, which is an adequate time to reveal the acute inflammatory response on the different surfaces. The reaction of the cells to the surfaces can be seen from cell spreading, ruffled membranes, and extended filopodia. On the smaller 30nm diameter and flat Ti, there seemed to be more filopodia extensions, with a spiky appearance of the cell, as shown by the arrows in **Fig.2.3-15**. Larger spreading areas with less filopodia are seen on the 50-100nm TiO₂ surfaces. Both the cell spreading and filopodia extension are typical signs of macrophage activation.

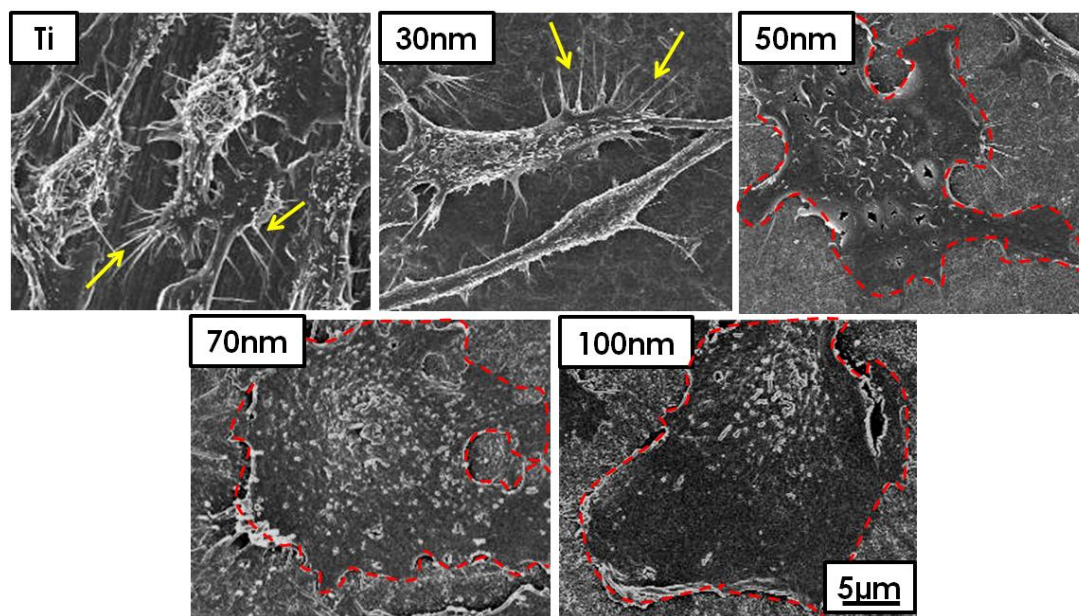


Figure 2.3-15. Macrophage morphology. The SEM images show macrophages on the experimental surfaces after 24 hours of incubation. Arrow indicate abundant filopodia extensions on the Ti and 30nm TiO₂ nanotubes. Dotted lines show cell outlines and large spreading area on the 50-100nm TiO₂ nanotubes surfaces.

Activated macrophages present a large number of morphologic, functional, and metabolic differences from normal resting cells. They are larger in size, and display pronounced ruffling of the plasma membrane, increased

capacity for adherence and spreading on surfaces, increased formation of pseudopods, as well as functional differences (136). Macrophage spreading is the preliminary step in of macrophage activation and considered to be an important marker of this event (137). The active process of spreading represents alterations including filopodia and rough plasma membranes. It appears that cells present on the experimental surfaces had activated morphologies with ruffled membranes and filopodia extensions. The Ti and 30nm TiO₂ nanotubes had extensive filopodia, whereas less filopodia but greater spreading area with some membrane ruffling was observed on the 50-70nm TiO₂ surfaces (**Fig.2.3-15**). Because cells on the Ti and 30nm TiO₂ show more abundant and well-established filopodia extensions, it can be assumed that there was a higher degree of activation on these surfaces.

Tumor necrosis factor (TNF) is a cytokine involved in inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. This cytokine was used to test the inflammatory response of the macrophages to the different surfaces. As presented in **Fig. 2.3-16**, the production of TNF per cell varied on the different substrates. Mean channel fluorescence (MCF) values exhibited several significant differences for cells grown on the various substrates. MCF for cells grown on tissue culture polystyrene was 8.85, a value significantly lower than that for cells cultured on flat titanium surfaces (9.81). Cells grown on 30, 50 and 100nm surfaces had no significant differences in MCF values compared to other surfaces (9.43, 9.45 and 9.40, respectively). However, cells cultured on 70nm surfaces had significantly lower MCF values than flat Ti (9.13).

Percent positive data show no significant differences among the culture substrates.

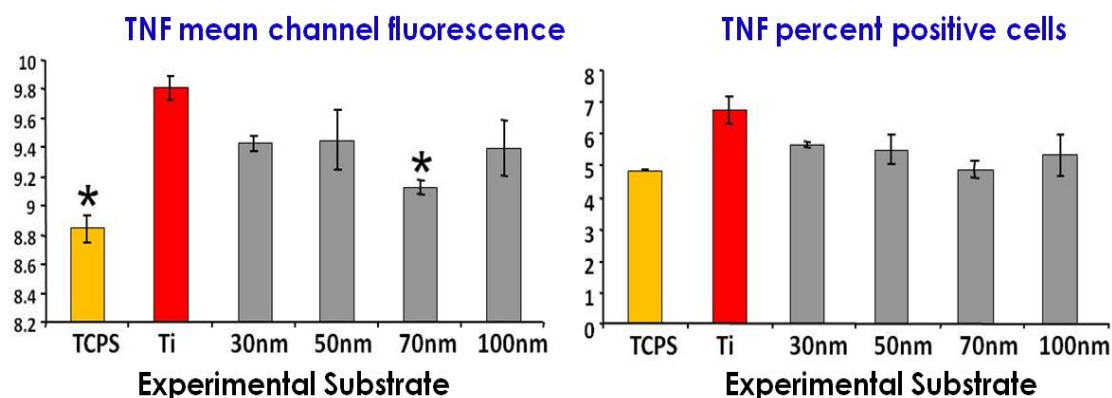


Figure 2.3-16. Inflammatory cytokine production per cell following 8 hours of culture on substrates. The production of the inflammatory cytokine TNF was dependent on the surface of the experimental substrate. * indicates statistical significance $p < 0.05$ compared to flat titanium surfaces.

A previous study on macrophage production of inflammatory cytokines showed higher production of many cytokines on flat Ti compared to nanostructured TiO_2 (130). In the current study on TNF expression on similar nanostructured surfaces with the same chemistry but varying geometries, we observed the same general trend of lower TNF production by cells on the nanotubes, suggesting that the nanotopography itself contributes to lowering the TNF production. No statistically significant correlation was observed as a function of the diameter size, but the largest decrease in TNF values was significant for macrophages cultured on 70nm nanotube surfaces (**Fig.2.3-17**). A possible reason for the lower inflammatory response in general of the TiO_2 nanotube surfaces could be due to radical quenching by the surface, a known property of TiO_2 (138-139), which was observed in similar results with higher levels

of oxygen radical quenching on TiO₂ nanotube surfaces compared to the other experimental materials (data not shown). Oxygen radical production is a common occurrence in inflammation (140), and the reduction of oxygen radicals at the surface of a biomaterial could reduce the local inflammatory response. Although the mechanism for oxygen radical reduction is not explained by the extent of this report, it was observed that the 70nm diameter nanotubes are most advantageous in terms of radical reduction for decreasing the inflammatory response to the surface, compared to Ti and 30, 50, 100nm diameter TiO₂. Interestingly, it was also found that the surfaces that had the most radical reduction or oxygen quenching, i.e. 50-100nm TiO₂ nanotubes (data not shown) showed the least amount of filopodia activation (**Fig.2.3-15**) and possibly a lower degree of inflammatory response of the macrophages. However, to shed further light on the complete understanding the effect of surface nanotopography on macrophage behaviors, additional studies are needed to determine the fate of the cells as well as the secretion of other growth factors.

In summary, this short study demonstrated that the TiO₂ nanotubes with 70nm diameter is the surface with the lowest level of macrophage morphological activation, the lowest macrophage production of inflammatory cytokines, and the greatest oxygen radical quenching capability. Hence, TiO₂ nanotubes in the ~70nm diameter regime is most promising for implant surfaces for decreased inflammatory response.

While TiO₂ nanotubes surfaces are well known for orthopedic implant technologies, it is advantageous and beneficial to consider this type of surface

nanostructure for other biomedical implant technologies including vascular stent applications, as revealed in the previous Section 2.3.2. In exploring the possibility of utilizing TiO₂ nanotubes as a possible Ti or NiTi surface modifications in arterial stents, it was found that the TiO₂ nanotube surface structuring is excellent for the growth, mobility, and endothelialization of vascular luminal surface (93).

Collectively, the reduction of inflammation and the quenching of oxygen radicals shown in the present study bolster the potential use of TiO₂ nanotubes for stents, especially because nitric oxide synthase has been shown to co-localize with atherosclerotic plaques (141).

2.4 TiO₂ Nanotubes by Hydrothermal Processing: Highly Bioactive 8nm Hydrothermal TiO₂ Nanotubes Elicit Enhanced Bone Cell Response

It is well known that Ti is the traditional and most popular material used in orthopedic implants, which was discussed in detail in Section 2.2. In terms of topographic effects on bone cell (osteoblast) behavior, it has been shown that ceramics, such as Ti oxide (TiO₂) the natural oxide on Ti metal, with different textures on the nanoscale exhibit enhanced effects on growth rates and bone forming ability (54, 121-122, 142-143). Many researchers utilize the common Ti surface nanotexturing technique of electrochemical anodization, focusing on the nanotubular structures' effects on osteoblast behavior, as was reported in Section 2.2. It has also been shown that anodized TiO₂ enhanced growth of osteo-progenitor cells (mesenchymal stem cells) on anodized TiO₂ nanotubes (68-69, 133). In this work, however, an alternative approach of hydrothermal

processing was chosen to create a nanostructure surface, eliminating the use of hydrofluoric acid and toxic electrolyte solutions which may impart impurities to the oxide surface during the anodization technique. The hydrothermal processing technique (144) is a simple and economic approach for creating a controlled nanotube architecture, with a unique one-dimensional nanostructure, and provides significantly smaller dimensions and larger surface areas, e.g., ~8 nm diameter TiO₂ nanotubes as compared to the 30 – 150 nm diameter for anodized TiO₂ nanotubes. Although many properties and functions of the hydrothermally processed nanotubes have been evaluated (145-148), this is the first study on the biological responses to hydrothermal Ti oxide (titania) nanotube surfaces.

Here the cell adhesion, morphology, bone forming ability, and bone matrix production of osteoblast cells grown on novel hydrothermal 8 nm diameter nanotubular titania surfaces is reported. With this study, a better understanding of the events at the bone cell-material interface can be attained, as well as aid in the development of new materials and approaches that promote osseointegration.

2.4.1 Mechanism of Nanotube Formation

Fabrication of nanotubular titania surfaces using a simple hydrothermal process is described in **Fig. 2.4-1**. A schematic of the hydrothermal autoclave chamber and experimental flow chart of the procedure are illustrated in **Fig. 2.4-1 (a)** and **(b)**, respectively. Under these hydrothermal conditions, the TiO₂

nanotube formation mechanism has previously been reported by Miyauchi and Tokudome (144):

- 1) An amorphous oxide or hydroxide layer is initially formed at the surface of the Ti metal by an oxidation reaction.
- 2) Then the surface region of the amorphous layer is dissolved.
- 3) The nuclei for the subsequent growth of the crystalline titanate nanotubes begin.

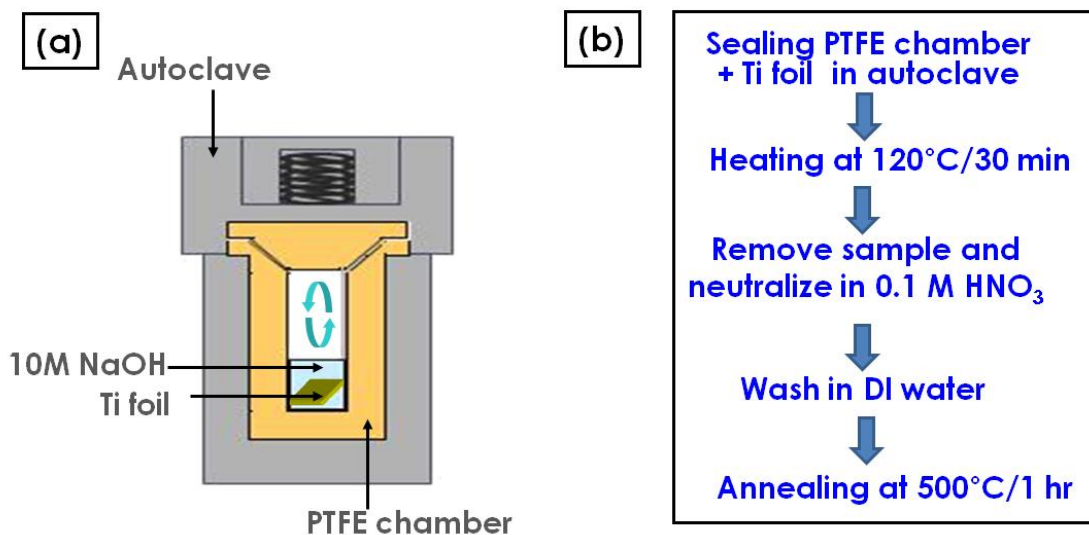


Figure 2.4-1. (a) Schematic illustration of PTFE lined autoclave for hydrothermal processing of Ti foils in 10M NaOH solution. **(b)** Experimental flow chart to form the 8nm diameter TiO₂ nanotube surface nanostructure.

2.4.2 Experimental

Preparation and Characterization of the TiO₂ Nanotube Arrays

Titania (TiO₂) nanotube arrays were synthesized by a hydrothermal treatment of titanium metallic substrates. The titanium substrates used for

nanotube production was a commercially available titanium foils (Alfa Aesar Co., Inc.) with ~ 0.25 mm thickness. To fabricate the TiO_2 nanotube arrays, these Ti foils were immersed into 10 M aqueous NaOH solution, heated at 120°C for 30 minutes in a PTFE-lined autoclave, and subsequently washed with 0.1 M HNO_3 aqueous solution and pure water. Then the samples were annealed in air at 500°C for 1 hour in order to transform the as fabricated amorphous nanotubes to anatase phase. Since the phase phenomenon of enhanced growth and function on anatase vs. amorphous TiO_2 nanotubes has already been shown, albeit on coarser 100 nm diameter anodized nanotubes (14), we have chosen to use heat-treated nanotubes for a similar anatase state in this study. The nanotubes were visualized under a SEM (scanning electron microscope) (XL30, FEI Co., USA). Transmission electron microscopy (FEI Tecnai Sphera 200KV TEM) was also used in order to image the structure of the TiO_2 nanotubes. The nanotubes were scraped off the Ti substrate, immersed into ethanol and agitated for separation and placed onto formvar support film 400 Cu grids and imaged at 200KV at various magnifications.

The atomic force microscope (AFM) was used to characterize the 3-D topography and surface roughness of the control Ti (untreated surface) vs. nanotube surface. The AFM apparatus was a Veeco scanning probe multi-mode microscope with a nanoscope IV controller. Since the AFM tip radius of curvature is typically 20-30 nm, the probe tip cannot penetrate somewhat vertically aligned and densely packed tangled 8 nm TiO_2 diameter nanotubes to provide true morphology of nanotube structures. The AFM metrology was

therefore used here mostly to get the general, macroscopic surface roughness characteristics. The root mean square roughness values (R_{rms}) and 3-D topographic images were measured in tapping mode using Micromasch tapping cantilever tips (NSC15/NoAl) over a $1.0\mu\text{m}^2$ scan area.

For adhesion strength of TiO_2 nanotubes on Ti foil, a simple adhesion set-up was utilized. Commercial thermosetting epoxy with relatively high viscosity (~ 3000 centa-poise) was attached on the surface of TiO_2 nanotube and hook-shaped metal wire-end was tightly embedded inside epoxy. After the epoxy was fully cured, uniaxial force via added weight was applied normal to the surface until fracture occurred. The radius of epoxy contacted to surface was 0.24cm. Adhesion strength was indirectly determined whether fracture occurs at epoxy/ TiO_2 NTs interface or TiO_2 /Ti foil interface.

The wettability (hydrophilicity) was evaluated by standard water droplet contact angle measurements. Small amount of D.I. water droplet ($\sim 3\text{mg}$) was placed the TiO_2 nanotube surface to measure static contact angle (CAM100, KSV instruments). Water droplet on the 8 nm diameter TiO_2 nanotubes was spread over the sample surface immediately after being placed on it, and it is evident that TiO_2 surface is of super hydrophilic nature as expected based on previously published observation(144).

The samples ($1.27 \times 1.27 \text{ cm}^2$) used for all experiments were sterilized by autoclaving before usage. A commercially available Ti sheet, with a native TiO_2 oxide of analogous composition to the TiO_2 nanotubes, cut into identical sized

pieces was used as a control after being chemically cleaned by acetone and iso-propanol for 10 minutes in an ultrasonic cleaner, dried, and autoclaved.

Osteoblast Cell Culture

For these studies, MC3T3-E1 mouse osteoblast (CRL-2593, sub-clone 4, ATCC, USA) were used. Protocol for osteoblast cell culture is described in Section 2.2.2.

MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) Assay

After the selected incubation periods, the samples were measured by MTT assay, description of methods is found in Section 2.2.2.

Cell Viability and Initial Cell Adhesion

Fluorescein diacetate (FDA; Sigma, USA) staining was conducted to as previously described in Section 2.2.2.

SEM for Substrate and Cell Morphological Examination

After 24 hours of culture, the cells on the substrates were fixed and analyzed by following the protocol given in Section. 2.2.2.

Immunofluorescence of Cytoskeletal Actin

After 24 hours of culture, the cells on the Ti and TiO₂ nanotubes were fixed and analyzed as described in Section 2.2.2.

Alkaline Phosphatase (ALP) Activity Test

To measure the bone forming ability of cells on the experimental surfaces, after the selected incubation periods, the samples (Ti vs. TiO₂ nanotubes and the control polystyrene dish) were analyzed as described in Section 2.2.2.

Bone Matrix Production

Calcium and phosphorus are the primary components of bone matrix. If cells have matured on the surfaces, they will begin to deposit bone matrix. In order to detect the presence of calcium and phosphorus on our surfaces, the cells were trypsinized by trypsin EDTA 0.25% (Invitrogen) and the samples were air dried for SEM and EDAX analysis. A Phillips XL30 FEI SEM was used to visualize the morphology of the deposited matrix. The Oxford EDX attachment and Inca Software determine elemental make up and composition of the deposited matrix.

2.4.3 Results and Discussion

This hydrothermal technique produces one-dimensional ~8nm diameter TiO₂ nanotube fibers, somewhat vertically aligned with ~150nm thickness (also revealed by (144)) on the Ti surface as shown in the SEM and TEM micrographs, **Fig. 2.4-2 (a-c)**. The TEM image also reveals a multiwalled nanotube configuration with about 2-6 parallel walls along the length of the elongated nanotubes. Uniquely, the overall morphology of the nanotube fibers showing densely packed and somewhat vertically aligned with a tendency of some tangled configuration could be advantageous for imitating the natural extra cellular matrix (ECM) environment of collagen type I fibers having fibrous and tangled nanoscale geometry. This is an important aspect to bone scaffold designs in order to present host cells with an innate ECM interface where they can repopulate and resynthesize a new matrix (149-150). Most research, like ours, has been focused on the nanometer level of precision for cell-material

interactions for enhanced bioactivity. While the hydrothermal temperature and time are very important parameters, it would be interesting to investigate potential time-temperature combinations to vary geometric length, diameter, and configuration of the nanotube films.

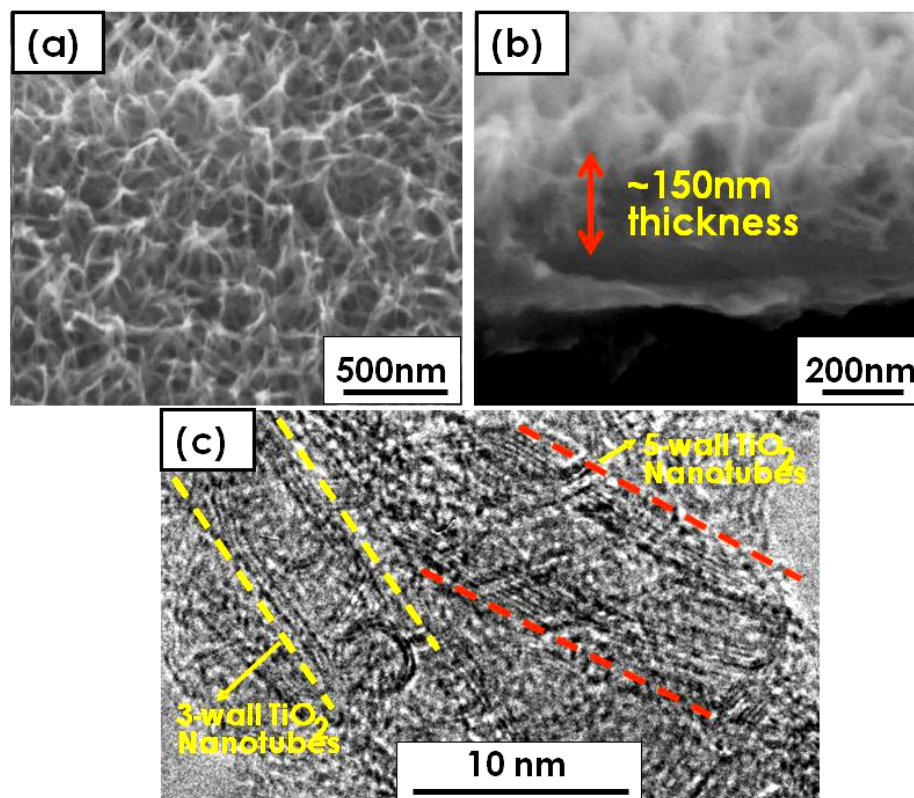


Figure 2.4-2. Scanning electron microscopy (SEM) images showing (a) top view and (b) cross-sectional view of the TiO_2 nanotube layer having $\sim 150\text{nm}$ layer thickness. (c) Transmission electron microscopy (TEM) image illustrating the multiwall nature of the $\sim 8\text{ nm}$ diameter TiO_2 nanotubes. Part of the nanotube lengths are indicated by dotted lines.

To estimate the macroscopic surface roughness and 3-D topography of the thin oxide film, AFM analysis was also conducted and shown in **Fig. 2.4-3**. In general, because the size of the AFM tip ($\sim 20\text{-}30\text{ nm}$ radius of curvature) is much larger than the feature size of the nanotubes in this research ($\sim 8\text{ nm}$ diameter),

the AFM is not capable of high resolution to depict the true roughness of the somewhat vertical and tangled nanotubes, but it still serves to describe the comparative macroscopic roughness. The overall roughness was increased slightly after the hydrothermal treatment, due to the chemical etching that took place. An increase in implant surface roughness is in general closely related to the degree of osseointegration (151).

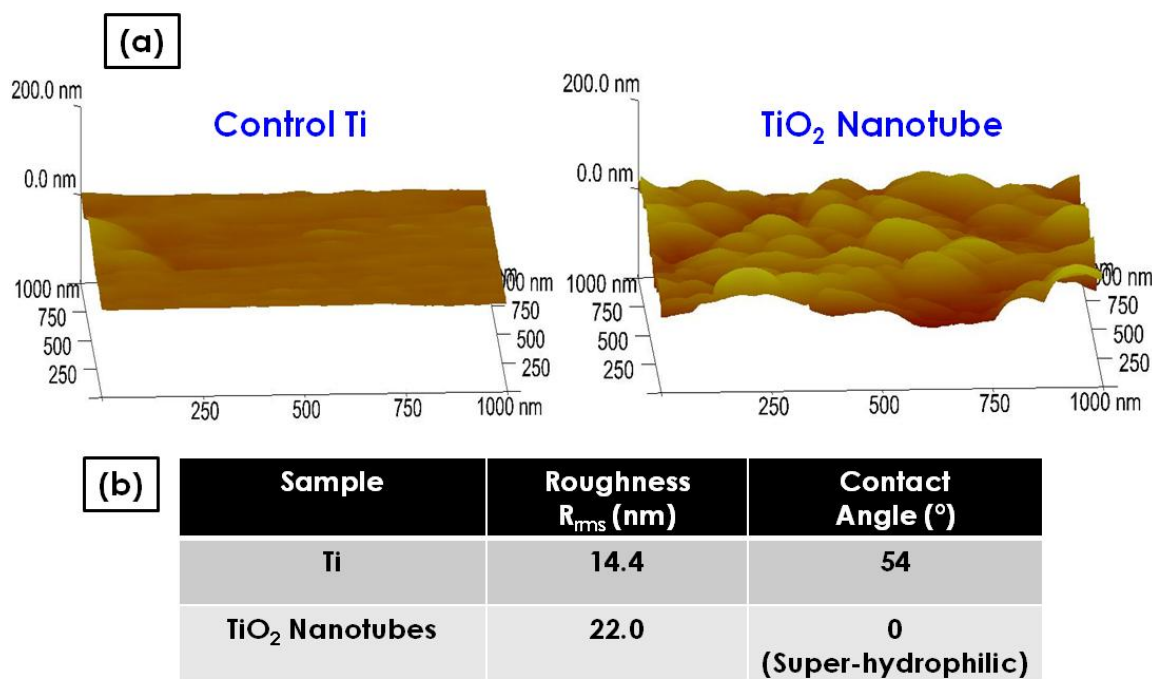


Figure 2.4-3. Macroscopic surface characterization of experimental substrates used for comparison bone cell culture. **(a)** 3-D Atomic force microscopy (AFM) images of control Ti foil surface vs. hydrothermally fabricated TiO₂ nanotube films **(b)** Table with roughness (R_{rms}) and surface wettability via contact angle measurements for Ti vs. TiO₂ nanotube surfaces.

Another physical advantage to this novel nanotube surface is that the oxide surface structuring is composed of and created directly from the native underlying Ti constituent, unlike foreign ceramic and spray coatings added on Ti or Ti alloy implant surfaces (e.g., hydroxyapatite layer plasma coated on Ti

implants), similar to the vertically aligned nanotube system created by anodization. In fact, the adhesion strength results indicated that the nanotube layer bond strength could withstand at least 309 psi (lb/in²) tensile force and the failure fracture occurs at the epoxy/TiO₂ nanotube interface, i.e. TiO₂ nanotube layer and Ti foil were still tightly adhered to each other, as it was the epoxy-connecting metal wire adhesion that failed. Other researchers have reported several problems with the addition of nanoscale coatings on implants stemming from cracking and separation from the metallic substrate (152-153). The adhesion results in this study revealed that we have developed a relatively robust and flexible nanoscale structure, without introducing any foreign ceramic or spray coatings, capable of the load bearing necessary for orthopedic implants.

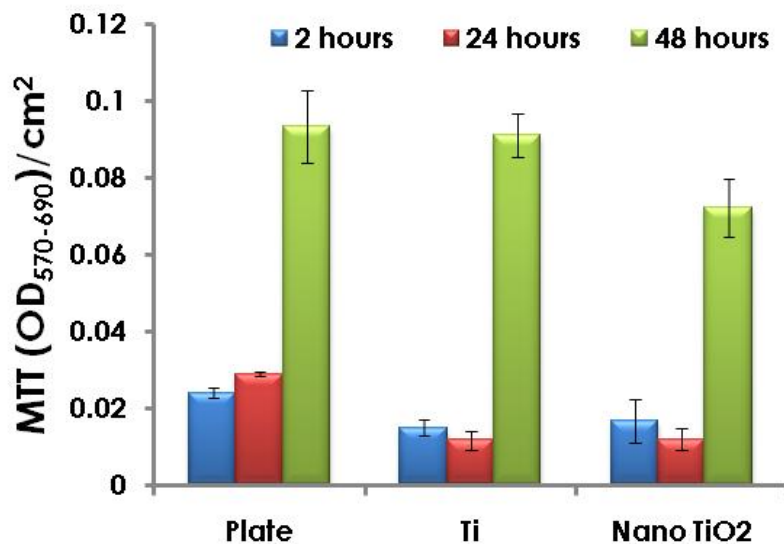


Figure 2.4-4. MTT assay cell viability test showing the optical density (OD) of reaction product of the MTT working solution (normalized by substrate area) with osteoblast cells cultured on polystyrene control dishes, control Ti, and 8nm TiO₂ nanotubular surfaces after 2, 24, and 48 h of incubation. The bar graph shows the average $\pm$ the standard error.

Fig. 2.4-4 reveals the MTT assay to estimate the density of viable cells on the experimental surfaces. The absorbance values for nanotubular and Ti surfaces are similar to that obtained from the polystyrene culture plate. Polystyrene is commonly used as a positive control for cell culture. The similarity between the absorbance values suggests that the cells are healthy and viable on all three surfaces. However, the initial (~2hrs incubation) adhesion and spreading of the osteoblast cells were seemingly different, as seen in **Fig. 2.4-5(a)**, for cells grown on the Ti vs. TiO₂ nanotube surface. In the initial stage of incubation, the cells seem to spread out more and form irregular edges, possibly more cellular extensions probing the surface on the nanostructured surface. The cells on Ti are more uniform and round. By determining the initial osteoblast attachment to the surface, we can attempt to correlate the cell attachment and spreading to the physical and mechanical properties of the scaffold (122). In the case of the nanotube surface, the surface is superhydrophilic with a 0°C contact angle compared to a moderately hydrophilic surface on control Ti with a 54°C (154) contact angle (**Fig. 2.4-3(b)**). The surface energy is therefore much higher on the nanotube surface. This may have implications in the increased cell spreading during the initial stages of adhesion. Hallab et al. (155) reported that the surface energy might be a more important determinant during cell adhesion and more useful than surface roughness for generating cell adhesion on engineered scaffolds. As well, Suh et al. (156) reported that a higher surface energy induced a stronger osteoblast cell attachment on hydrothermally treated anodic oxide films, which might be the case on our hydrothermally processed

nanotube surface as well. A stronger adhesion mechanism, with cells anchoring into the surface nanofeatures, would help ensure mechanical interlocking between implant and growing bone.

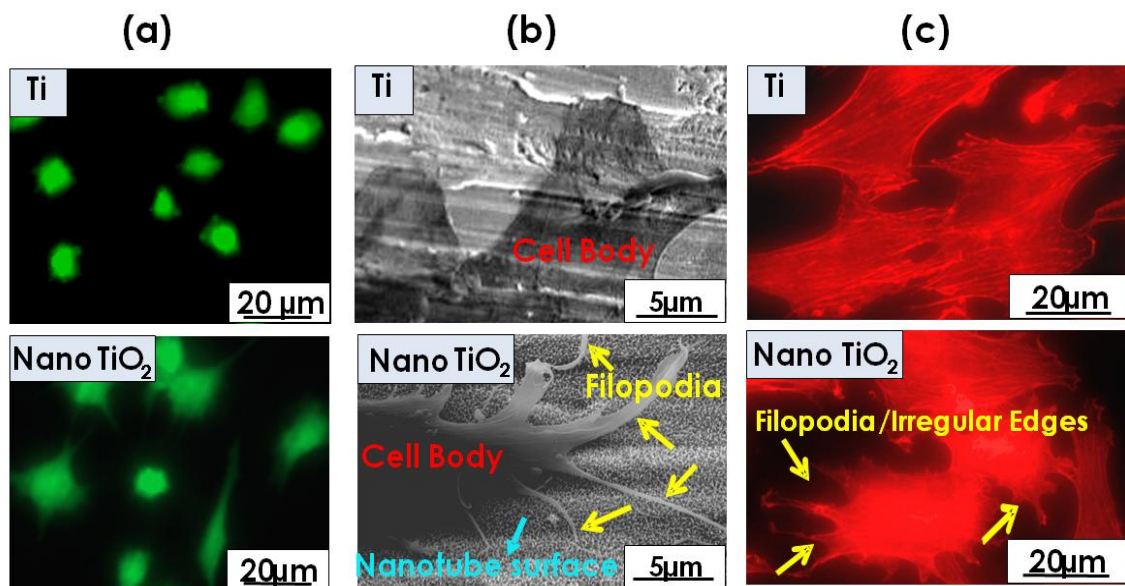


Figure 2.4-5. Cell adhesion behavior. **(a)** Initial adhesion and spreading (2 hrs incubation). FDA viability marker, which stains living cell cytoplasm green, shows that osteoblast cells spread out and begin expanding their cytoplasm as early as 2 hours of culture on the nanotopographic TiO₂ nanotube surface. In contrast, cells on control Ti appear to remain spherical on the surface with less adhesion spreading area. **(b)** High magnification SEM micrographs of osteoblasts on control Ti vs. 8 nm TiO₂ nanotube surfaces after 24 h of incubation. A much more pronounced protrusion of filopodia with a significantly longer configuration is seen on the 8 nm TiO₂ nanotube surface. **(c)** Immunofluorescent images of cytoskeletal actin (red) for osteoblasts on Ti vs. 8 nm TiO₂ nanotube surfaces after 24 h of culture incubation. The nanotube image reveals more filopodia extensions and protrusions (arrows) at the outer edges of the cell body.

High magnification SEM images in **Fig. 4.5-5(b)** and actin cytoskeleton images in **Fig. 4.5-5(c)** revealed a difference in cell morphology on the nanotube vs. Ti control surfaces. The cells adopted a flattened and spreading form, but also extended filopodium-like processes with irregular edges that were intimately adapted to the nanotube surface, which was lacking on the Ti control.

Quite possibly, the induction of increased filopodia extensions is a good sign that the cells are able to anchor more soundly using the nanotube architecture as anchorage sites as previously reported (54, 157).

To assess the bone forming ability, alkaline phosphatase (ALP) activity was measured. During periods of active bone growth, ALP activity levels are elevated in osteoblast cells so it is beneficial to design an implant surface that would enhance the ALP activity to initiate the formation of new bone. A standard colorimetric assay was employed to determine the ALP levels and the values were normalized based on total amount of protein content to account for the difference in number of cells on each of the surfaces.

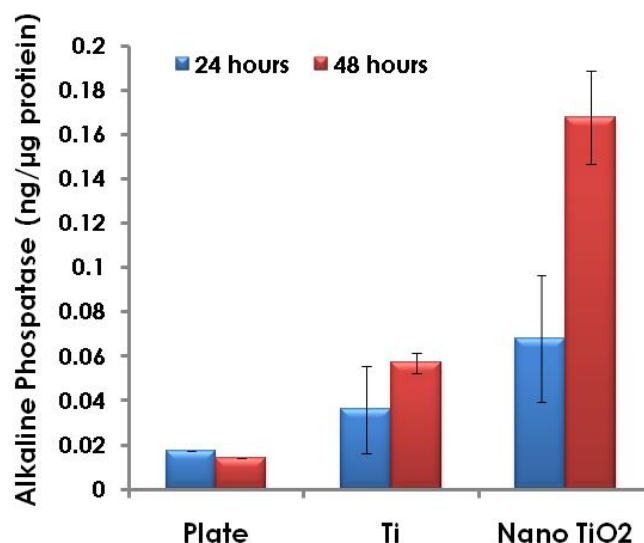


Figure 2.4-6. Alkaline phosphatase (ALP) activity of osteoblast cells cultured on control Ti, and 8 nm diameter TiO₂ nanotubes after 24 and 48 h of incubation. The bar graphs show the average $\pm$ standard error bars. The nanotube surface enhanced the alkaline phosphatase activity.

Fig. 2.4-6 reveals that the nanotube surfaces enhanced the ALP activity of osteoblasts cells grown on the surface. At 48 hrs, the nanotube surface had

almost 2.5 times higher level of ALP activity as compared to the control Ti. This up-regulation in ALP suggests that the nanotube surface has an advantage as a bioactive implant surface because it stimulates early bone formation better than unmodified Ti surfaces.

After two weeks of culture, the experimental surfaces were visualized by SEM to investigate the deposited bone matrix, **Fig. 2.4-7**. Large areas of granular deposits were revealed on the nanotube surface (**Fig. 2.4-7(b)**), which was not present on the control Ti (**Fig. 2.4-7(a)**). This agrees with previously reported findings showing increased extra cellular matrix production stimulated by a coarser, 100 nm diameter TiO₂ nanotube surface (93, 122). Clearly this type of nanotopography has stimulated increased matrix deposition, allowing granular material to fill in-between spaces and covering over the nanotube surface, possibly a sign that indicates improved osseointegration at the material interface. Calcium and phosphorous are the two main components of bone matrix. EDAX surface analysis in **Fig.2.4-7(C)** revealed an increase in the amounts of calcium and phosphorous on nanotubular surfaces, compared to a negligible amount on titanium surfaces. This corresponds to greater bone matrix deposition on nanotubular surfaces as compared to titanium surfaces, which was apparent in the SEM images as well. EDAX analysis under SEM examination confirmed that the deposited material was primarily made up of calcium and phosphorus, which are important bone matrix constituents. However, because of the EDAX detection limit, further experimentation with more advanced surface analysis such as XPS would be beneficial to aid in the investigation of the deposited

matrix. It has been commonly reported in the literature that nanoscale surfaces have been reported to induce higher amounts of calcium and phosphorus mineral deposition in vitro and in vivo (122, 158-159). The nanotube surface in this study has a similar effect on osteoblast cells, suggesting that the 8 nm TiO₂ nanotube surface provides a favorable interface for matrix production.

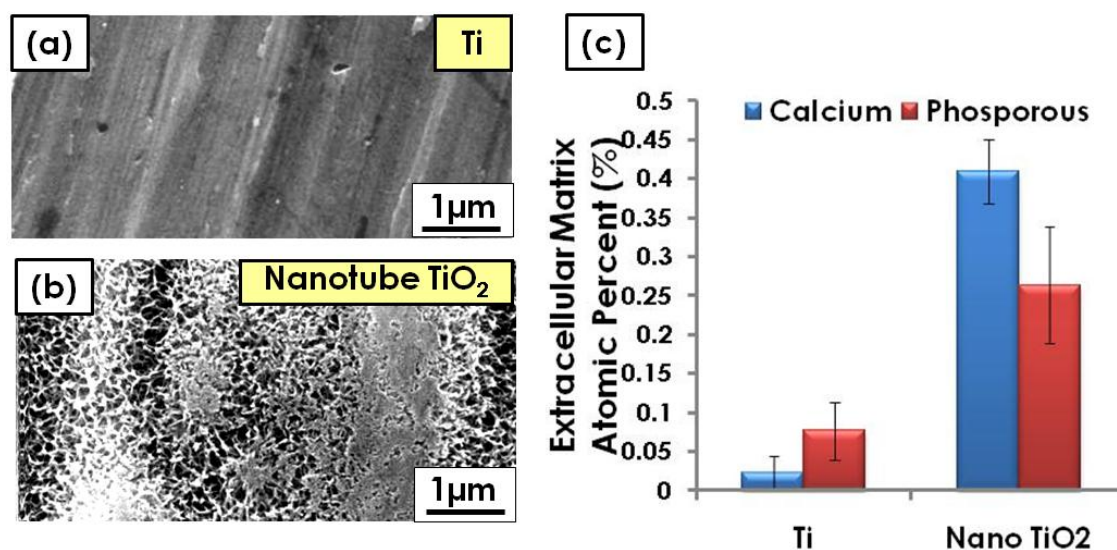


Figure 2.4-7. Extracellular matrix (ECM) mineral evaluation after 2 weeks of culture. **(a)** SEM observations of the ECM deposition on the control Ti, and **(b)** on 8 nm TiO₂ nanotube surface. **(c)** EDAX evaluation of the atomic percent of calcium and phosphorous mineral elements present at the surface of the Ti vs. TiO₂ nanotube. The bar graphs show the average $\pm$ standard error bars.

This study was intended to help facilitate the osseointegration and the design of the oxide layer on Ti implants. It appears that creating 8 nm diameter, ~150 nm long, one-dimensional nanotubes on the surface of Ti is advantageous for stimulating a superior osteoblast response. The development of nanostructured platforms based on novel metal-oxide films, as demonstrated in

this work, can provide insight into the design of bone cell and implant interactions.

2.5 Conclusions

2.5.1 Considerations for Further Development

In the future, nanostructured ceramics will be created that demonstrate even higher degrees of integration between materials and biology (160). Future ceramic nanostructures may possess even more precisely tailored grain and/or pore sizes in order to obtain specific tissue interactions. For instance, loading nanoporous structures can provide an implant with biological functionalities (161) are key prospects in deriving therapeutic based nanostructures that integrate and for wound healing, bone repair, and cardio vascular restoration, to name just a few. For example, silver and zinc-containing zeolites, marketed under the trade name AgION®, are currently being assessed for use in wound dressings. **Fig. 2.5-1** shows the idea of a “nanodepot” using the nanopores of the TiO₂ nanotubes for loading biological agents.

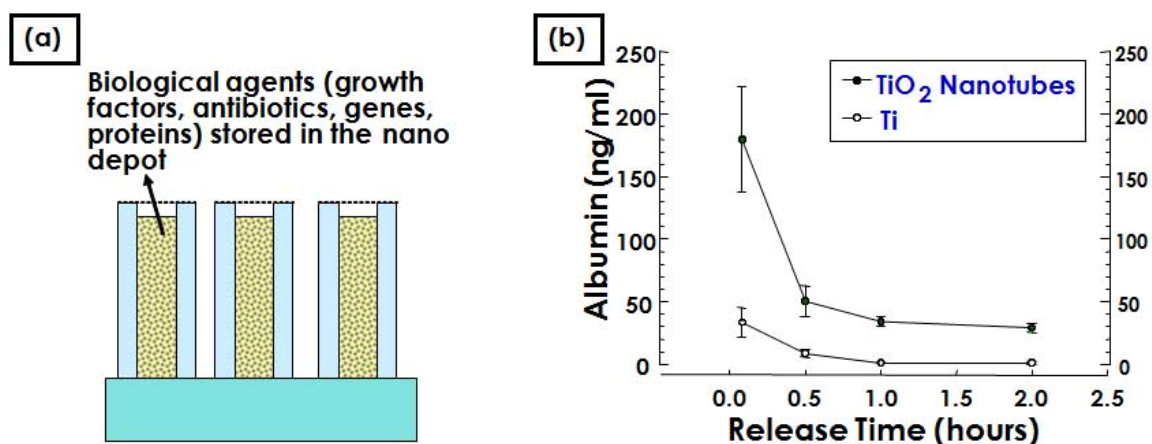


Figure 2.5-1. (a) Schematic illustration of “nanodepot” concept in TiO₂ nanotubes. **(b)** Example of albumin protein elution from inner pores of TiO₂ nanotube structures.

Chapter 5 will shed further light on the potential of nanoporous surfaces as drug carriers for therapeutic coatings on orthopedic implants.

Secondly, during the last few years, there has been remarkable progress in applying the process of electrospinning to the fabrication of ceramic nanorods and nanofibers. Ceramic nanofibers are becoming useful and niche materials in several applications owing to their surface dependant and size dependant properties (162). The use of ceramics nanostructures that more closely mimic the extracellular matrix, for example resembling the hydrothermal nanotube structure in Section 2.4, holds beneficial applications in future tissue engineering schemes. Nanotubes, nanorods or nanofibers of this nature could be advantageous for imitating the natural ECM environment of collagen type I fibers having fibrous and tangled nanoscale geometry. This is an important aspect to bone scaffold designs in order to present host cells with an innate ECM interface where it triggers repopulation and resynthesizing a new matrix. On the

basis of this theory, the electrospinning technique could be used as an alternative method for creating TiO_2 fibers with a 3-D network of interconnecting fibers with varying porosity and high surface area which mimics the niche-like unit ECM fibrils for facilitating proper cell behavior. The electrospinning technique accepted bottom-up methods for producing nanostructures with high aspect-ratios (fibers, tubes, ribbons, mesoporous, and coated structures). For the electrospinning process [162]:

- 1) A polymer solution is injected from a needle in the presence of electric field.
- 2) When the applied electric field overcomes the surface tension of the liquid a continuous jet is ejected which upon subsequent solvent evaporation and bending produces nanofibers on the collector surface.
- 3) Generally, ceramic nanofibers are made by the electrospinning of ceramic precursors in the presence of polymer followed by calcination at higher temperatures.

The resultant fibers can be randomly or aligning on the surface based on ground collection plate. For the process of TiO_2 nanofibers, described in **Fig. 2.6-2**, a polymer precursor using titanium propoxide and poly vinyl acetate is fed through the syringe and collected as shown in **Fig. 2.5-2 (a)**. After baking the as-deposited fibers to burn out the polymer, the final ceramic TiO_2 nanostructure is revealed in **Fig. 2.5-2 (b)**.

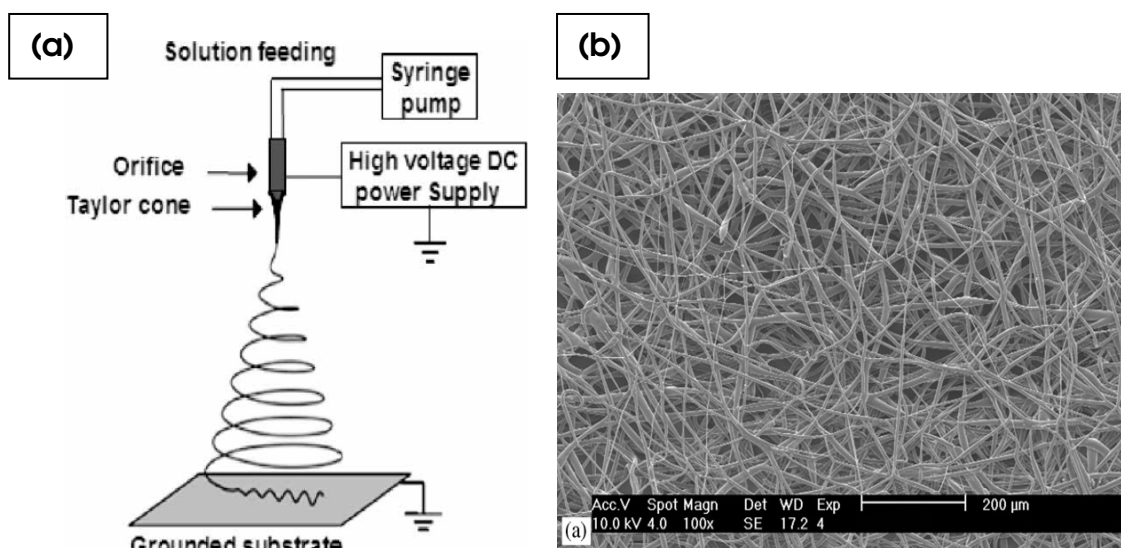


Figure 2.5-2. (a) Electrospinning apparatus(163) **(b)** TiO₂ ceramic electrospun nanofibers.

Electrospinning allows for engineering fibers at the nanolength scale with many opportunities to tailor the physical, chemical, and biological properties of a material for specific applications and cellular environments (164). Aspects of scaffold design, including architectural properties, surface functionalization and materials selection need further exploration in terms of controlling cell behaviour and differentiation.

Another suggestion would be to directly compare the behaviour of cells on the different ceramic nanostructures side by side in order to optimize the geometry and structure for better understanding of architecture on a nanoscale. For instance, it would be interesting to compare anodized vertically aligned, hydrothermal uni-directional, and electrospun fiber structures of TiO₂. A systematic evaluation comparing all three would shed light on the optimal structure for implementing desired cell behaviour.

Chapter 2 is a reprint of the material as it appear in Acta Biomaterialia Volume 5 (8), 2009, Page 3215 written by Karla S. Brammer, Seunghan Oh, Christine J. Frandsen, Lars M. Bjursten, Henri van der Heyde, and Sungho Jin; and in Nano Letters Volume 8 (3), 2008, Page 786 written by Karla S. Brammer, Seunghan Oh, John O. Gallagher, and Sungho Jin; and the Journal of Materials Science and Engineering C Volume 30 (4), 2010, Page 518 written by Karla S. Brammer, Seunghan Oh, Christine J. Frandsen, Shyni Varghese, and Sungho Jin; and the Journal of Advanced Engineering Materials published online Jan. 17th 2011 (DOI: 10.1002/adem.201080103) written by Karla S. Brammer, Hyunsu Kim, Kunbae Noh, Mariana C. Loya, Christine J. Frandsen, Li-han Chen, Laura S. Connelly, and Sungho Jin. The dissertation author was the primary investigator and author of this paper.

CHAPTER 3

3. Stem Cell Differentiation via Surface Nanostructures

3.1 Adult Stem Cells

Stem cells, which have the potential to differentiate into multiple cell types, provide great promises in advances in regenerate medicine. The differentiation of stem cells into the appropriated lineages is temporal- and spatial-specific, with the surrounding microenvironment playing critical roles in governing the stem cell fate. Two important characteristics of stem cells are 1) they are unspecialized cells that renew themselves for long periods through cell division and 2) under certain physiologic conditions, they can be induced to become cells with specialized functions. There is ongoing research to identify the signals that cause stem cells to become specialized (lineage specific) cells.

For the purposes of this work the focus is on adult stem cells, or mesenchymal stem cells (MSCs), that are derived from bone marrow. It is well known that MSCs are multi potent stem cells that can differentiate into stromal lineages such as adipocyte, chondrocyte, fibroblast, myocyte, and osteoblast cell types by generating the appropriate intermediate progenitors for each. **Fig. 3.1-1** illustrates a schematic of MSC differentiation.

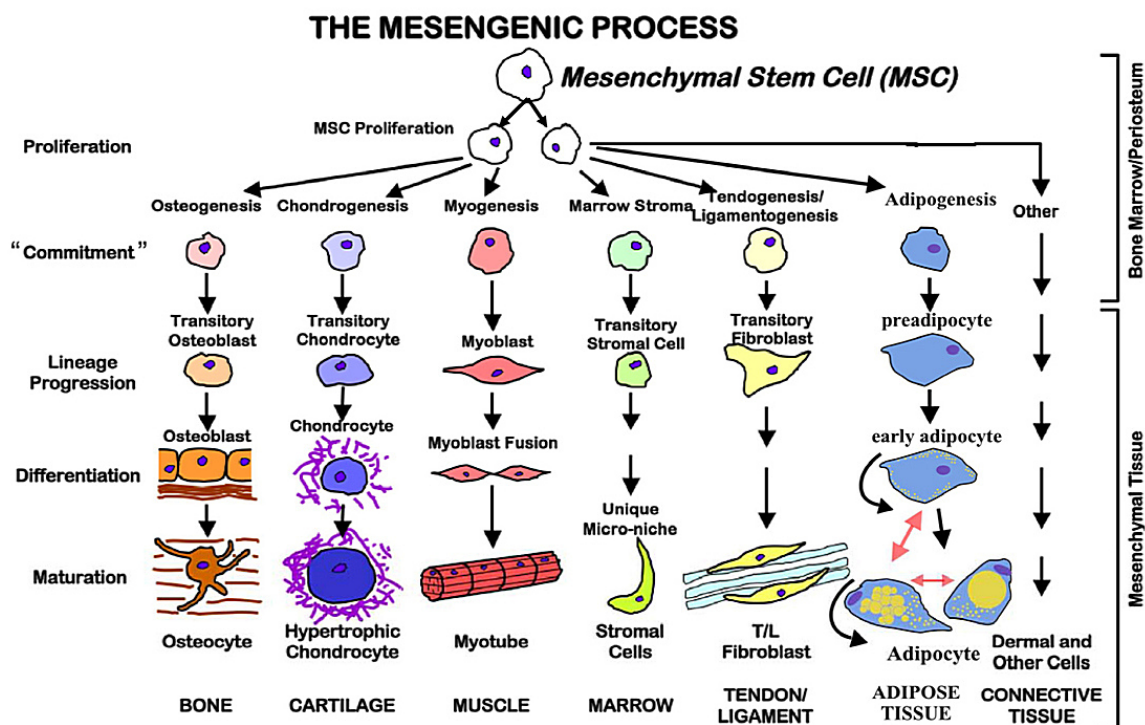


Figure 3.1-1. Mesenchymal stem cell differentiation. Mesenchymal stem cells (MSCs) can be cultured *in vitro* to generate a variety of differentiated cells and demonstrate their multi-potent capacity and differentiation plasticity (165).

By examining the cellular behavior of MSCs cultured *in vitro* on nanostructures, it can provide some indication and understanding of the effects that the nanostructures may have on the stem cell's response, and possibly how to direct the formation of selective tissue *in vivo*, i.e. bone. Most previous studies have used the combination of osteogenic inducing media (with growth factors, vitamins, and hormones) and nanostructured culture substrates to accelerate osteogenesis and bone formation of MSCs *in vitro*, but it is difficult to truly distinguish if the nanostructure by itself can achieve MSC differentiation or not. Additionally, the aspect of additional biochemical factors is very risky since they

are usually synthetic molecules, foreign to the human body, and the use *in vivo* would be unpredictable. In this work it is demonstrated that nanostructures have a guided effect on mesenchymal stem cell (MSC) differentiation into osteoblasts in the absence of osteogenic inducing factors. Because orthopedic implants encounter two types of cells, osteoblast cells in bone tissue (Section 2.2) and MSCs present in bone marrow, it is advantageous to look at the differentiation potential of orthopaedic implant surfaces in order to initiate a mature population of bone building cells for enhanced osseointegration. This is key principle in terms of orthopaedic implant technologies, to initiate the differentiation of bone in the absence of chemical factors.

3.1.1 Role of the Surface in Stem Cell Research

An approach to further advance the orthopedics technology is to introduce a combination of nanotechnology and stem cell treatment. For generation of osteoblasts, the MSCs need to be guided to selectively differentiate to osteoblasts, rather than differentiating into other types of cells such as myocytes, adipocytes or neural cells.

In particular, nanotopography has recently been proven to have significant and favorable effects on stem cell commitment into mature lineages. The ability to control stem cell differentiation using topography alone has focused attention on elucidating the mechanism by which a cell perceives these topographical cues and relays this information into the nucleus to initiate a cellular response. In terms of bone cell differentiation, Dalby et al. (166-169) have shown that random circular nanostructures promote and direct osteoblast

differentiation of MSCs. This finding shows that the effect of nanoscale geometries alone can induce differentiation in the absence of any biochemical inducing factors. These studies have demonstrated that different degrees of symmetry or disorder induces change in adhesion formation, which impacts on cytoskeletal tension, affects indirect mechanotransduction pathways (described in a later section) and imposes morphological changes on cells. The surface nanotopography directly induces pronounced changes of cell shape, and consequently gene expression, which can potentially mediate differentiation of stem cells into various cell types.

A reoccurring theme is that the cell morphology/spreading dominates cell fate. McBeath et al. showed that commitment of stem cell differentiation to specific lineages is dependent upon cell shape (170). In a single cell experiment with micropatterned surfaces the critical role of cell spread/shape in regulating cell fate was determined. Another study also showed MSCs in multi-cell islands differentiated into different lineages depending on the MSC location in population (170). These findings have been interpreted on the basis that cells at the edges of the micropatterns experience higher tension (higher stress) than the cells at the center. The cellular tension/stress due to morphological control may provide the mechanical cues for controlling cell fate. Investigations with the manipulation of cell substrate rigidities have led to the same conclusion: MSCs on rigid surfaces (high tension) differentiated into osteogenic lineage, whereas MSCs on soft surfaces expressed neuron markers (171). All these recent

evidences clearly demonstrated that the MSC fate is determined by their responses to the physical nature of the substrate.

By combining nanotechnology, cell biology and bioengineering to guide stem cell osteogenesis *in vitro* and *in vivo* the goal of cell-based therapy for bone repair can be achieved. The realization of the full potential of MSCs in regenerative medicine requires selective differentiation. In the Jin Lab, recent studies on nanotechnology osteogenic-related MSCs treatments have also shown the effects of nanotopography on specific differentiation by using only the geometric cues of the surface (172). On substrates made of TiO₂ nanotube surface structures selective osteoblast differentiation was induced by creating stem cell elongation accompanied by increased cytoskeletal stress using large dimensions of nanotube diameters (~100nm). It is hypothesized that different nanotube diameters control MSC fate by having different effects on the cell morphology and consequently cytoskeletal stresses which in turn modulates differentiation.

3.1.1.1 Mesenchymal Stem Cell Elongation and Osteogenesis on TiO₂ Nanotubes

A novel discovery that the exposure of MSCs to substrates containing TiO₂ nanotubes with various diameters (Fig. 8.3 (a)) can induce differential fates has recently been reported by the Jin Lab. For TiO₂ nanotubes with a small diameter such as ~30 nm, the stem cell differentiation was suppressed while adhesion and growth was accelerated. Such cell enrichment and proliferation without differentiation is a beneficial application of stem cell technology to cell therapy. In contrast, when the MSCs were exposed to a larger diameter nanotube

substrates (100 nm), the cells were significantly stretched with ~10-fold elongation as observed by SEM analysis and by live cell imaging using FDA (fluorescein diacetate) staining, as shown in **Fig. 3.1-2 (a and b)**. This type of morphological determination by altered nanotube dimension with osteoblast cells was also observed and discussed in Section 2.2. The osteo-differentiation of these stretched MSCs was determined by PCR (**Fig. 3.1-2 (c)**) and immunostaining (data not shown) of the osteogenic markers alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OCN). Uniquely, MSCs on the largest 100nm diameter TiO₂ nanotube surfaces had the highest up-regulation of the osteogenic RNA transcription levels (**Fig. 3.1-2 (c)**) and were the only experimental samples that stained positively for OPN and OCN at two weeks of culture. The stem cell elongation and osteo-differentiation trends seem to correlate as the diameter increases there is an increase in cell elongation and up-regulated osteogenic expression. The suggested mechanism is in agreement with the general notion that the morphological elongation induced increased cytoskeletal stress which in turn modulated differentiation into the specific osteoblast lineage.

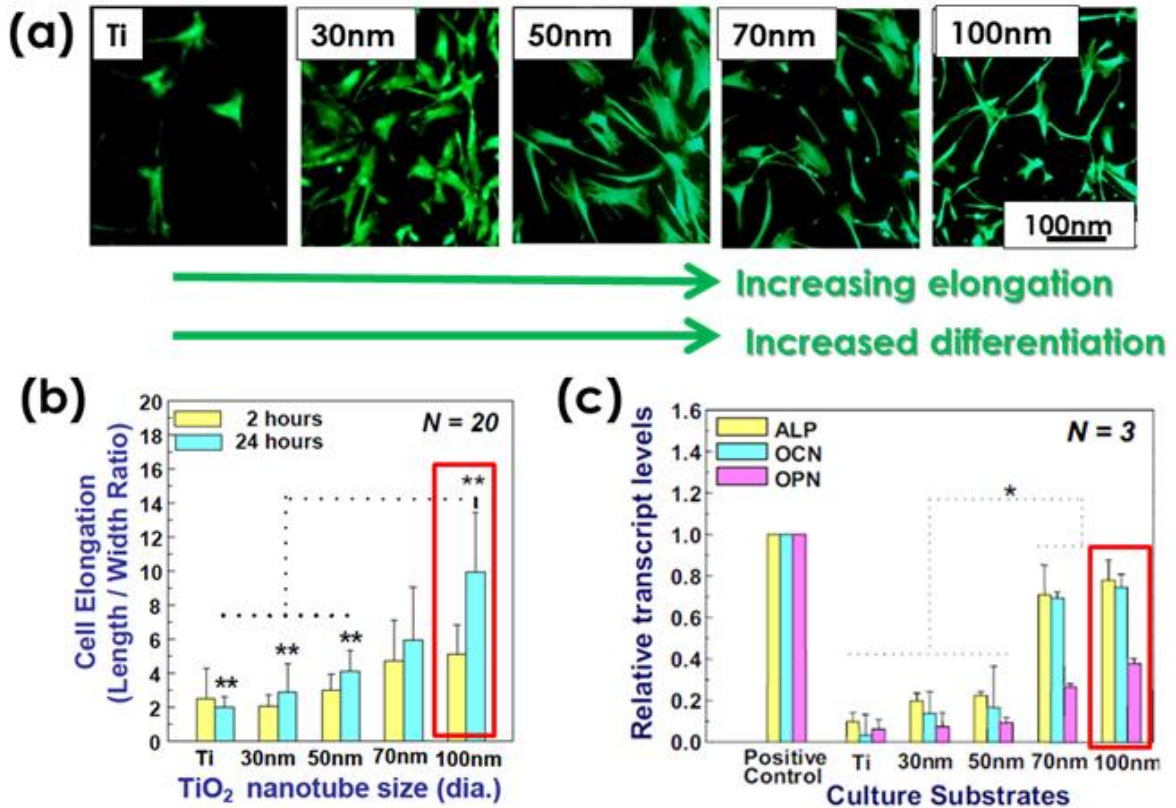


Figure 3.1-2. *In vitro* mesenchymal stem cell (MSC) response to different sized nanotube surfaces. **(a)** Morphological observation of cells by FDA live staining. MSCs increase in elongation with increasing nanotube diameter. **(b)** Quantification of MSC elongation. **(c)** Comparative bar graph showing osteogenic differentiation on the different sized nanotube surfaces evaluated by PCR relative transcription levels for the primary osteogenic markers alkaline phosphatase, osteocalcin, and osteopontin (ALP, OCN, and OPN).

The overall results indicated that the MSC fate was determined by nanotube diameter because the osteogenic differentiation was facilitated most proficiently by large-diameter nanotubes. Thus a concept was developed in determining stem cell fate based solely on the geometric cues of the surface nanostructure and size of the nanotube diameter.

3.1.2 Mechanotransduction

There is a need to better understand the mechanism by which such nanosurfaces direct MSC osteogenesis, and to optimize the culture conditions in order to maximize MSC expansion and differentiation. For bone growth, it requires cell proliferation and selective differentiation, and these processes are found to occur at different but discrete nanosurface topography conditions such as variations in nanotube diameter. A concept developed by Dalby et al. (167), suggested that MSC differentiation was determined by mechanotransductive pathways that were impacted because the cell was under tension caused by way the cell was adhering to the underlying nanostructure surface. Furthermore, it has been reported that cells are exquisitely sensitive to forces of varying magnitudes, and they convert mechanical stimuli into a biochemical response, this is known as *mechanotransduction*. Mechanotransduction provides a simple means by which cells and organisms can ensure structural stability, as well as a way to regulate morphogenetic movements to generate precise functionality; for instance bone is shaped by forces of gravity and muscle contraction(173).

The schematic in **Fig. 3.1-3** represents the forces on the cell the proposed mechanism of mechanotransduction.

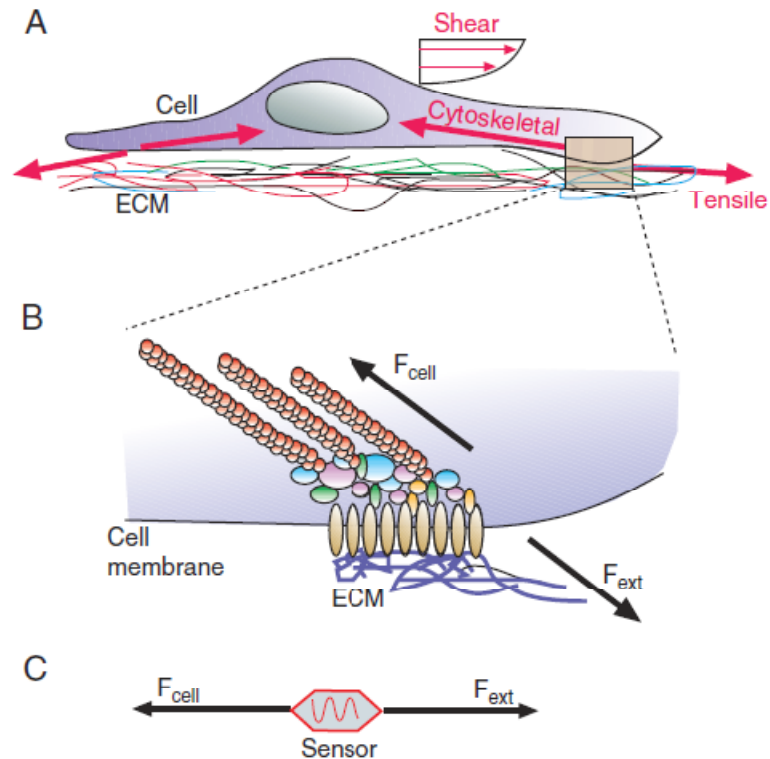


Figure 3.1-3. Application of force to a cell. **(A)** Cells can be exposed to multiple types of forces, such as shear forces, through fluid flow over the cell, tensile forces acting through the ECM, and cytoskeletally generated contractile forces. Depicted is a single cell attached to a complex ECM (illustrated as a multicoloured fabric). **(B)** Close-up of a focal adhesion showing the balance of external and internal forces (F_{ext} and F_{cell} , respectively) in driving stress at a mechanosensor. Depicted are actin stress fibers (red) anchored into focal adhesions (multicoloured array of proteins) that bind to the ECM (blue) through integrins (brown). **(C)** This balance of forces provides the stress necessary for mechanical sensing. (174)

Clearly, the integrin–cytoskeleton linkage is sensitive to force. This triggers a cascade of reactions that alter the balance of anabolic / catabolic events within the cell, ultimately differentiation. Modulation of focal adhesions will alter cellular mechanotransduction. Mechanotransduction is the process by which external mechanical stimuli (including cell stretch, compression, and interaction with topography) are transmitted into the nucleus, resulting in adaptive gene-

and protein-level changes. Signaling cascades can be induced at focal adhesions, leading to indirect downstream effects on gene expression. As part of a sensory system, these integrin receptors, together with a multitude of associated proteins, including real signaling elements (for example, kinases, phosphatases and adaptor proteins), respond to particular biochemical and physical characteristics of the microenvironment by initiating a cascade of events (**Fig 3.1-4**) (175). In the case of the MSCs on TiO₂ nanotubes, it is hypothesized that the cells are in tension, meaning that they experience a tensile force (or stress) from the adhesion/integrin complexes attached to the outside nanotubular surface, acting as the perceived ECM *in vitro*, which pulls to elongate the cell.

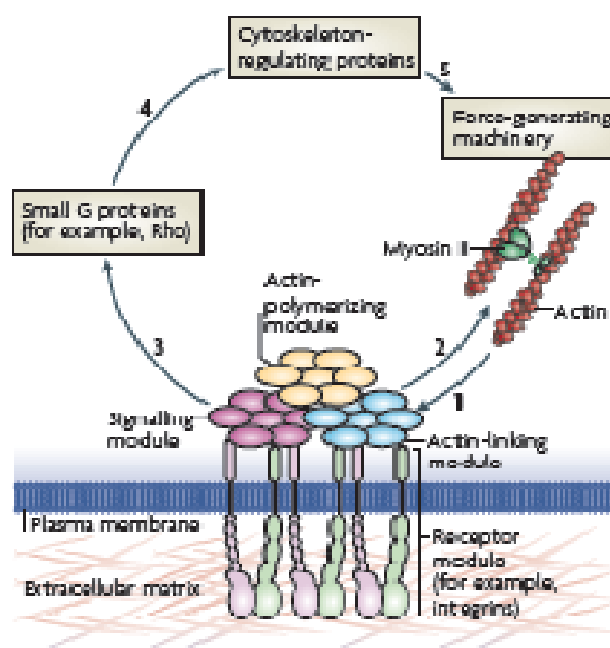


Figure 3.1-4. Actin cytoskeleton–focal adhesion interplay. A schematic depicting the feedback loops that interconnect the actin machinery and integrin-mediated adhesions (175).

3.1.3 Strain Energy Involved in Stem Cell Stretching on Nanotube Surfaces

In this section, the role of mechanical forces during osteogenesis on the nanotube substrates is explored. This will allow further insight to how these forces might specifically regulate basic cellular processes (such as proliferation, differentiation and organizational changes). Although the biochemical events that occur upon stem cell differentiation are well characterized, there is limited knowledge of the physical nanomechanics of undifferentiated vs. differentiating MSCs.

It generally concluded from the previous sections that, the cell modifies its internal mechanical scaffold, the cytoskeleton, in order to adapt to its nanotube environment by elongation. External forces from the external nanostructure influenced the fundamental functions of MSCs such as shape, proliferation and osteo-differentiation. For this mathematical model, the mechanical strain energy due to stretching will be determined. This difference in strain energy is biologically important for understand cell behavior during, differentiation, regeneration, etc. The present approach to examining cell mechanics are motivated by recent advances in both the fields of cellular micromechanics and cell-based tissue engineering. Despite these advances, a fundamental aspect of MSCs has been understudied, their biophysical characteristics in response to mechanical stresses. This proposed model will shed light on the physical response and mechanical stress of MSCs during osteo-differentiation on TiO₂ nanotubes with varying diameters. Because MSCs give rise to all skeletal tissues such as bone, cartilage, skeletal muscle, tendons, and ligaments, mechanical

modulation of MSCs is of paramount importance to differentiation behavior (176). Hence, there is much interest in investigating the effects of the nanostructural and nanomechanical characteristics of MSCs.

Establishing possible connections between mechanical properties of MSCs in differentiation is valuable to the field of mechanobiology. It can be speculated that natural forces that MSCs encounter in a physical environment does not need a strong cytoskeleton, however upon osteogenic differentiation, in which differentiation of MSCs become part of a larger bone structure that functions to provide both form and strength, the supporting structure of the cells are enhanced to enable function and withstand load bearing wear that bones endure. Understanding the physical characteristics of MSCs during differentiation may aid in the development of new biomaterials, which can potentiate the necessary mechanics of the cells for the advancement of tissue engineering. The underlying engineering problem involved in this section is defined in **Fig. 3.1-5**. A direct relationship between MSC stretching/elongation, deformation, and shear force acting on the cell during elongation is proposed. In this case, shear force was chosen as the primary force acting on the entire cell body during cell elongation; this is based on the principle that there was so called slippage and translation of the cell body during shape transformation. It is hypothesized that increased stretching results in an increase in deformation as well as increased shear force felt by the cell. The differences of shear stress acting on the cell therefore have implications for regulating osteoblastic differentiation. Since mechanical forces of the cell are essential in determining cell fate, here we can

determine the relative shear force and deformation ratio needed to induce osteo-differentiation of MSCs. The strain energy transferred during elongation can subsequently be determined by a force vs. displacement curve and the differentiation energy for different stages of differentiation on the altered diameter nanotube surfaces based on our previous work (134) will be deduced.

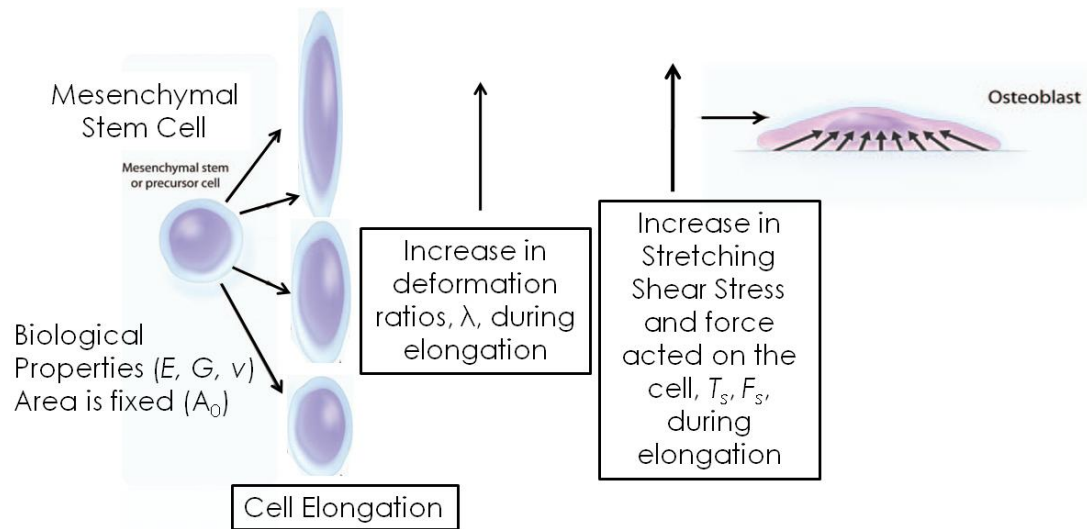


Figure 3.1-5. Schematic representation defining the mechanism based analysis for the observations of osteogenic differentiation on nanostructured surfaces. The variables in the problem are emphasized as deformation and stretching shear stress. The main query in this problem is determining the force acting on the cells based on the morphological deformation responsible and necessary for osteogenic differentiation.

The following are the constituent equations used in this model. The strain energy potential function, U , describes nonlinear elastic stress-strain behavior when deformation from the equilibrium shape. The visco-elastic nature of biological tissues is assumed for this model. Additionally, this model follows a similar cell deformation model by Lim et al. (177).

$$U = \frac{G_0}{2} (\lambda_1^2 + \lambda_2^2 + \lambda_3^2 - 3) \quad (\text{Eq. 3.1-1})$$

where G_0 is the initial shear modulus, and λ_i ($i=1$ to 3) are principal stretches. The incompressibility condition implies that $\lambda_1 \lambda_2 \lambda_3 = 1$ because we assume that the volume does not change. We can simplify **Eq.3.1-1** for a two dimensional deformation model of the cell as shown in **Fig. 3.1-6**.

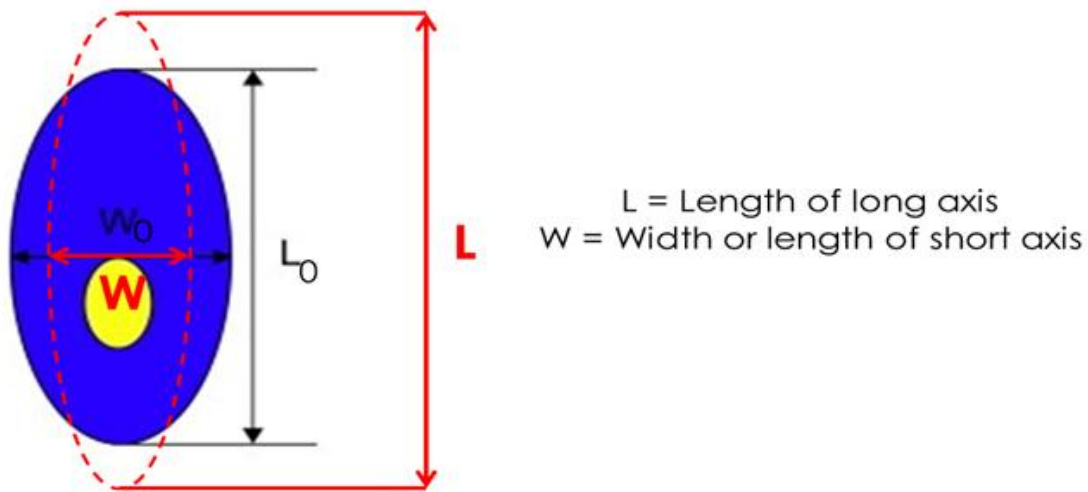


Figure 3.1-6. Schematic illustration of a cell body with a length, L , and a width, W . Dotted lines represent deformed cell body after elongation with new length and width, respectively. This demonstrates the deformation of the cell according to the morphological stretching of the cells during elongation on a nanotube sample surface.

The shear stress expressed in units of force per unit area,

$$T_s = 2\mu\gamma_s = \frac{\mu}{2} (\lambda_1^2 - \lambda_2^2) \quad (\text{Eq.3.1- 2})$$

$$T_s = \frac{1}{2} (T_1 - T_2) \quad (\text{Eq. 3.1-3})$$

$$\gamma_s \equiv \frac{1}{2}(\varepsilon_1 - \varepsilon_2) = \frac{1}{4}(\lambda_1^2 - \lambda_2^2) \quad (\text{Eq. 3.1-4})$$

$$\lambda_1 \lambda_2 = 1 \quad (\text{Eq. 3.1-5})$$

where T_1 and T_2 are the in-plane principal membrane stresses, ε_1 and ε_2 are in-plane principal strains on the membrane, and μ is shear modulus (assumed to be constant). **Eq.3.1-5** describes the condition that the area is also kept constant. In addition, it can be assumed that there is uniform deformation so that λ_1 and λ_2 approximate the ratios of current axis length to initial axis length in the axial and transverse directions. This can be related to the major and minor axis of a cellular shape, described as the width, W , and length, L in **Fig. 3.1-6**, which shows the cell draw schematically as an ellipse with two principle axes. The cell shear modulus μ is equivalent to the material property G ,

$$G = \frac{E}{2}(1 - \nu) \quad (\text{Eq.3.1-6})$$

where E is Young's modulus of the cell and ν is Poisson's ratio. Based on our model we assume that the deformation ratio in the longitudinal direction is much more dramatic than in the width direction, i.e.

$$\lambda_1 = \frac{L}{L_0} \quad \lambda_2 = \frac{W}{W_0} \quad \lambda_1 \gg \lambda_2 \quad (\text{Eq. 3.1-7 (a-c)})$$

Therefore we can approximate **Eq. 3.1-2** as the following:

$$\frac{2T_s}{\mu} = (\lambda_1^2 - \cancel{\lambda_2^2}) \rightarrow \frac{2T_s}{\mu} = \lambda_1^2 \quad (\text{Eq. 3.1- 8})$$

Atomic force microscopy (AFM) is a new tool for probing the mechanical properties of living cells. To obtain the elastic modulus, E , for MSCs during differentiation, the Hertz model is the most straightforward mathematical derivation for describing the elastic responses of an indented sample by the tip of the AFM (176). The Hertz model is described as:

$$E = \frac{3F_{AFM}(1-\nu^2)}{4\sqrt{R\delta^{3/2}}} \quad (\text{Eq. 3.1-9})$$

where E is the Young's modulus of elasticity, F is the applied nano-mechanical load applied on the cell by the AFM, ν is the Poisson's ratio, R is the radius of the curvature of the AFM tip, and δ is the amount of sample indentation. The amount of applied load and the indentation depth can be derived from force versus distance plots that are recorded by the AFM each time the tip approaches and retracts from the sample surface (176). The Young's modulus for various materials is shown in **Fig. 3.1-7** (178). Young's modulus for living cells is in the range of 1-100kPa. **Using Eq.3.1-6**, the shear modulus can then be deduced using a fixed value of E and ν for MSCs.

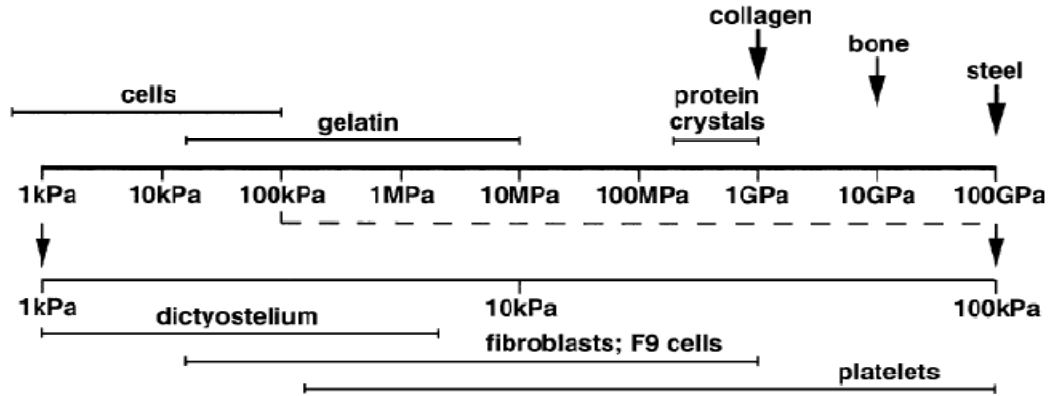


Figure 3.1-7. Young's (elastic) moduli of different materials (178). The diagram shows a spectrum from very hard to very soft: steel > bone > collagen > protein crystals > gelatin, rubber > cells.

The stretching force F_s in pN defines the force acting on the cell from the deformation of cell stretching/elongation on the nanotube surfaces during osteo-differentiation is defined as,

$$F_s = T_s A_0 \quad (\text{Eq.3.1-10})$$

where A_0 is the area of the cell. Consequently, F_s felt on the cell during cell morphological changes were graphed as a function of the principle stretch ratio for a range of forces up to 300pN in **Fig. 3.1-7**. By plugging in this range of force values into **Eq.3.1-8**, the corresponding principle stress ratio (λ_1) was calculated. The curve on the graph of F_s vs. λ_1 shows a shape of a second order polynomial relationship between the force and deformation respectively, as predicted by **Eq. 3.1-8**. The experimentally observed principle stretch (134), λ_1 , for each cell

population on the nanotube surfaces were approximated (see values in **Table 3.1-1**) and marked on the graph in **Fig. 3.1-8**.

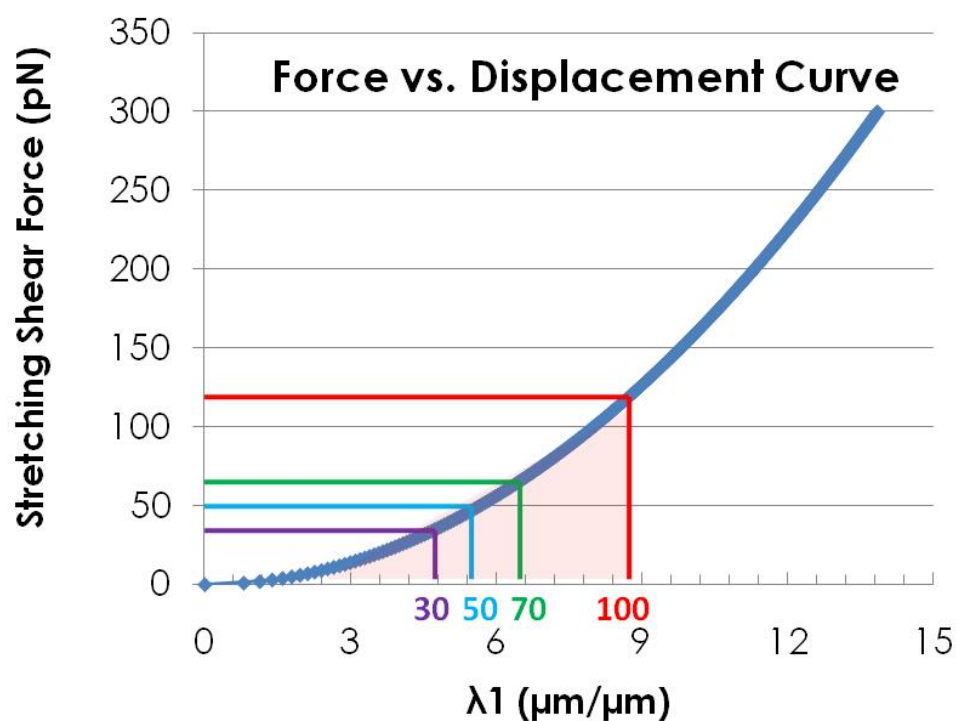


Figure 3.1-8. Mathematical analysis for determining the applied stretching shear force on the cell as a function of the stretch factor in the longitudinal direction or the ratio of current and initial major axis length, λ_1 . Based on experimental data and assumptions, the experimental observed λ_1 for each nanotube size (30-100nm) and associated stretching shear force is shown on the graph. The area under the curve, which is shaded in lightly for the 100nm substrate, represents the potential energy required to deform the cells.

Table 3.1-1. Associated stretching force and energy generated in the cells according to nanotube diameter as observed in experimental data and the theoretical values graphed in **Fig.3.1-7**. Energy is generalized as a triangular area under the force vs. displacement curve for each nanotube condition respectively.

Nanotube Diameter Size (nm)	L Observed Longitudinal Axis (μm)	λ_1 For $L_0 = 20\mu\text{m}$	F_s Associated Stretching Force (pN)	U Strain Energy Involved $\approx 1/2 yx$ (pJ)	Osteo-Differentiation Factor
100	178	8.9	124	552	1
70	130	6.5	66	215	0.73
50	110	5.5	48	132	0.61
30	95	4.8	36	86	0.53

Based on the curve of the graph, the stretching force felt by the cell can be extrapolated for each case of nanotube diameter (30-100nm). In addition, the area under the curve can be used to find relative strain energies involved for each cell morphological deformation according to nanotube size. The area symbolizes transferred energy, work done in order to stretch the cell on the surface during the deformation. A summary of all the relevant, estimated, calculated and graph values for the MSC mechanics, including the forces acting on the cell during deformation as well as the energy involved in stretching the MSC during morphological elongation according to observations using nanotube surfaces (**Fig. 3.1-2 (b)**) (134) are shown in **Table 3.1-1**.

The degree of differentiation for each nanotube substrate was also listed in **Table 3.1-1**. In this fashion one can compare the degree of differentiation with the mechanical values achieved in this model to correlate physical forces on MSC differentiation. From the model, it is predicted that increased levels of stress

resulted in high deformation stretch ratios which in turn modulated the internal networks of the cell to up-regulate the osteogenic behavior. The greater deformation that occurred on the cell (with increasing nanotube diameter size) was a direct indicator of the degree of induced osteogenesis.

The following conditions were assumed in determining the mechanical characteristics of the MSCs at various induced deformation:

i) The area of the cell during deformation is kept constant. The area of an ellipse, with initial L and W dimensions is described as,

$$A_0 = \pi \left(\frac{1}{2} L_0 \right) \left(\frac{1}{2} W_0 \right) \quad \text{Eq. 3.1-11(a-d)}$$

$$L_0 = 20 \mu m \quad W_0 = 15 \mu m \quad A_0 = 236 \mu m^2$$

ii) The applied stretching force (pN) on a cell during morphological changes is in a defined range such that,

$$0 < F_s < 300 \quad \text{Eq.3.1-12}$$

iii) The biological material properties of the cell are considered for a cell undergoing osteogenic differentiation with elastic modulus $E=54\text{kPa}$, $G=13.5\text{kPa}$, Poisson's ratio $\nu=0.5$ (176).

- iv) Current lengths (L) due to stretching/elongation for the different cell populations on the nanotube surfaces from experimental cell elongation data (134) can be used as a reference for determining the cell deformation ratio λ .
- v) The degree of osteogenesis (0-1) is determined by the gene expression ratio of ((nanotube sample of interest)/100nm diameter) from PCR transcription levels for osteogenic gene markers where the 100nm diameter cell population is considered to be fully differentiated into osteoblasts with the value of 1.

In terms of limitations to the model it is difficult to estimate, however, how far the stretching shear stress can be applied to the living cell. The application of this model implicitly assumes the existence of the cell equilibria arrived at almost instantaneously in a homogeneous reversible system. The cell is most likely a heterogeneous system with heterogeneous conditions. Nonetheless with this model we present experimental and theoretical results of large deformation of MSCs subject to a stretching force during observed elongation on our nanostructures. Clearly, the amount of stress endured during differentiation increases as the cells become more and more osteoblastic-like. This mechanical process in this study is of paramount importance to discovering cellular mechanisms for control of stem cell fate. With this knowledge we can better understand how to simulate the characteristic “osteo-force” to be acted on the cell that directs and regulates the cell for an osteoblast lineage.

3.1.3.1 Further Considerations for Future Development

In terms of the dimensions of the nanotubes in the osteoblast (bone cell) and mesenchymal stem cell (osteo-progenitor cell) studies, it was reported that

a unique variation in cell behavior even within a narrow range of nanotube diameters (65, 121). The results of the previous research can be simply summarized: osteogenic functionality, both biochemical activity in osteoblasts and internal gene regulation of osteo-progenitor cells, were altered by the size/diameter of TiO_2 nanotubes, the trend is revealed in **Fig. 3.1-9**.

Such a trend can be utilized for improvement and control of the bone forming functionality for advanced orthopaedic implant technologies.

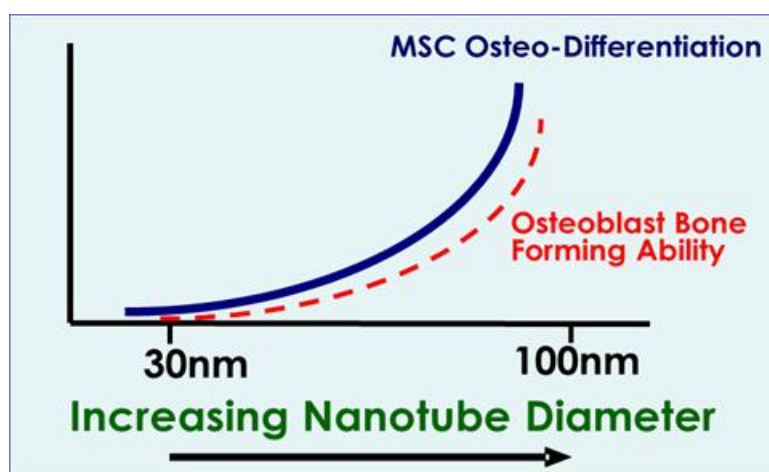


Figure 3.1-9. Overall all trend for diameter effect on osteogenic cell behavior. As the nanotube diameter increases the osteogenic cell behavior is enhanced.

In these studies however, TiO_2 nanotubes having a 1: 3 diameter: height aspect ratio was used, which was determined by the electrochemical anodization conditions including electrolyte solution, voltage, time, etc. While this current-state-of-the-art self assembly process of TiO_2 anodization does not easily allow fabrication of TiO_2 nanotubes with the diameter larger than $\sim 100\text{nm}$ with the electrolyte used in this study, it would be interesting to study the effect of

even larger diameter TiO_2 nanotubes, possibly using a modified chemical process, on osteogenic cells.

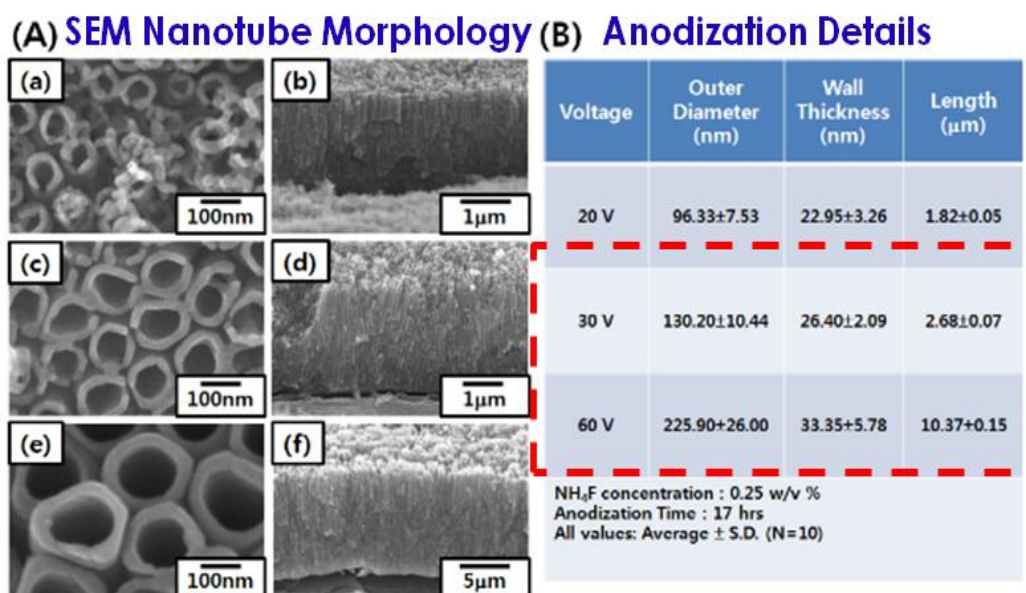


Figure 3.1-10. Large diameter nanotubes prepared in 0.25 w/v% NH_4F with various applied voltages. **(A)** SEM micrographs showing nanotube morphology by top view **(a, c, e)** and cross-sectional view **(b, d, f)**. **(B)** Chart describing the effect of applied voltage on the physical nature of the nanotube dimensions.

Other methods for making large diameter ($>100\text{nm}$) TiO_2 nanotubes using aqueous organic electrolytes with a future potential use as orthopaedic implant surfaces have been explored. Previously the anodization electrolyte method included aqueous dilute hydrofluoric acid. In an alternative fabrication method ammonium fluoride (NH_4F) in an ethylene glycol solution as an electrolyte can be investigated. **Fig. 3.1-10 (a)** illustrates the SEM figures of TiO_2 nanotubes prepared by 0.25 w/v% NH_4F electrolyte at various anodization voltage for 17 hrs. The table in **Fig. 3.1-10 (b)** indicates the variations in applied voltage and the resultant physical dimensions of the fabricated nanotubes. By controlling the applied

voltage, the diameter of the nanotubes could also be controlled. Nanotubes with diameters from ~130-225nm have been successfully prepared. Future work should include the use of large size nanotubes to find the most optimal stem cell osteogenesis and bone cell/tissue function.

Another idea for further consideration in developing a cell culture scheme for exact control and systematic evaluation of cell elongation lies in the design of generating precise topographies for physically guiding cell elongation. For instance, patterned ECM to manipulate MSC shape can be performed by micropatterned parallel array strips with the width of 10, 20 and 30 μ m can be fabricated using ECM (Collagen I) by micro-contact printing or nano/micro imprinting of ECM proteins and stamp-transfer to a substrate. Such pre-shaped ECM available sites are likely to force the MSCs to be elongated to the desired aspect ratio as the substrate regions outside the strips will be made to contain no ECM for restricting cell attachment. MSC could be cultured on ECM strips with growth medium without the osteogenic chemical stimuli, so that only the forced elongation of stem cells can induce the osteogenic differentiation. With this type of systematic assessment of the aspect ratio, deformation, and cell area, the optimal parameters to direct MSC differentiation for osteogenic lineage could be identified. An example of the micro-patterned surface is show in **Fig. 3.1-11**.

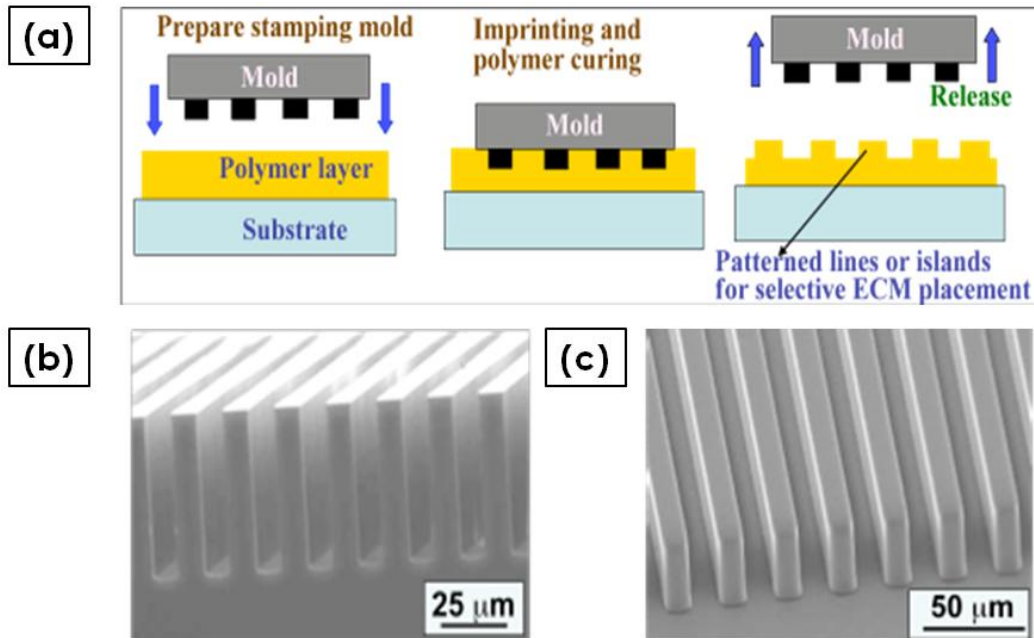


Figure 3.1-11. Si stamp (mold) with (a) schematic illustration of stamping operation (179-180). PDMS stamp with (b) 10 μm and (c) 12 μm lane width.

If the cell shape control is not sufficient to direct differentiation, active mechanical forces (181), such as cyclic stretch and shear stress, can be applied to the MSCs to increase cellular strain level and polarity. In addition, the traction forces generated by cells on different micropatterned surfaces to understand the underlying mechanisms by which cellular stress with different morphology governs cell differentiation could be investigated. The results generated from this can be used for scale-up culture conditions to organize stem cell structures *in vitro*, which in turn, control cell fate.

3.2 Si Nanopillar Arrays: Hydrophobic Nanopillars Initiate Mesenchymal Stem Cell Aggregation and Osteo-Differentiation

The ability to control MSC differentiation, using only the topographical factors, has a profound effect on both MSC biology and tissue engineering. This study aims to highlight the importance of the physical material carrier in stem cell based tissue engineering schemes. Surface engineering approaches that alter the physical topography of a substrate could be used as an effective tool and as an alternative to biochemical means of directing stem cell interactions and their subsequent differentiation. Although much has been studied on the effects of biological factors, such as growth factors, hormones, biochemicals, and vitamins, in the induced differentiation of mesenchymal stem cells (MSCs), the roles of the physical environment in the differentiation process are just starting to emerge. Analyses of biochemical characteristics of MSC differentiation do not necessarily translate to *in vivo* applicability and interactions with complementary or competing signaling pathways active under the physical physiologic conditions may be overlooked (182). Therefore, by examining the cellular behavior of MSCs cultured *in vitro* on various micro- and nanotopographies, it can provide some indication and understanding of the effects that the physical environment may have on the stem cell's response, and possibly how to regulate control of stem cell fates. A key principle in stem cell engineering is to reduce the use of potentially toxic, unpredictable biochemical inducing reagents or uncontrollable soluble factors by stimulating differentiation locally by use of the scaffold materials instead(183). In this section hydrophobic micro- vs. nanopillar

type fabrication techniques for probing MSC interaction with the surface physical environment is compared.

Various surface micro- and nanostructures have been used to create different effects in cells, including cell orientation, morphology, cytoskeletal arrangements, proliferation, gene expression, and most recently differentiation (68, 93, 121, 134, 168, 184-191). In fact, topography has been proven to have significant and favorable effects on stem cell commitment into mature lineages as described earlier in Section 3.1, and for a review see ref. (192). Most studies involve grooved, pore, or short (<100nm tall) pillar structures, but in this study micro- and nanoscale diameter pillars with a height of ~2.5 μ m were investigated. As well, a hydrophobic metallic surface, namely gold (Au) coated silicon substrates, seemingly different from the polymeric (188, 193-197), hydrophilic type surfaces investigated in the realm of MSC differentiation was chosen for this study.

Adipogenic, chondrogenic, and osteogenic differentiation, the three prominent MSC differentiation paths, were examined on the experimental surfaces. In this comparative report, flat vs. micro- vs. nanopillar configurations were studied and it was found that pillars with nanoscale diameters have greater impacts on MSC growth and lineage specific, osteogenic (in our case) differentiation. The aim was to explore the effects of not only the size of the topographic features, but also the hydrophobic physical nature of the Au surface on MSC culture.

3.2.1 Substrate Pillar Fabrication and Formation Mechanism

Micro- and nanoscale fabrication techniques are particularly suitable for probing stem cell interactions with their microenvironment because they allow for levels of precision compatible with the delicate regulatory control necessary to determine stem cell fates (198).

3.2.1.1 Micropillar Array Fabrication

A typical photo-lithography process for fabrication of the Si microtemplates is schematically illustrated in **Fig. 3.2-1**. The microtemplates were fabricated on (100) silicon wafers patterned with photo-lithography and a cryo reactive-ion-etch (RIE) process with a metal mask to achieve the desired finescale geometries as described below. A positive photoresist was spin coated on the silicon wafers (See **Fig. 3.2-1(a)**). Then it was exposed by UV to create a pattern of holes, which was performed by a Karl Suss MA6 Mask Aligner system (**Fig. 3.2-1(b)**). After exposure, the resist was developed (**Fig. 3.2-1(c)**). Ni thin metal layer (~50nm thickness) is sputter deposited on the whole surface of Si (**Fig. 3.2-1(d)**). Only the Ni layer in the "holes" have direct contact with Si substrate remain on the surface after a lift-off process (**Fig.3.2-1(e)**). A metal mask of 50 nm Ni was used to transfer this fine lithographic definition by cryo RIE steps (**Fig.3.2-1(e)**). After RIE process, the Ni layer was etched with Ni etchant (**Fig.3.2-1(f)**). Micropillars with ~2 μm diameter and 2.5 μm height were fabricated by photo lithography and RIE (**Fig.3.2-1(g)**). Finally, Ti 2 nm\Au 20 nm were tilted sputter deposited on the micropillars (**Fig.3.2-1(h)**). A thin titanium pre-coating was used as an adhesion layer between the gold layer and silicon substrate. The

morphology of the Au coated micropillar arrays were visualized using a FEI field emission scanning electron microscopy (SEM).

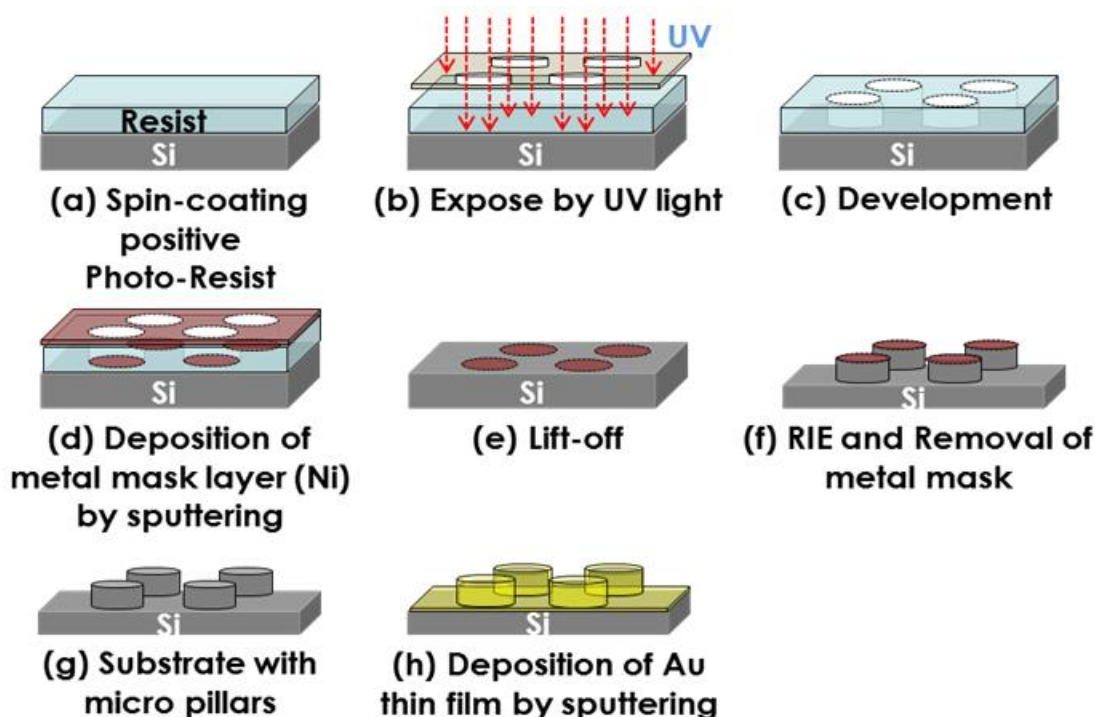


Figure 3.2-1. Schematic illustration of a typical photo-lithography process for fabrication of the micropillar surfaces used as one of the MSC experimental culture substrates.

3.2.1.2 Nanopillar Array Fabrication

First, silicon nanopillar arrays were prepared using the electroless chemical etching method (199-201). Briefly, the samples were immersed in a solution composed of 4.6M HF/0.02M AgNO₃ for 20 min. The etching procedure was performed at 50°C. After the etching process, the samples were immersed in H₂SO₄/H₂O (1:1) to remove the silver dendrites formed during the etching process. The samples were washed several times with distilled water and ethanol. During these steps, the samples were kept in the solution. The regular drying process for the wet etched nanopillar samples was performed using nitrogen gas

environment. To further describe the process of fabricating the nanopillar samples, **Fig. 3.2-2** illustrate the mechanism involved,

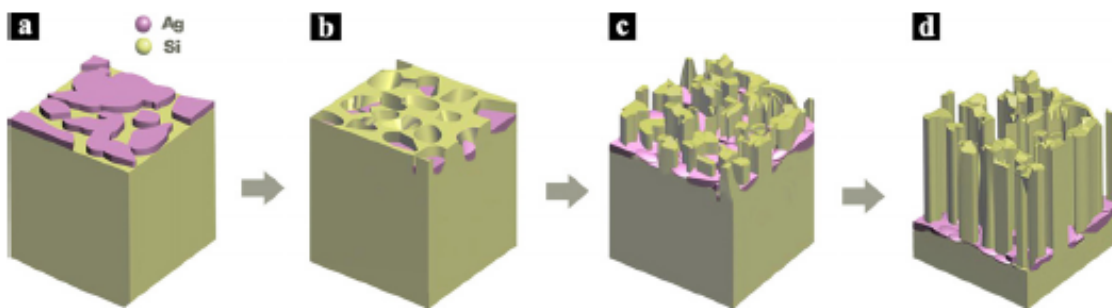


Figure 3.2-2. Schematic diagram of the possible formation process of SiNW arrays on an Ag dry-deposited Si substrate in $\text{H}_2\text{O}_2/\text{HF}$ solution (199).

- a) After thermal deposition on the silicon wafer most Ag clusters form islandlike networks on the silicon wafer.
- b) At the initial stage of etching, collective sinking of the net of Ag clusters would scratch the Si surface together with some big clusters breaking up into small clusters or particles, to give rise to the formation of porous silicon.
- c) The subsequent dissolution of the thin pit walls would result in the formation of freestanding silicon nanostructures.
- d) When the stable Ag nanoparticle network forms, interaction among clusters causes the whole net to collectively sink vertically downwards, and finally to fabricate SiNWarrays vertical to the substrate surface.

For the purposes of this study, a thin layer of gold (Au) was tilt sputtered on the surface the in the same manner as the micropillars. The morphology of the Au coated nanopillar arrays were visualized using a FEI field emission scanning electron microscopy (SEM).

A flat Si wafer surface with the same Au coating was used as a control surface without topographic features for the cell assays. All experimental samples (flat, micro-, and nanopillar) were cut into identical size pieces (0.5x0.5in²) and autoclaved before use.

3.2.2 Experimental

Contact Angle Measurement

The measurement of contact angle for the experimental surfaces was carried out by a video contact angle measurement system Model No. VSA 2500 XE (by AST Products, Inc.). Details are described in Section 2.2.2.

Mesenchymal Stem Cell Culture

Rat marrow stromal cells, or mesenchymal stem cells (MSCs), isolated from rat bone marrow (Cell Applications, Inc.) were used. The growth media consisted of alpha minimum essential medium (α -MEM; Invitrogen, USA), 10 % fetal bovine serum (FBS; Cellgro, Mediatech, Inc. USA) and 1 % penicillin-streptomycin (PS; Invitrogen, USA). Cells were cultured under a 37°C, 5 % CO₂ environment. The cells were seeded onto the experimental substrate of interest at a density of 5×10^4 cells/well of a 12-well polystyrene plate (Nunc) in 1ml growth media, and stored in an incubator until assayed at various time points. Cells were used at passage 4 for all experiments. For a control, the polystyrene culture dish (12-well plate) optimized for cell culture was utilized.

MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) Assay

After the selected incubation periods, the samples were measured for MTT as described in Section 2.2.2.

Scanning Electron Microscopy (SEM) for Cell Adhesion and Spreading Analysis

After a culture time of 24 hours, the cells on the substrates were fixed according to the methods described in Section 2.2.2

Immunofluorescence of Cytoskeletal Actin

After 24 hours of culture, the cells on the experimental surfaces were fixed and analyzed as described in Section 2.2.2

Assessment of Differentiation

For positive control cultures on polystyrene culture dishes (Nunc 12-wells), after 1 week of culture in regular MSC growth media, described above, the media was changed to lineage specific media to induce three different types of differentiation: adipogenesis, chondrogenesis, or osteogenesis. The induction media was changed every three days for the next two weeks. All cultures were maintained in 37°C, 5 % CO₂ environment.

Measurement of Adipogenesis: Adipogenic media was prepared by using DMEM (Invitrogen) supplemented with 10% FBS (Cellgro, Mediatech, Inc.), 1 mM dexamethasone (Sigma), 0.5mM 3-isobutyl-1-methylxanthine (Sigma), 1 mg/mL insulin (Sigma), and antibiotics (PS, Invitrogen) (202). The lipid accumulation representative of adipogenesis was stained using oil red O. The cultures were washed with PBS, fixed for 10min with 4% paraformaldehyde in PBS and stained for 1 hour using 3 vol. of a 0.5% stock solution of oil red O (Sigma) in isopropanol plus 2 vol. of distilled water. To quantify retention of oil red O, stained cultures were extracted with 1 ml of 4% Igepal CA-630 (Sigma) in isopropanol for 15 min, and absorbance was measured by spectrophotometry at 520nm (203).

Measurement of Chondrogenesis: Chondrogenic chemically defined inducing media was prepared by containing serum-free DMEM (Invitrogen), 50 mg/mL ascorbate (Sigma), 0.1 mM dexamethasone (Sigma), 40 mg/mL L-proline (Sigma), 100 mg/mL sodium pyruvate (Sigma), ITS-plus (Sigma), antibiotics (PS, Invitrogen), and 10 ng/mL recombinant human transforming growth factor- β 1 (TGF- β 1; R&D Systems, USA) (202). The degree of chondrogenesis was evaluated by staining with Alcian blue, which stains positive for cartilage matrix. Cultures were washed with PBS, fixed for 10min with 4% paraformaldehyde in PBS, stained with 0.5% alcian blue (8GX, Sigma, USA) in 0.1 N HCl overnight at 4°C, and rinsed with distilled water 3 times for 10 minutes each. To quantify the amount of sulfated glycosaminoglycans representative of the cartilage matrix, the alcian blue-stained cultures were extracted with 200 μ l 6 M guanidine•HCl (Sigma, USA) for 2 hours at room temperature (204). Optical density of the extracted dye was measured at 650nm by spectrophotometry.

Measurement of Osteogenesis: Finally, osteogenic inducing media was prepared by adding 10 nM dexamethasone (Sigma), 150 μ g/ml L-Ascorbic acid (Sigma), and 10 mM beta-glycerophosphate (Calbiochem) to cell growth media (205-206). The degree of osteogenesis was evaluated by staining cultures with alizarin red. To detect mineralization (calcium deposits), cells were fixed for 20minutes with 4% paraformaldehyde and stained overnight at 4°C with 2% (w/v) alizarin red S (Sigma, USA) solution in distilled water, with the pH adjusted to 4.1-4.3 using 0.5% ammonium hydroxide. Afterward, the samples were washed with distilled water with gentle rocking 3 times for 10 minutes each. To quantify the

amount of calcium deposition representative of an osteogenic matrix, methods have been previously published elsewhere(207) . Briefly, the dye stained monolayer is extracted by acetic acid and neutralization with ammonium hydroxide followed by colorimetric detection at 405 nm by spectrophotometry. For further confirmation of osteogenesis, after three weeks, the cultures were fixed and permeabilize as described above for cytoskeletal actin, then incubated for 1 hour at room temperature in 1% BSA/1X PBS followed by the addition of anti-osteopontin (OPN) antibody (1:100, AKm2A1, Santa Cruz Biotech. Inc.), and incubated overnight at 4 0C. After incubation, cells were washed three times for 5 minutes each wash with 1X wash buffer. Goat anti-mouse IgG-FITC (1:100, Santa Cruz Biotech., Inc.) in 1X PBS was added and the cells were incubated again for 1 hour at room temperature. The cells were washed three times with 1X wash buffer for 5 minutes each wash. The samples were then inverted onto cover-slips mounted, visualized and photographed using a green filter by an fluorecence microscope (DM IRB, Leica Microsystems Inc., Bannockburn, IL).

3.2.3 Results and Discussion

Scanning electron micrographs in **Fig.3.2-3** illustrate three surfaces and their topographic features used in the mesenchymal stem cell (MSC) studies. A gold (Au) coated flat silicon (Si) wafer was used as one type of control surface without topographic features. Micro- and nanopillar structures were fabricated by simple lithography (micropillar) and wet etching (nanopillar) techniques with subsequent Au deposition. The lithographic process flow chart for fabricating

micropillar surfaces is illustrated in **Fig.3.2-2**. The steps include a typical photolithography process, mask layer deposition, and subsequent REI removal. The electroless etching technique was utilized for obtaining oriented single crystalline nanopillar arrays using hydrofluoric acid (HF) solution containing silver nitrate (AgNO_3).

The Au layer was chosen as the surface chemistry for this work because it is a noble metal, biocompatible, and essentially bioinert with low solubility (208-209), and is often useful for bio-related research. Several biomaterial studies utilize the Au surface chemistry to tailor substrates for analyzing protein monolayer assembly, cell patterning, and cell attachment(210). By coating all surfaces with a thin conformal layer of Au, the surface chemistry is held constant among all experimental surfaces in order to eliminate the possible effect of chemical composition on the initial serum protein adhesion (which leads to different cell behaviors) and thus evaluate only the modification of the physical topography. This type of approach has become a trend in order to focus on topography vs. chemistry independently (211). Further studies on the synergetic effects of surface chemistry and topography would be valuable to optimize biomaterial surface interactions with proteins, cell response, and ultimately differentiation.

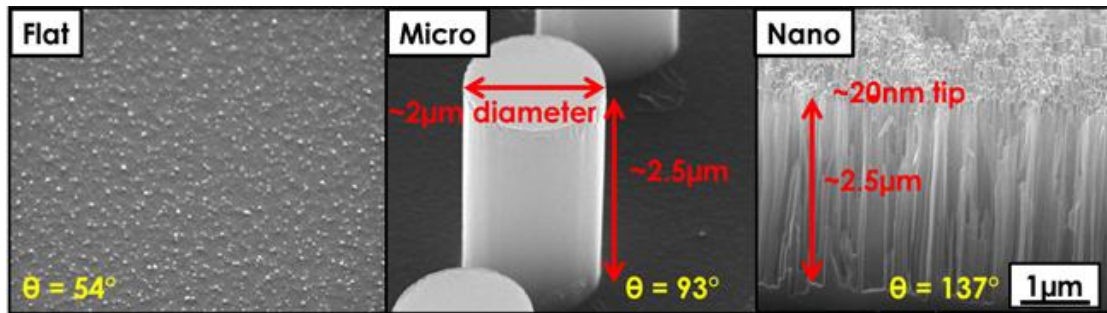


Figure 3.2-3. Scanning electron microscopy (SEM) images of the culture substrates depicting the gold (Au) coated flat, micropillar, and nanopillar silicon (Si) substrates. Shown in yellow is the contact angle for each surface indicating a significant increase in hydrophobicity of Au-coated surface from 54 to 137° with increasing pillar aspect ratio and density from the micropattern to the nanopattern.

Furthermore, both structures have a height $\sim 2.5\mu\text{m}$ but the diameters of the pillars are made significantly different, $\sim 2\mu\text{m}$ and 20nm for the micro- and nanopillars, respectively. To further describe the surface features, the micropillars have an aspect ratio (diameter: height) close to 1:1, but the nanopillars have an aspect ratio of about 1:100. While the spacing in-between pillars is proportional to the diameter of the pillars for both micro- and nano surfaces, the density of pillars is much greater on the nanotopographic surface and the surface area is certainly much greater on the nano surface by orders of magnitude as surmised from the relative ratio of the height to diameter (a factor of $\times 100$). In addition, the surface wettability measured by the contact angle of a water droplet was greatly affected by the surface topography. The contact angle increased with decreasing size scale going from 54° on the flat surface into the hydrophobic range of 93° on the micro- surface, and then to 134° on the nano surface. This increase in hydrophobicity can most likely be explained by the Wenzel

model(212) for the less hydrophobic case and the Cassie-Baxter model(213) for more hydrophobic case, which describes the wetting of textured surfaces.

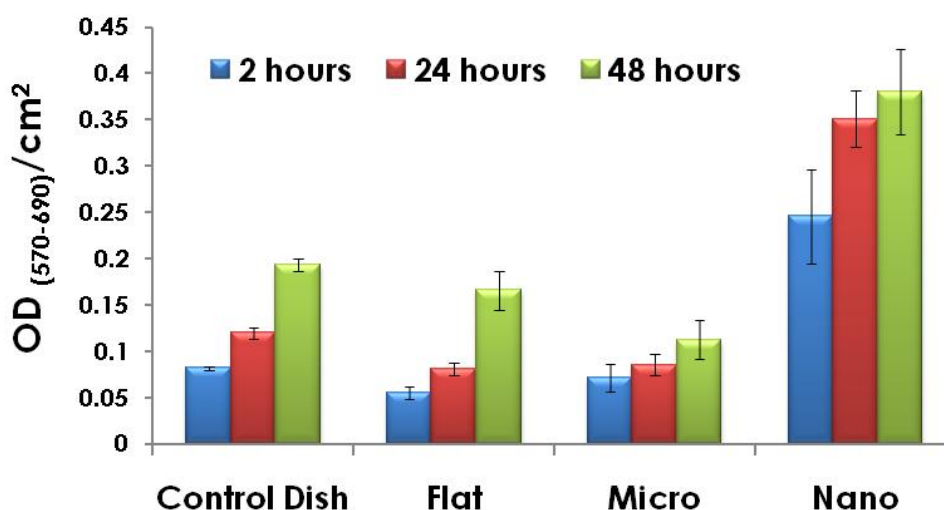


Figure 3.2-4. Cell viability test. MTT assay showing the optical density (OD) of reaction product of the MTT working solution with MSCs cultured on polystyrene control dishes, flat Au, micropillar Au, and nanopillar Au after 2, 24, and 48 h of incubation. The bar graph shows the average $\pm$ the standard error. The nanopillar surface significantly increased the viable cell density for each time point.

Fig.3.2-4 depicts the MTT test for MSCs cultured on control polystyrene tissue culture dishes (12-well plate), flat Au, micro-, and nanopillar surfaces as a function of incubation time. The MTT test is directly correlated to the number of viable, or living, cells on the surfaces. While the flat and micropillar surface showed similar cell adhesion and growth, the nanopillar surface had significantly greater cell adhesion at 2 hrs, as well as increased growth after 24 and 48 hours. In general, nanosized features have been proven to improve cell adhesion and growth over micrometer features (34-35). Although there has been contradictory

reports on cell adhesion and the hydrophobic nature of a surface (possibly due to different materials and topographic features), Bauer et al. recently reported improved adhesion and growth on hydrophobic nanotopographies with contact angles over 120°, which is in agreement with our study(214). In addition, the physical features of increased hydrophobicity and decreased feature size on the nanopillar surface may have an effect on the density and organization of adsorbed proteins that ultimately influence cell adhesion. It has been shown that hydrophobic Au surfaces increase the amount of adsorbed collagen (215). As well, as the diameter feature size decreases, from ~2µm to ~20nm (micro vs. nanopillars), the nanopillar dimensions become comparable to the typical size of globular proteins which may induce locally increased protein density (216-217) for enhanced adhesion. Our recent efforts in stem cell research have also shown that decreasing dimensions of nanotubular shaped topography (from 100nm to 30nm) had a major effect on the density of globular proteins, including serum albumin and fibronectin, adsorbed to the surface with increasing agglomerations on the smallest 30nm diameter nanotube size (134).

Not only growth but also spreading morphology was directly affected by the topography of the surface. As seen in the SEM and actin images (after 24 hours of incubation) represented in **Figs. 3.2-5 and Fig. 3.2-6(a)**, MSC spreading on the nanopillar surface was greatly reduced. When the spreading area was quantified (**Fig. 3.2-6(b)**), the micropillar surface did not seem to influence the cell spreading as drastically as the nanopillar. It seems that the cells grew over the micropillars and in between as if they were unaltered by the features. On

the nanopillar surface however, the cells seem to interplay more with the ~20nm tips of the nanopillar and have rearranged their shape based on the finescale cues of the pillar features. As well, the actin distribution is well pronounced and well organized with long fibers extending across the cell body on both flat and micropillar surfaces, however prominent actin fibers are not observed on the nanopillar surface. The nanopillars seemingly restrained the cell spreading. It is possible that the nanotopographic features inhibit some focal adhesion complexes and reduce spreading ability. This could be due to the curvature of the nanopillar tips as well as the interfacial energy which may be very different from a flat, or micro-sized surface(218). Furthermore, this difference in initial morphology on the nanopillar surface could play a significant role in the stem cell fate at later stages of culture as it has been shown before that initial cell morphologies on nanotopographic surfaces set the mode for upregulated differentiation because the cytoskeletal stress and resultant mechanotransductive pathways are affected by the change in morphology(134).

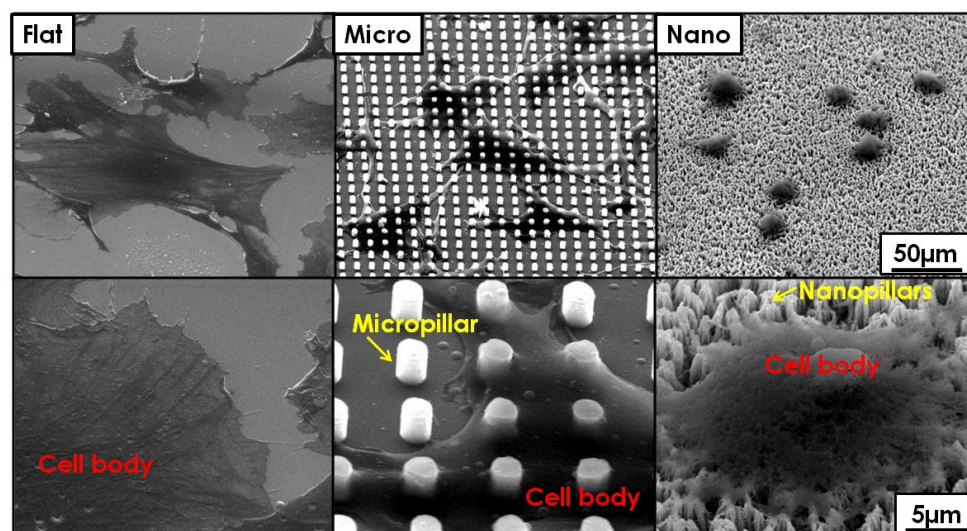


Figure 3.2-5. SEM micrographs of MSCs (which appear dark) on flat Au, micropillar and nanopillar surfaces. Two levels of magnification are illustrated by the top panel (low mag) and bottom panel (high mag).

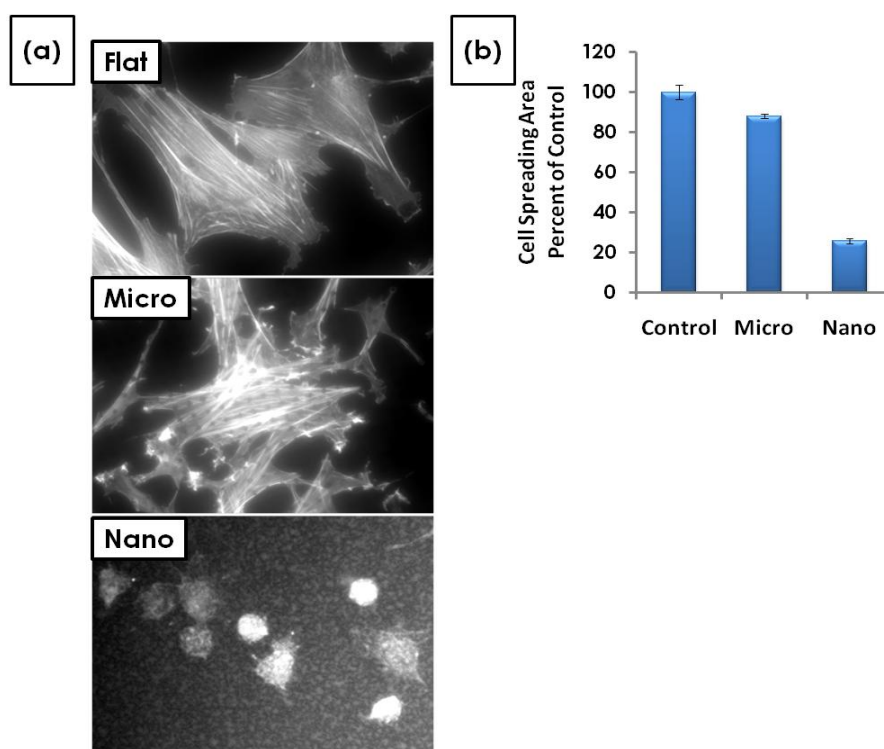


Figure 3.2-6. (a) Actin staining (white color) for MSCs on flat, micro- and nanopillar surfaces. **(b)** Quantitative cell spreading area for actin staining on the different surfaces. Clearly the cellular shapes, cell size, cell spreading, and cytoskeletal actin distribution is much different on the nanopillar surface.

In the initial stages (up to 48 hrs) of culture it appeared that the micropillar surface did not have much involvement in changing the growth behavior or morphological appearance of the MSCs compared to flat surfaces without topography, however the nanopillar surface made great impacts on all areas of cell behavior. At even longer periods of culture time (3 weeks or 21 days) when the cells on the experimental surfaces were observed, once again a topographically specific phenomenon was discovered on the nanopillar surface. The MSCs had formed large aggregates as illustrated by SEM in **Fig.3.2-7 (a)**. On contrary, cells on the flat and micro surface did not have this type of cellular arrangement (data not shown). In a study by Mao et al., it was reported that undifferentiated mesenchymal stem cells have long and thin cytoplasmic arms on both ends (219), similar to the cells seen in monolayer sheets on the flat and micropillar surfaces. Because the cells were morphologically different and formed aggregates on the nanopillar surface, it was hypothesized that cells were possibly differentiating based on these morphological changes, which were not apparent on the other surfaces. The aggregates were so distinct, they could even be noticed by the human eye and a photograph of the actual sample surface (after CO₂ critical point drying for SEM preparation) is shown in **Fig. 3.2-7(b)**, with the arrows indicating aggregates. The hydrophobic nature of the nanopillars may provide a local microenvironment that promotes the development of cell aggregation. It has been previously reported that hydrophobic surfaces encourage stem cell aggregation behavior as well as enhanced differentiation in the cell aggregates (220). Increased cell growth and

colony expansion are additional possibilities that could lead to cell aggregation, however further experimentation may be necessary to determine the exact mechanism of aggregation.

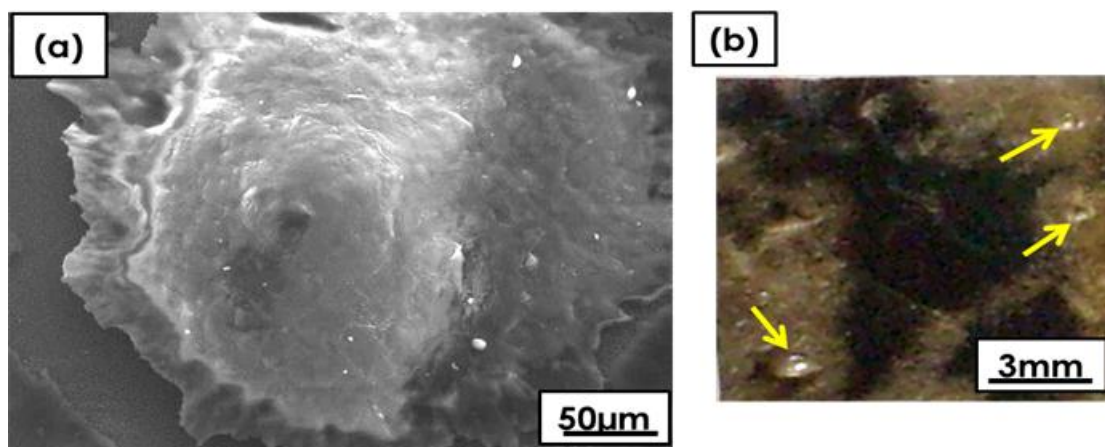


Figure 3.2-7. (a) SEM micrographs illustrating large cell aggregates present on the nanopillar surfaces after 3 weeks. **(b)** Photograph depicting the actual size of the cell aggregates present on the nanopillar surface.

To investigate and identify possible differentiation in the cell aggregates found on the surface of the nanopillars, the three potential lineages of adipogenesis, chondrogenesis, and osteogenesis were measured by quantifying the extractable amount of oil red O, alcian blue, and alizarin red, respectively. These three simple and convenient staining methods were chosen to assess relative differentiation of the three MSC lineages; oil red O detects the adipogenic fat deposition, alcian blue labels chondrogenic glycosaminoglycans, and alizarin red identifies the osteogenic mineralization in differentiating MSCs (221-222). Compared to control MCSs that were treated with positive biochemical inducing reagents, the aggregates (having no biochemical

inducing reagents) appeared to have dominant osteogenic differentiation (**Fig.3.2-8 (a)**), with significantly enhanced alizarin red quantification. To confirm osteogenic differentiation further, we evaluated the expression of osteoblast-specific protein osteopontin, **Fig.3.2-8(b)**. Intense positive staining was observed throughout the aggregates with more intense bone protein deposits within. Therefore, it seems that we have demonstrated that a nanostructure and physical environment itself can potentially guide the domination of osteo-differentiation, bone mineralization and protein deposition of mesenchymal stem cells without chemical inducing reagents.

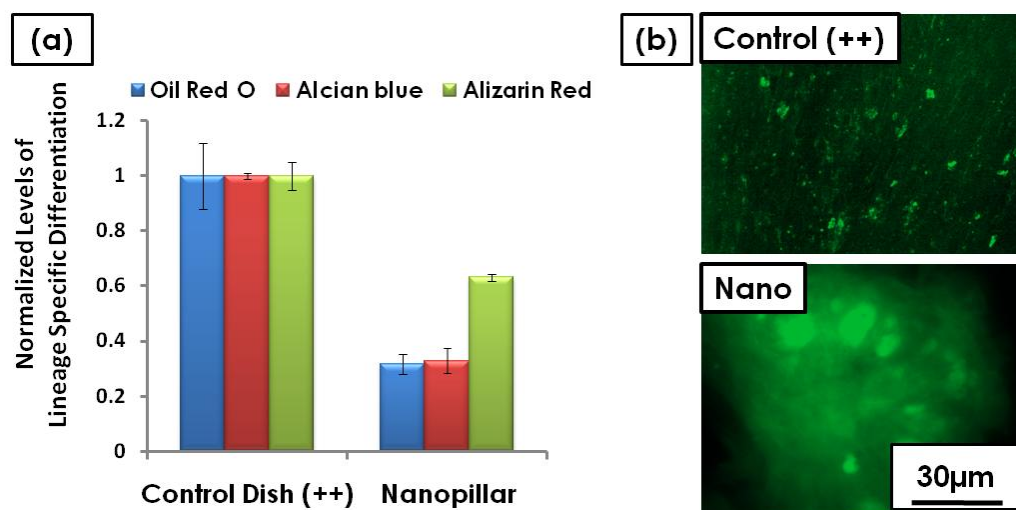


Figure 3.2-8. (a) Quantification of lineage specific matrix staining on positive control dishes (with induction media) and nanopillar surfaces after 3 weeks of culture. Oil Red O was used as an adipocyte matrix marker, Alcian blue for a chondrocyte marker, and alizarin red was used for osteoblast-like matrix marker. **(b)** Immunofluorescence staining for osteopontin, a bone matrix protein, for positive control cells and the cell aggregates on the nanopillar surface.

It can be hypothesized that the apparent cell aggregation on the hydrophobic nanopillar surface is responsible for the induced lineage specific osteogenic differentiation. These observations can possibly be explained based on the fact that the coalescing cells of this nature have major impacts from neighboring cells. Cell-cell interactions, in the form of morphogenic and mechanical factors, can be utilized to regulate the commitment and differentiation of stem cells(223). Tang et al. studied the extent of MSC osteo-differentiation based on the extent of cell-cell contact (224). It was determined that the extent of differentiation was fairly linearly related to the extent of contact characterized by coordination number, or neighboring cells. As the cell-cell contact increased, osteogenic differentiation also increased. The largest cell “microislands” had the highest relative differentiation. In our case, the cell aggregates created by the surface hydrophobicity and nanopillar structure allowed for much increased cell-cell contact. It has been suggested that the cells regard each other as a surrounding “material” (225) and therefore the mechanical cues, or stiffness of the material, from neighboring cells can be presented as a way of creating impacts that stimulate differentiation (172, 224, 226-227). It should also be noted that cultures undergoing osteogenic differentiation begin to form multilayered nodular structures (228), which the aggregates on the nanopillar surfaces closely resemble. It is possible that sub-populations in mesenchymal stem cell cultures could contribute to the apparent aggregation or “bone nodule” formation per se on the nanopillar surface, however under the same growth conditions, the aggregates were not observed

on flat or micro surfaces, indicating that the surface may be responsible for this phenomenon.

It is interesting that the initial cell morphology and lack of spreading (**Fig.3.2-6(b)**) does not appear to be typical for inducing osteogenic cell differentiation based on previous studies (170, 188), which claims that the opposite, increased spreading, induces osteogenesis. However, cell spreading was confined in this study. This may indicate that different signaling may be implicated for the osteogenic differentiation on our nanopillar surface. One possibility is the nanoscale disorder of the nanopillar array. The nanopillars are made by an etching technique which gives the structure some intrinsic nonuniformities, as revealed in the SEM images of **Fig. 3.2-3**. Dalby et al. (188) has shown that random circular nanostructures with varying degrees of disorder promote and direct osteogenic differentiation of osteo-progenitor cells, revealing that nanoscale disorder alone can induce differentiation. It was claimed that the disordered nanotopography induced changes in the adhesion formations and cell morphology which impacts on cytoskeletal tension and affects indirect mechanotransductive pathways. Future experimentation on the initial binding and adhesion formation events on the nanopillar surface would be helpful to evaluate the cell-substrate interaction and subsequent differentiation.

It was successfully demonstrated that cells are capable of growth, proliferation, and differentiating into the three lineages on the nanopillar surface, thus a new platform for stem cell technologies employing nanotechnology was discovered. It has been successfully attempted to utilize the physical nature and

nanotopographic cues already available in the hydrophobic nanopillar surface to probe the MSC response and regulate differentiation. Although all three lineages of adipogenic, chondrogenic, and osteogenic were observed, the osteogenic lineage was the most prevalent. The use of simple lithography and wet etching can be easily applied to various materials and directly used in experiments with stem cells as we have demonstrated. This study was designed to help revolutionize the understanding of the physical environment on stem cell fate. The findings in this work may contribute to the specific design of efficient biomaterials for stem cell regulation.

This study was conducted to probe the mesenchymal stem cell (MSC) response and differentiation using only the topographic features of the culture substrate by holding the chemistry of the surface constant. Micro- vs. nanosize diameters were investigated to determine if the size regime of the tall hydrophobic pillars utilized in this biomaterial surface examination effected growth and differentiation of MSCs. It was found that the nanopillar surface had significantly greater impacts with increased adhesion and growth. In addition, cell aggregation was induced on the nanopillar surface which enhanced the osteo-differentiation of the MSCs. Osteogenic mineralization measured by alizarin red staining was considerably up-regulated in cell aggregates occurring on the nanopillar substrates, as well as intense osteopontin staining, the osteoblast specific protein marker. Thus, a surface engineering approach that utilizes the physical topography of a substrate for use as an effective tool in regulating stem cell fate for advanced tissue engineering schemes was successfully attempted.

3.3 Conclusions

3.3.1 Considerations for Further Development

Influencing cell behavior, from proliferation to differentiation, using the material design of the substrate or implant topography is a desirable approach for many regenerative medicine applications(33). The main goal of the research in this Section 3 is to utilize the physical properties of nanostructured surfaces to establish a method for optimal control of MSC niches for control of differentiation fate, in order to develop a highly active surface, thus providing a solid and practical foundation of effective substrates for industrial cell culture schemes.

Additionally, many clinical conditions require regeneration or implantation of bone. MSCs represent a unique new approach for bone regeneration. MSC-based therapies (229) on bone implant materials have been explored. This work helps to enable the design of improved nano-surfaces for selective MSC osteogenesis, thus providing a novel platform for MSC based, advanced implants. There are large numbers of orthopedics patients in the United States that need improved methods for implant and prosthetic treatments that would reduce their morbidity and enhance their quality of life. Engineering new materials to conveniently incorporate stem cells into implants for accelerated repair of bone, and to enable new prostheses that integrate directly into bone is clinically desirable.

One objective is to develop a new generation of highly active orthopedic implant materials that significantly improve osseointegration using MSC therapy and transplantation. Some preliminary *in vivo* study with rabbits demonstrated

that implants with TiO₂ nanotube surface greatly improved bone bonding with significantly increased interface bond strength and tighter osseointegration with minimal trapping of soft tissue (230). It would be extremely beneficial to perform further experiments utilizing the TiO₂ nanotubes with precise geometrical dimensions in the presence or absence of MSC seeding, and perform comparative osseointegration studies in rat tibia to shed light on the *in vivo* clinical application of nanostructured surfaces and MSC therapy.

Another idea, such as for the purpose of developing surfaces for probing different differentiation pathways, one could systematically test different surface chemistries of nanopatterns, which will be further discussed in Section 4 in terms of TiO₂ nanotubes with different chemistries. By holding the underlying topography constant, chemical coatings could be applied nanometers thick in order to test different surface chemical properties on differentiation. For instance further develop should be made to exploring nanopillars with different chemistries. There have been several studies that have demonstrated the role of surface chemistry in combination of topography on osteogenic differentiation of cells, because these types of surface properties have a prominent effect on biomineralization and structure of the formed hydroxyapatites. It was determined that it was the integrin binding specificity that regulates biomaterial surface chemistry effects on cell differentiation, one reasoning was that certain chemical surfaces exhibit positive charges at pH 7.4 (231-232).

Biomaterial surface chemistry has profound consequences on cellular and host responses, but the underlying molecular mechanisms remain poorly

understood. In a brief study, when the Si nanopillars were coated with Au and other biocompatible materials, such as Ti and Zr, or oxidized to form a surface chemistry of SiO_2 , the cell attachment, cell spreading area, as well as differentiation was dramatically changed based on surface chemistry. **Fig. 3.3-1** illustrates the different cell morphology after 24 hours of incubation on Si nanopillars with different chemistries.

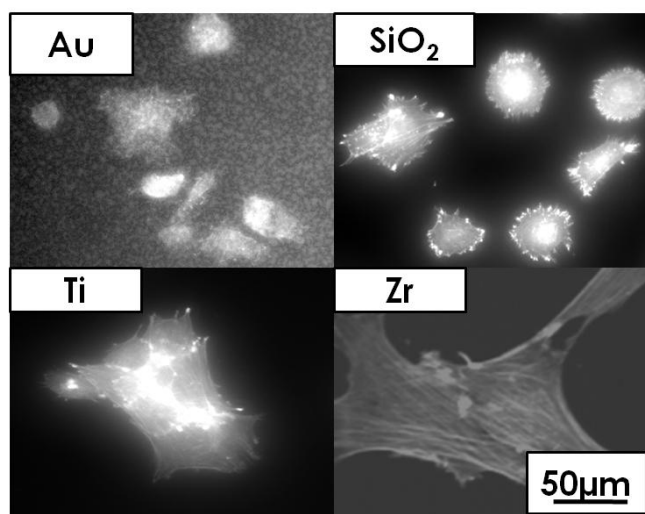


Figure 3.3-1. Cell morphological observations of MSCs on Si Nanopillar substrates with different surface chemistries, Au, SiO_2 , Ti and Zr. The images show the actin cytoskeletal organization (white color), which also reveals the spreading pattern of the cells on the different chemical nanostructures.

When nanopillar surfaces are designed with different surface chemistries the initial cell adhesion morphology becomes quite different depending on the chemistry. The nanopillars with different surface chemistries have distinguishable differences in the influence of cell shape. Au and SiO_2 nanopillars had similar influence on prohibiting the cell spreading area, where the Ti and Zr surfaces had much more pronounced spreading comparatively. While Ti and Zr are trans-

metals, and their isoelectric points are neutral at pH 7.4, this may have some implications on the behavior of cell spreading. Oppositely the surface of Au and SiO₂ may possess surface charges, as gold forms cations Au³⁺ and SiO₂ readily forms MO⁻ (M=metal) on the surface in solution. While this is only a speculation, it has been shown that surface charge has impacts on cell spreading (12-13, 231-232). These differences in cell shape and spreading may have implications on influencing cell behavior.

To investigate the osteogenic differentiation of cells on the nanopillars with different chemistries, immunofluorescent (IF) staining for a specific osteogenic protein expression, osteopontin, was utilized to test the differentiation after 3 weeks of culture. It appears in **Fig. 3.3-2** that MSCs have developed very different patterns on the different chemical surfaces. On Au and SiO₂ the cells appear aggregated, multi layered and coalescing. Oppositely, the cells have spread in a monolayer fashion on the Ti and Zr coated surfaces. Additionally, osteopontin staining intensity (green color in **Fig. 3.3-2**) was much greater for the aggregated cells on Au and SiO₂ nanopillars, while faint staining was shown on Ti and Zr.

While the mechanism for aggregation and intensity of osteo-differentiation on the Au and SiO₂ nanopillars is not discussed in this report, it seems that there was a correlation between the initial cell shape and local chemical environment that promotes the development of cell aggregation and osteo-differentiation. It would be beneficial to design further studies to elucidate the effects of surface chemistry on MSC differentiation.

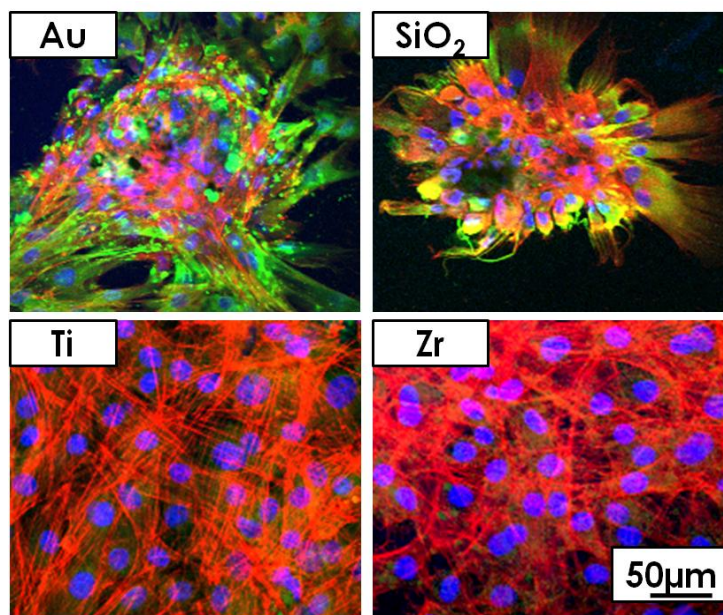


Figure 3.3-2. Immunofluorescent staining for actin (red), osteopontin (green), and DAPI nuclear stain (blue) after 3 weeks of incubation. MSCs on the Au and SiO₂ surface have formed large nodules highly expressing osteopontin, an extracellular structural protein and an organic component of bone.

Chapter 3, is in part a reprint of the material as it appears in Acta

Biomaterialia Volume 7 (2), 2011, Page 683 written by Karla S. Brammer, Chulmin

Choi, Christine J. Frandsen, Seunghan Oh, and Sungho Jin. The dissertation

author was the primary investigator and author of this work.

CHAPTER 4

4. The Effects of Nanostructure Chemistry on Cell Behavior

4.1 Comparative Cell Behavior on Carbon Coated TiO_2 Nanotube Surfaces for Osteoblasts vs. Osteo-progenitor Cells

In the previous sections it has been shown that the physical environment of nanotopography has positive effects on cell behavior, yet direct comparisons of nanotopographic surface chemistry has not been fully explored. Amorphous and crystalline carbon films deposited on metals have been studied as possible candidates for biomedical applications mainly because of their chemical inertness and the natural presence of this element in the human body (233-235). Carbon films, with a large range of properties, have shown a variety of desirable biomaterial properties including no toxicity toward living cells, no inflammatory response or loss of cell integrity, and no cellular damage, for review see ref. (233). Several groups have shown that carbon films are a convenient way of modifying materials that are sensitive to wear, such as Ti or Si, and it has been shown that many variations of thin film carbon coatings had much potential in implant coating applications (236-242), especially orthopaedic related implants. Most of the *in vitro* cell culture with osteoblast cells or osteo-progenitor, mesenchymal stem cells (MSC), research has varied either (i) the type of diamond materials (i.e. grain size or chemical composition) or (ii) the underlying topography of the substrate, holding the carbon chemistry constant. While studies show carbon is

beneficial in terms of orthopaedic considerations, direct comparison of the carbon (C) coatings vs. common ceramic oxide coatings (i.e. TiO_2), which is extensively used as orthopaedic implant material, has not yet been evaluated. Therefore this study was designed to directly compare TiO_2 and C chemistry for potential surface chemistries for advancing orthopaedic implant technology. In this section the concept of holding the geometric properties of the underlying nano-morphology constant, i.e. vertically aligned laterally spaced 100nm diameter nanotubes, while two surface chemistries, TiO_2 vs. C, were compared and evaluated *in vitro* using osteoblast cells and mesenchymal stem cells (MSCs) (the two main cell types involved in orthopaedic implant research) was implemented. Among several nanoscale factors, surface chemical properties, is one of the most important parameters that can influence the cell/tissue-materials interactions (157), thus directly affecting bone bonding after implantation. Here within we chose to evaluate the surface chemical properties for optimal bone growth and implant success. The results reveal that surface chemistry influences specific cell functions, such as alkaline phosphatase (ALP) activity in osteoblasts and protein expression in mesenchymal stem cells, and that different cell types may have different chemistry preferences for optimal function.

4.1.1 Experimental

Nanotube Substrate Fabrication

TiO_2 nanotube surfaces were created using a two-electrode setup, anodization process as described previously in Section 2.2.2. The Oxford EDX

attachment and Inca Software determine elemental make up and composition of the surface, where the carbon coated surface showed a clear carbon peak and there was an increased weight percent of elemental carbon on the surface after deposition. The TiO₂ and C coated nanotube samples (1.27×1.27 cm²) used for all experiments were sterilized by autoclaving before usage.

Contact Angle Measurement

The measurement of contact angle for the TiO₂ and C nanotube surfaces was carried out as described in Section 2.2.2. In this case, we also wanted to verify that the carbon (more hydrophobic in nature compared to TiO₂) was deposited and observe the change in surface energy after deposition.

Osteoblast Cell Culture

For these studies, MC3T3-E1 mouse osteoblast (CRL-2593, sub-clone 4, ATCC, USA) were used as described in Section 2.2.2. For long term culture, media was changed every 3 days.

Mesenchymal Stem Cell Culture

Human mesenchymal stem cells (hMSCs) were obtained from Lonza Corporation. The cell growth media were composed of α -MEM (Invitrogen), 10 % FCS (Invitrogen), 100 units/ml penicillin and 100 μ g/ml streptomycin (Invitrogen). Growth media was used on all experimental surfaces. For preparing positive control for PCR purposes, osteogenic inducing media were also prepared by adding 10 nM dexamethasone (Sigma), 150 μ g/ml L-Ascorbic acid (Sigma), and 10 mM beta-glycerophosphate (Calbiochem) to cell growth media (134, 205-

206). Culture protocol was identical to the osteoblast culture methods as described previously.

MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) Assay

MTT test was used to determine the amount of adhered viable cells and cell proliferation. After the selected incubation periods, methods described in Section 2.2.2 were followed accordingly.

Immunofluorescence of Cytoskeletal Actin

After 24 hours of culture, the cells on the experimental surfaces were fixed and analyzed as described in Section 2.2.2.

Scanning Electron Microscopy (SEM) for Cell and Substrate Observation

In order to observed high resolution cell features of cells grown on the nanotube surfaces, SEM was employed as described in Section 2.2.2.

Alkaline Phosphatase (ALP) Activity Test

To measure the bone forming ability of cells on the experimental surfaces, after the selected incubation periods, the samples analyzed as previously described in Section 2.2.2.

ALP Staining and Semi-Quantification of Osteoblast Cells

ALP staining kits (Sigma, USA) are intended for the semi-quantitative demonstration of alkaline phosphatase activity. For ALP staining, the cells grown on the experimental substrates and stained following the manufacturer's instructions. Briefly, the cells were incubated in a mixture of naphthol AS-MX phosphate alkaline solution with fast blue RR salt. The resulting blue, insoluble, granular dye deposit indicates sites of alkaline phosphatase activity. The samples

were then visualized (through a blue filter) and photographed using a fluorescence LEICA DM IRB microscope. In order to quantify the sites of ALP activity based on the positively stained area on the experimental surface, the microscopic fields were analyzed using Image J software, a public domain image processing and analysis program developed by the NIH.

Alizarin Red S Staining of MSC cultures

The degree of osteogenesis was evaluated by staining cultures with alizarin red after. To detect mineralization (calcium deposits), cells were fixed for 20 min with 4% paraformaldehyde and stained overnight at 4°C with 2% (w/v) alizarin red S (Sigma, USA) solution in distilled water, with the pH adjusted to 4.1–4.3 using 0.5% ammonium hydroxide. Afterward, the samples were washed with distilled water with gentle rocking three times for 10 min each. Then the samples were visualized and photographed using a red (actin) filter by a fluorescence LEICA DM IRB microscope. In order to quantify the alizarin red area on the experimental surface, the microscopic fields were analyzed using Image J software, a public domain image processing and analysis program developed by the NIH.

Real-time PCR

After 3 weeks of culture, total RNA of the cells on the TiO₂ and C coated nanotube substrates were extracted with Trizol (Sigma), and reverse-transcribed into cDNA by qScript™ cDNA Synthesis Kit (Quanta BioSciences) Real-time PCR was performed by Taqman® Gene Expression Assays (Applied Biosystems), and the information of Taqman® PCR primer is as follows; GAPDH (Hs99999905_m1,

Amplicon length : 122), OCN (Hs00609452_g1, Amplicon length : 74) and OPN (Hs00960942_m1, Amplicon length : 63). Real-time PCR were carried out using Taqman® Fast Universal PCR Master Mix and 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). cDNA samples (1 μ L for total volume of 20 μ L) were analyzed for gene of interest and for house-keeping gene GAPDH. The comparison test of cycle-threshold point was used to quantify the gene expression level of each sample. In this study, all levels of expression were normalized by the level of expression of positive control (hMSCs cultured with osteogenic inducing media).

4.1.2 Results and Discussion

The high quality biocompatibility and ideal implant properties of TiO₂ nanotubes have been repeatedly shown and discussed in this report, but the question has been raised to whether cell behavior is specific to the TiO₂ nanotube system or if cell behavior is universal to other nanotube materials. Therefore, in this section the idea of introducing a carbon coating on the nanotube surface for comparison to the TiO₂ surface chemistry is investigated.

Fig.4.1-1 reveals a schematic illustration and SEM images of the TiO₂ and C coated nanotubes used in this study. The SEM images show near identical structures, having a ~100nm outer diameter, ~10nm wall thickness, ~10nm spacing, and ~300nm height as previously described in Section 2.3 (53, 121, 185). The nanotube surface was conserved and it should be pointed out that the DC sputter system used for creating the C coating allows for a conformal layer with the advantage of controlling the C thickness as well as underlying substrate such

as other metals, alloys, glass, or polymer materials. The C layer is expected to have extremely high stability under biological fluids *in vivo* and *in vitro* as it was been reported to have a high mechanical strength and very good adhesion to Ti substrates (243). C coatings also protect metal implants against corrosion (244).

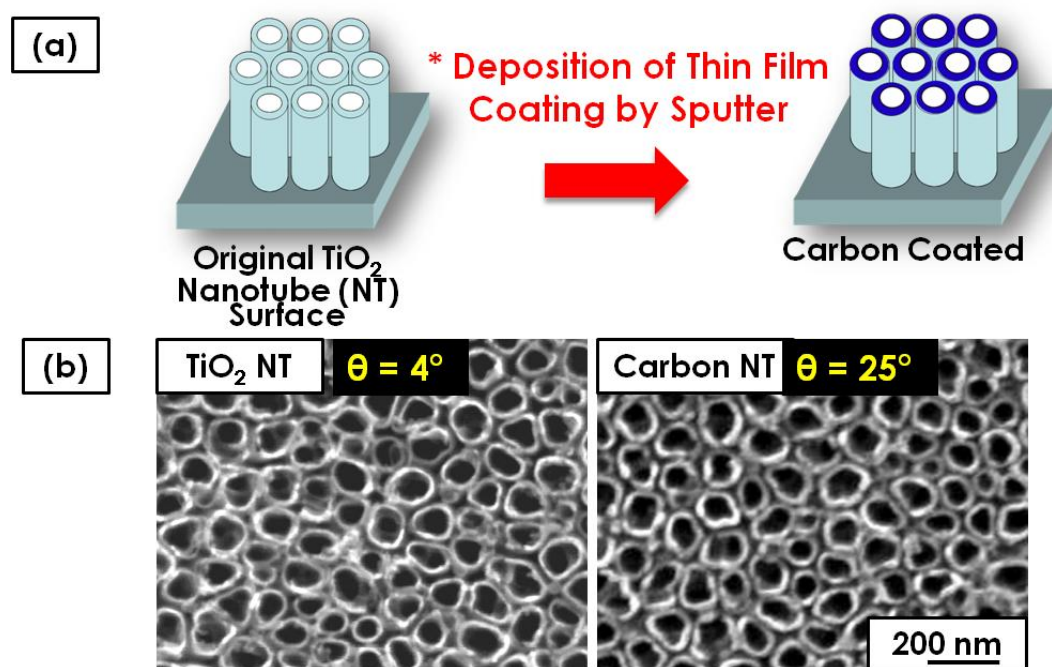


Figure 4.1-1. (a) Schematic illustration of nanotube surfaces with different surface chemistry. The original TiO₂ nanotube surface was sputter coated with a thin carbon (C) film. **(b)** Scanning electron microscopy (SEM) images of the nanotube substrates. The images depict preservation of the nanotube geometry and structure post carbon coating. Shown in yellow is the contact angle for each surface indicating a decrease in hydrophilicity on the C-coated surface from 4 to 25°.

The C coating induced a slight decrease in hydrophilicity from 4 to 25° on the TiO₂ and C coated surfaces, respectively. However, it has been reported that the cell-biomaterial culture studies of longer duration, as in our study (2-3 weeks), are often influenced by multiple factors, including surface chemistry, rather than solely surface wettability (238). The nanometer roughness may have also been

affected by the carbon deposition however, there has been reports that osteoblast and osteo-progenitor cells were not affected by the surface roughness of the surface (245).

From the results of the MTT test for estimating the number of viable osteoblast cells and the cell proliferation, the TiO_2 and C nanotube surfaces were practically the same, **Fig.4.1-2 (a)**. It can be hypothesized that the adhesion and proliferation of osteoblasts was not affected by the chemistry of the nanotubes. As well, the osteoblast morphology and cell organization seem to follow the same scheme on the TiO_2 and C nanotube surfaces as shown in **Fig.4.1-2(b)**. Crisscross actin cytoskeleton patterns (**Fig.4.1-2(b)**, arrows) were prominent regardless of surface chemistry. The mesh like actin structure (**Fig.4.1-2(b)** *upper panel*) and finger like filopodia extensions observed in the SEM images of **Fig. 4.1-2(b)** (*lower panel*) were consistent on both TiO_2 and C nanotube surfaces. These cytoskeletal arrangements are most likely influenced by the substrate structure (246), independent of the chemistry. This is in agreement with recent studies on diamond like carbon (DLC) coatings on μm grooved cpTi (239); regardless of chemistry, osteoblast cells had the same adhesion, proliferation and morphology on both DLC and cpTi grooved surfaces.

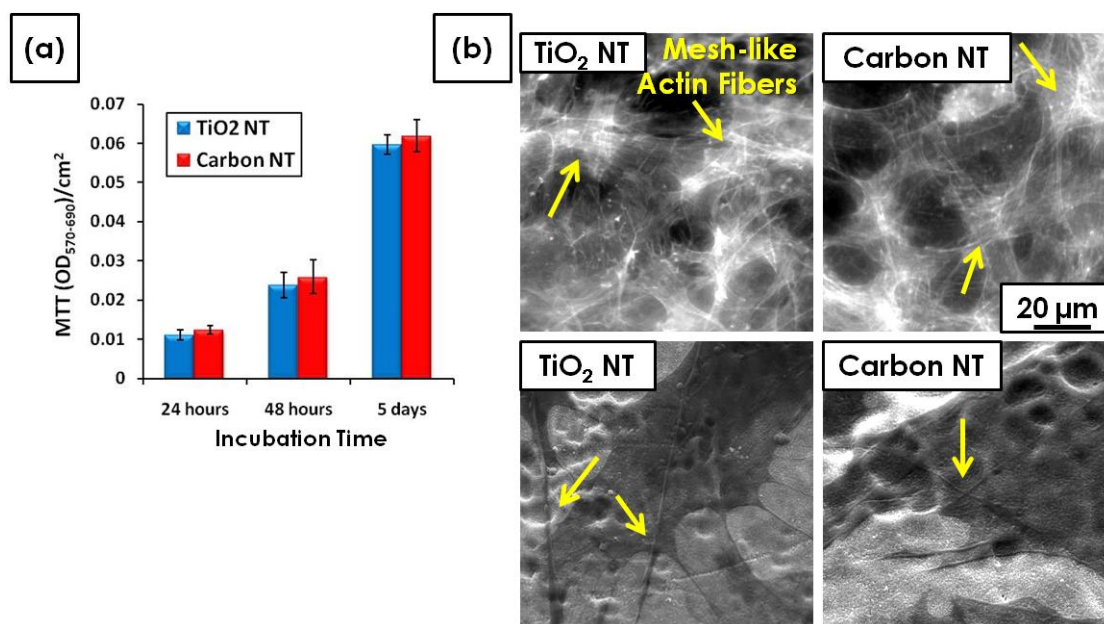


Figure 4.1-2. Osteoblast cell adhesion behavior, growth and morphologic observations. **(a)** MTT assay for cell viability and proliferation showing the optical density (OD) of reaction product of the MTT working solution (normalized by substrate area) with osteoblast cells cultured on nanotubular surfaces as a function of incubation time. The bar graph shows the average $\pm$ the standard error. **(b) Upper Panel:** Immunofluorescent images of cytoskeletal actin (white color) for osteoblasts on nanotube surfaces after 24 h of culture incubation. A criss-cross pattern of mesh-like actin (yellow arrows) is observed for both TiO₂ and C nanotube surface chemistries. **Lower Panel:** High magnification SEM micrographs of osteoblasts on nanotube surfaces after 24 h of incubation. Finger-like filopodia seem to extend across neighboring cells (yellow arrows).

Fig.4.1-3 illustrates the alkaline phosphatase activity, known as the bone forming ability, of the osteoblast cells on the different nanotube surfaces. The time dependent ALP line graph in **Fig.4.1-3(a)** shows that TiO₂ and C nanotube surfaces induced the same trend of ALP regulation as a function of time, but there was slightly higher ALP activity at 24hrs and significantly higher activity at 2 weeks on the TiO₂ surface. After 3 weeks of culture, the TiO₂ nanotubes had much more positively stained ALP present on the surface which was confirmed both visually (**Fig.4.1-3(b)**) and in the semi-quantification of several microscopic

views (**Fig.4.1-3(c)**). One possible explanation for this observation could be the difference of crystalline vs. amorphous character of the TiO_2 vs. C coating, respectively. The as-deposited C film is expected to be amorphous in nature, while more studies may be necessary for confirmation. Studies on the effect of crystallinity on cell behavior is somewhat contradictory, the evidence to date is that cell adhesion is independent of crystal phase of the material, as was observed in **Fig.4.1-2**, but that subsequent cell functions such as ALP and extracellular matrix secretion is dependent on crystal phase (247-249).

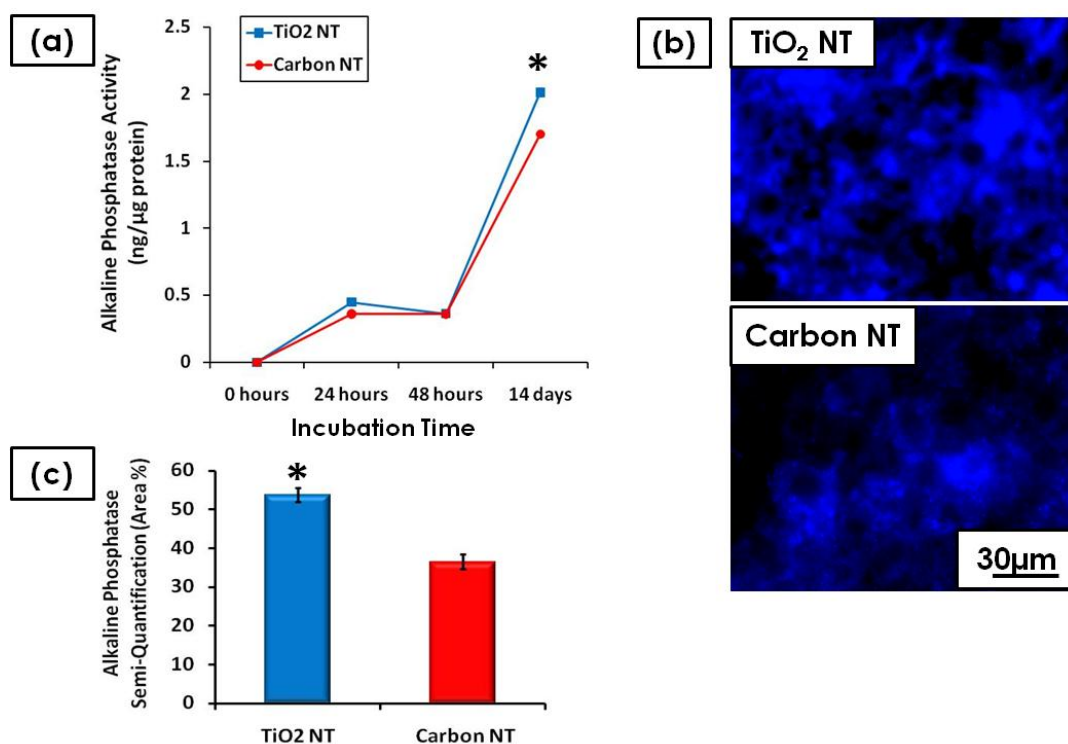


Figure 4.1-3. Alkaline phosphatase (ALP) activity of osteoblast cells cultured on the nanotube surfaces. **(a)** ALP activity as a function of time. **(b)** Fluorescent images showing ALP staining after 21 days. **(c)** Semi-quantification of ALP staining representing how much cell surface area was covered by ALP activity based on microscopic fields (N=4). The bar graph shows the average $\pm$ the standard error. P values after performing t-tests reaching statistical significance $P < 0.05$ are marked on the graphs.

Both TiO₂ and carbon coated nanotubes enhanced the osteoblast growth and function over flat controls of smooth Ti and carbon coated smooth Ti (data not shown), however TiO₂ nanotubes have a slight advantage over C coated nanotubes in terms of ALP activity. Therefore it can be hypothesized that it is the nanotopographic structure that affects osteoblast behavior in terms of adhesion and morphology, but chemistry/material properties may play a role in functionality. It should be noted that titanium, with its natural oxide surface TiO₂, is the oldest and most popular orthopedic implant material because of its superior biocompatibility and osseointegrating ability. In the case of Ti base biomaterials, the high bone-binding ability has been attributed to a spontaneous modification of the passive TiO₂ surface by calcium and phosphate ions upon exposure to the biological environment (250-251). In addition, electrostatic attraction of Ca²⁺ ions on the TiO₂ surface and subsequent adsorption of Ca-dependent proteins has been shown (252-253). The isoelectric point of the TiO₂ ceramic is close to the physiological pH.

Since most bone implant materials are implanted into adult bone in direct contact with bone marrow tissue, it is advantageous to use bone marrow cells, also known as mesenchymal stem cells to investigate new implant materials. Bauer et al. reported on AuPd and ZrO₂ nanotube surface effects on mesenchymal stem cell behavior(133). While a similar trend of cell behavior, including cell adhesion and proliferation was observed, based on changing the AuPd and ZrO₂ nanotube diameter size, the osteogenic functionality or directed differentiation was not addressed. This section sheds light on optimizing the

nanotube surface material by directly comparing the osteogenic function of mesenchymal stem cells on orthopedic implant related TiO₂ vs. C surface chemistries, holding the diameter and geometry constant, since nanotube size effect has already been studied in our lab (134).

The degree of osteogenesis was evaluated by alizarin red, a simple and convenient staining method for detecting calcium mineral deposition for assaying relative osteogenic differentiation. **Fig.4.1- 4** shows the alizarin red staining and semi-quantification for mesenchymal stem cells (MSCs) cultured on the experimental surfaces after 3 weeks of incubation. The C nanotube surface appears to have induced a greater deposition of bone like mineral, but it was not significantly different. In **Fig.4.1-5**, ALP activity and osteocalcin and osteopontin bone matrix protein expression was up-regulated on the C coated nanotube surface, showing significant differences in terms of ALP and osteopontin expression. The ALP activity and PCR experiments were used as additional markers of bone formation, the relative osteogenesis of the MSCs. Osteocalcin (OCN) and osteopontin (OPN) are two major noncollagenous protein component of bone extracellular matrix, synthesized and secreted exclusively by osteoblastic cells, and is considered indicator of osteogenic differentiation. In **Fig. 4.1-5** it appears that the C nanotubes induced a significant increase in relative osteogenic differentiation over the TiO₂ nanotubes. These results reveal that cells can discriminate between remarkably different surface chemistries and crystallinity as other research groups have also observed, for review see ref. (254).

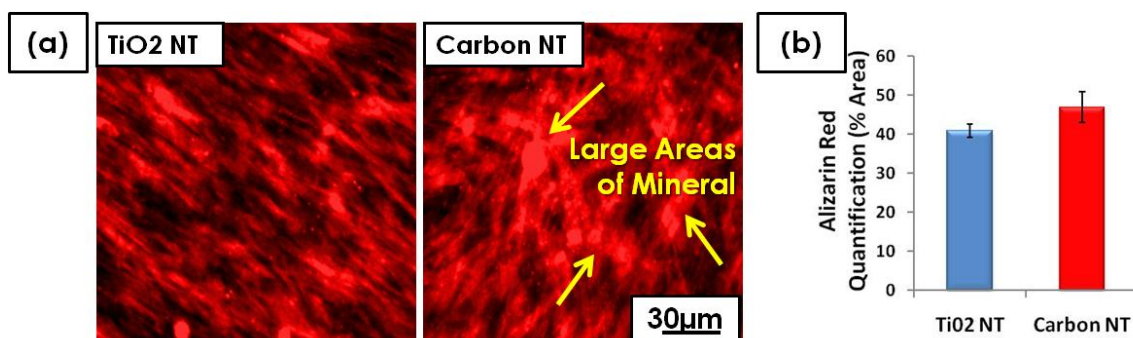


Figure 4.1-4. Alizarin red staining for mineral deposition of hMSCs cultured for 3 weeks. **(a)** Fluorescent images showing alizarin red staining (bright red) for the different experimental surfaces. **(b)** Semi-quantification of alizarin red representing how much surface area was covered by bone like mineral matrix based on microscopic fields (N=4). The bar graph shows the average $\pm$ the standard error.

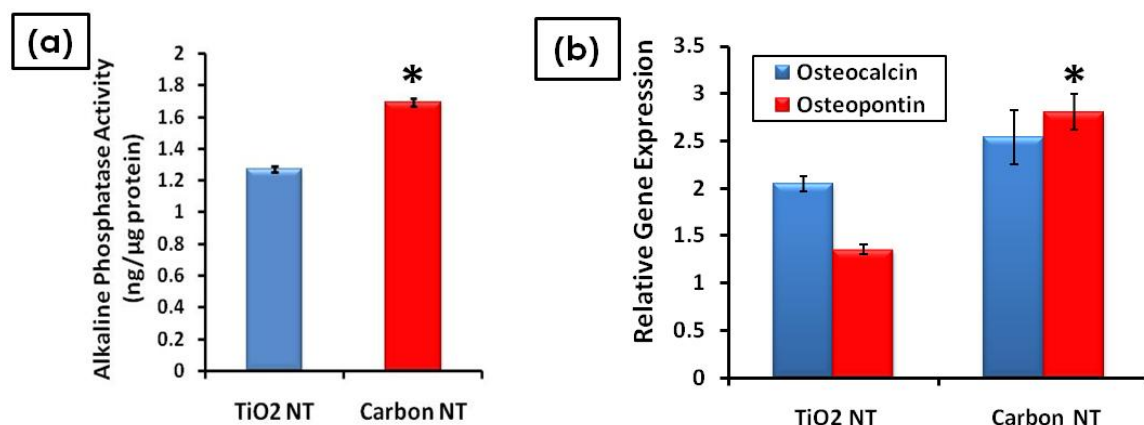


Figure 4.1-5. Degree of osteoblastic differentiation and maturation in late stage mesenchymal stem cell (MSC) culture (3 weeks). **(a)** ALP activity for MSCs cultured on the nanotube surfaces, TiO₂ vs. Carbon chemistry. **(b)** Quantitative PCR analysis for osteocalcin and osteopontin. The graph shows the average $\pm$ standard error bars. P values after performing t-tests reaching statistical significance $P < 0.005$ are marked on the graphs.

The natural environment of the cell has complex chemical and topographical cues. Mesenchymal stem cells are present in bone marrow as

highly sensitive cells, unprogrammed, undifferentiated and readily influenced by environmental factors. Osteoblasts on the other hand are mature adult cells, specific to bone tissue. It seems that the different cell types have a different chemical preference for optimal cell function. The osteoblast cells had higher ALP activity on the TiO_2 surface, while the MSCs had the highest osteogenic differentiation on the C surface. It could be speculated that the MSCs prefer a C surface while in programming mode, but once matured in the bone tissue as an osteoblast cell, the preference changes to TiO_2 , possibly due to the cells' natural environmental differences in the body. MSCs are in bone marrow, which contains many organic carbon rich components. The bone tissue is made up of a more alkaline environment with higher ceramic/mineral rich components, similar to TiO_2 .

This study was conducted to aid in the understanding of surface chemistry effects on orthopedic implants. Biomedical devices and implant materials have defined features, thus introducing new research on different types of surface features would be helpful for design rationale.

4.2 Conclusions

The effects of TiO_2 vs. carbon coated nanotube surface chemistries on osteoblast and osteo-progenitor cell behavior was compared. It was revealed that the chemistry of the nanotube surface had a direct effect on the functionality of the cells and that different cell types had different chemical preferences for optimal functioning. These results show the potential for the use of experimenting with different surface materials with different chemistries as a

means of influencing cell behavior and lineage commitment for advancing biomedical implant surfaces.

4.2.1 Considerations for Further Development

An interesting topic of consideration would be to investigate the use of TiO₂-C composite surface coatings effects, since individual chemistries have their own advantages as discussed in this work, and a composite surface may have additional benefits. The use of plasma source ion implantation- ion beam enhanced deposition (255) could be a valuable method for creating TiO₂-C composites with varying degrees of compositions to further optimize or tailor to specific cell types and applications.

As well, investigating other metal coatings, such as tantalum, which are well known orthopedic implant materials would be interesting to investigate with the nanotube geometry. Recently tantalum is gaining more attention as a new metallic biomaterial as it has been shown to be bioactive and biologically bonds to bone (256). However, the relatively high cost of manufacture and an inability to produce a modular all Ta implant has limited its widespread acceptance (257). Ta coatings have been successfully deposited on Ti using laser engineered net shaping (LENS™) to enhance the osseointegration properties (258). It was shown that the enhanced cell attachment and proliferation on the Ta surface were a direct consequence of its high wettability and surface energy. Such beneficial properties of surface chemistry can be utilized for its potential for enhanced/early biological fixation.

Chapter 4, is a reprint of the material as it appears in Acta Biomaterialia published online Mar. 2011 (DOI:10.1016/j.actbio.2011.02.039) written by Karla S. Brammer, Chulmin Choi, Christine J. Frandsen, Seunghan Oh, Gary W. Johnston, and Sungho Jin. The dissertation author was the primary investigator and author of this work.

CHAPTER 5

5. Nanostructured Templates for Drug Delivery

The lack of proper control over a drug release rate and target delivery area is a huge disadvantage for conventional drug tablets. Tablets tend to provide rapid and immediate release of therapeutic agents and require more frequent and repeated dosages for maintaining therapeutic levels, causing unwanted fluctuations in drug amounts delivered to the blood and tissue(259). In order to circumvent problems in drug adsorption, metabolism, and irregular concentrations and to optimize the therapy itself, a controlled release dosage is advantageous over conventional tablets(260). Biomaterials with nano-scale features have become increasingly popular as controlled release reservoirs for drug delivery. Using nanoporous structures, for example, for controlled release reservoirs of drugs is advantageous over drug tablets/injection because nanostructures are on the same nanoscale as the drug molecule and a steady state release controlled by geometry of the nano-system can be achieved.

5.1 Concepts and Advantages of Nanoscale Drug Delivery

Providing a controlled and localized release of therapeutics within the body are key objectives for increasing efficacy and reducing the risks of potential side effects of drug tablets/injections. Nanotechnology can help because there is complete control over morphology, dimension, and interfacial

properties of the nanostructures. **Fig. 5.1-1** depicts a graph schematic of the difference between daily tablets vs. nonporous implant drug release, revealing the advantage of nanostructured carriers. Nanoscale drug delivery systems can be devised to:

- Tune release kinetics
- Regulate biodistribution
- Adjust bioavailability over time
- Minimize toxic side effects
- Enhance the therapeutic index of a given drug

Nanostructures could lead to smaller, smarter and more-interactive implants in the human body.

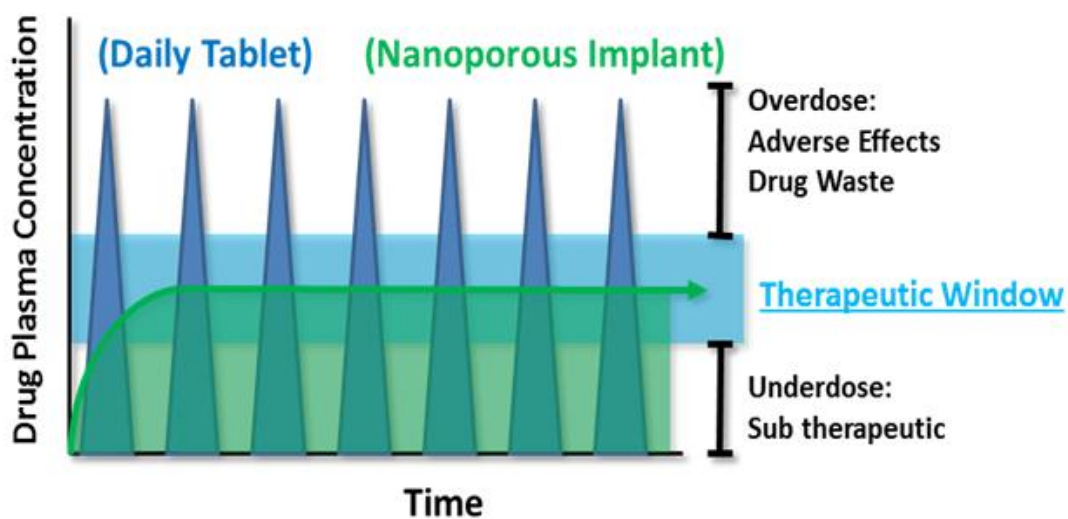


Figure 5.1-1. Schematic drawing illustrating drug concentration vs. tablet (blue spikes) and nanostructured implants (solid green).

5.2 Anti-Biofouling, Sustained Antibiotic Release by Si Nanowire Templates

Localization, controlled release, and sustainability of drugs over long periods of time within the body are key challenges in the design of effective drug therapies. Various nanostructured material systems have been developed to meet these challenges. Porous Si templates in particular hold several ideal properties that have been found to be remarkably beneficial (261-266).

Biocompatible porous Si is an attractive material for controlled drug delivery applications for many reasons including the versatility and capability of tailoring the pore sizes (from microns to nanometers) and volume of the reservoir, there are numerous existing and convenient chemistries for surface modifications and surface reactions, it can be used as a template for designing polymeric nanostructures, it has unique optical properties and exceptional biosensing potential(261).

Here a drug release behavior from a quite different nanostructured porous Si template, namely Si nanowire (SiNW) arrays with a high porosity and surface reactivity, and a relatively large reservoir volume is reported. These structural features have spurred our interest in the use of this system as a host or “mothership” for the controlled release of drugs. The electroless etching (EE) approach is used to obtain oriented single crystalline SiNW arrays on silicon substrates which has proven a simple and efficient method to prepare large area samples (199-201) that we have utilized in this study as an antibiotic type drug carrier.

This *in vitro* experiment was undertaken to determine the release profile of common antibiotics, penicillin/streptomycin (P/S), from SiNW arrays. While sustained drug release from SiNW was the primary aim of this study, we have also observed biodegradability of SiNWs as well as anti-biofouling characteristics of such SiNWs with strikingly suppressed cell and protein adhesion on SiNW surface, which indicates a reduced probability for biofouling and drug release impediments.

5.2.1 Experimental

Silicon Nanowire (SiNW) Array Fabrication

SiNW arrays were prepared using the electroless chemical etching method described in Section 3.3.1. For the purposes of this study, a bare silicon wafer surface was used as a control surface for all drug and cell assays. All samples were cut into identical size pieces (0.5x0.5in²) and autoclaved before use.

Drug Loading, Release, and Collection

Insertion of liquid into nanostructures is not always easy as the surface tension of the liquid has to be overcome. At room temperature (25°C), the SiNW samples were placed in a vacuum ($\sim 10^{-4}$ torr) for approximately 5-10 minutes to rid nanopores (between Si nanowires) of any trapped air. Remaining under vacuum, by opening of a control chamber valve, an antibiotic solution was loaded into the samples. The high concentration antibiotic solution loaded was 1ml of combination antibiotics consisting of 100,000 units/ml penicillin + 100,000 µg/ml streptomycin (P/S) (from Invitrogen), which is primarily used to prevent

bacteria in animal cell cultures. The samples were incubated overnight under vacuum to allow sufficient time for the drug to fully penetrate into the SiNW pore structure. The drug-loaded SiNW samples were washed 3X with ice cold (to restrict diffusion from the reservoir) phosphate buffered saline (PBS) pH 7.4 (Invitrogen). Next, the samples were individually placed in new wells (Nunc, 12-wells) incubated in a humidified 95% air/5% v/v CO₂ incubator at 37°C in 1ml fresh simulated body fluid (PBS was used in this study) for one day. The solution was collected at daily time points (days 1-7) and weekly time points thereafter (up to day 42 or 6 weeks) and 1ml fresh PBS was added. Drug concentration was determined by measuring the collected solution with a commercial BCA Protein Assay Kit (Pierce) and ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA U.S.A.). Briefly, 25µl of sample or standard replicate was pipetted into a microplate 96 well (Nunc), 200µl of BCA working reagent was added to each well, the plate was mixed on a plate shaker for 30 seconds, incubated at 37°C for 30 minutes and the solution was read at 560nm using the ThermoMax microplate reader. The assay was calibrated by use of reagent blanks and standards containing 20-2000µg total antibiotics (P/S solution (Invitrogen)). Each sample was read in triplicate. Four samples for each time point were measured and the average values $\pm$ standard deviation was graphed to obtain release profiles.

Cell Culture

For this study, MC3T3-E1 mouse osteoblast (CRL-2593, sub-clone 4, ATCC, USA) were used and methods are described in Section 2.2.2.

Scanning electron microscopy (SEM) for cell adhesion and spreading analysis

After 2 and 24 hours of culture, the cells on the substrates were fixed and analyzed according to methods described in Section 2.3.3. In order to quantify the amount of adhered cells and spreading area on experimental specimen SEM images at 2 and 24 hours after plating, the cells were analyzed in Image J software, a public domain image processing and analysis program developed by the NIH.

5.2.2 Results and Discussion

The SiNWs are generally aligned vertically parallel to one another as shown in **Fig.5.2-1(a)**. Although many studies have been conducted based on porous Si and drug delivery, this is the first study with this novel nanowire structure. Not only are etched pores present, the interconnectivity between the nanowire bundles allows for increased surface area and drug loading capacity. This unique nanostructure has increased relative porosity with a more readily reactive surface due to the etching process. It is known that reactive hydride species are left on the surface after fabrication which are available for various reactions and determines the dissolution rate in aqueous media(261). In this drug delivery system, the antibiotics most likely adsorb and chemically bond to the surface. Especially, it has been observed that sulfate SO_4^{2-} ions, streptomycin sulfate for instance used in this study, chemically adsorb onto the surface of Si through hydrogen bonds(261). One advantage to this system (and porous Si in general) is that this type of surface nanostructure has potential to concentrate the drug into the pores for increased loading capability. The contact angle of water droplet

on SiNW was measured and it was found that the SiNW surface is super-hydrophilic, with the contact angle being essentially 0° (the measurement of contact angle was carried out by a video contact angle measurement system Model No. VSA 2500 XE (by AST Products, Inc.). When a water droplet is added to the SiNW surface it is completely instantaneously absorbed in-between the nanowire pores. This surface allows for complete and sufficient wetting of the penicillin-streptomycin (P/S) solution for possibly easier and enhanced drug loading capabilities as well (**Fig. 5.2-1(b)**).

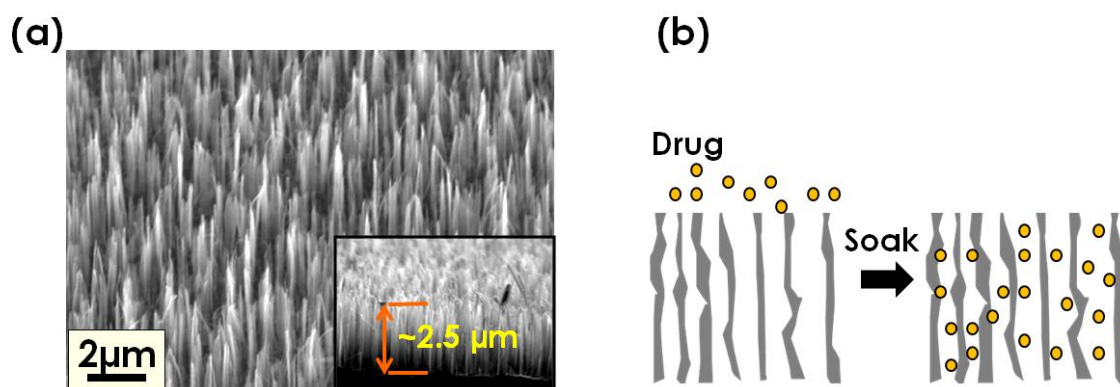


Figure 5.2-1. (a) SEM micrograph depicting the vertically aligned Si nanowire (SiNW) morphology (45° tilt) with cross-section shown in the inset. **(b)** Schematic of SiNW drug loading.

An antibiotic release profile from the SiNW filled with the (P/S) solution was obtained over 42 days, illustrated in **Fig. 5.2-2**. Bare Si wafer controls, without a nanostructured surface topography, showed almost zero antibiotic release as expected. This proved that it was the nanostructure reservoir and surface properties of the SiNW templates that were responsible for the sustained drug release. Note the wave-like aspect of the release in **Fig. 5.2-2** which describes the

total amount of antibiotic measured per time point. This shape of release profile is similar to another study using the antibiotic gentamicin (also in the aminoglycoside antibiotic family as streptomycin) release from porous implant materials(267). Nonetheless, a near steady drug administration is achieved over 42 days with an average release rate over all the time points was $\sim 219 \mu\text{g}$. The ideal release profile for most drugs would follow this type of a steady release rate so that the drug levels in the body remain constant while the drug is being administered (268).

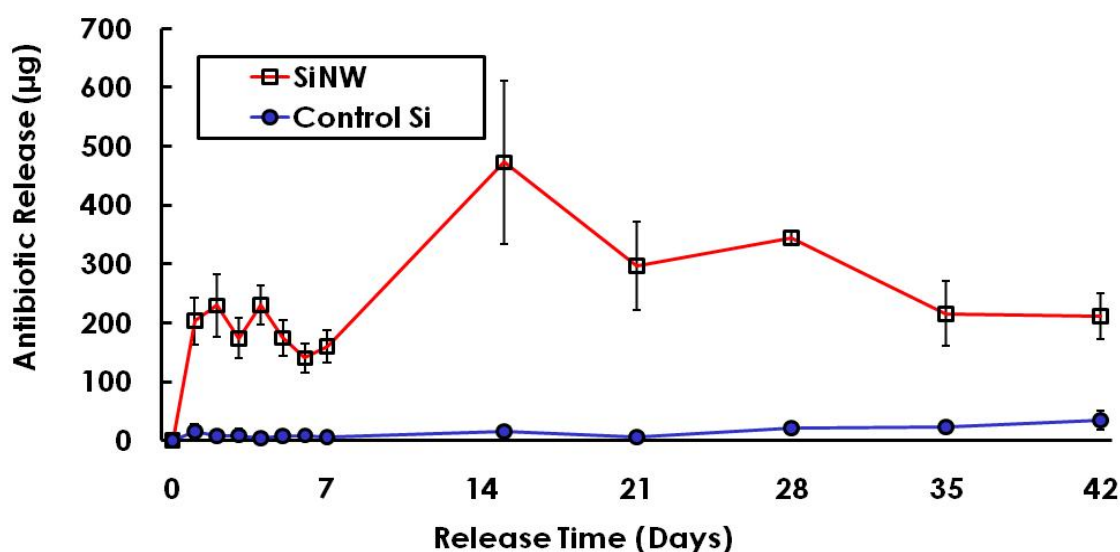


Figure 5.2-2. Absolute release of antibiotic measured as a function of sampling time. Mean $\pm$ standard deviation is given ($n = 4$). A near constant release is shown over 42 days (6 weeks) and the average amount of antibiotics released for all time points was approximately $219 \mu\text{g}$ indicated by the dotted line.

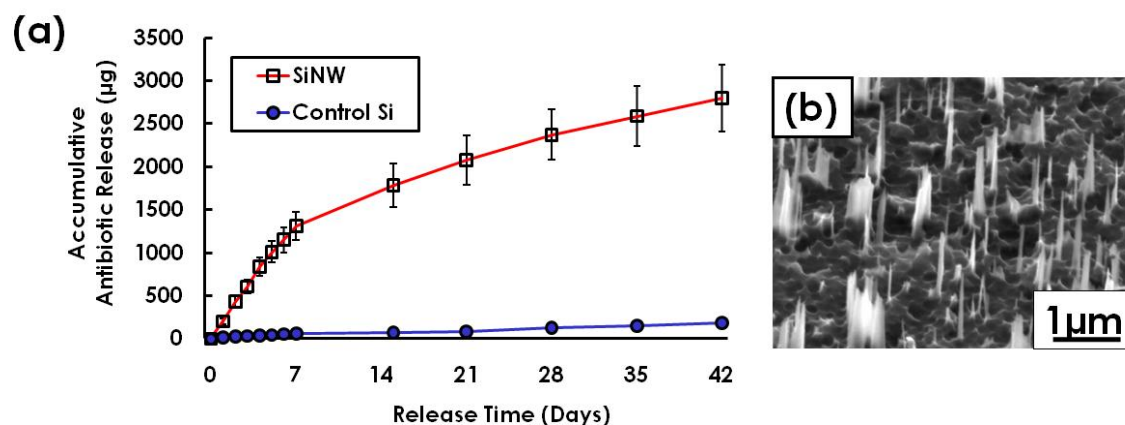


Figure 5.2-3. (a) Accumulative release amounts of antibiotics as a function of time showing prolonged release and increased bio-availability from SiNW arrays which are still releasing antibiotics up to day 42. Mean $\pm$ standard deviation bars is given ($n = 4$). **(b)** SEM micrograph from a portion of the sample surface depicting the SiNW erosion after 42 days in simulated body fluid.

Fig. 5.2-3(a) illustrates the accumulative release curve measuring up to 3mg of antibiotic over 42 days showing near zero-order kinetics over time. The drug is most likely being released as the supporting porous Si matrix is being slowly degraded. Not only is Si biocompatible, but in a biological context it is also bioresorbable. In a recent article it was pointed out that the low toxicity, degradation properties, and solubility of the degradation byproducts of porous Si all combined have generated much interest in its use in controlled drug delivery systems (261). After 42 days in simulated body fluid (SBF) the substrate has clearly eroded as shown in **Fig. 5.2-3(b)**. The dense SiNW structure of **Fig. 5.2-1** has been substantially deteriorated. The reactions of a variety of porous Si structures with SBF have been monitored similarly using SEM showing significant dissolution (269-270). This dissolution is mostly due to the high porosity and reactive surface of the

nanostructure which can be tailored for a range of biodegradability. For some biomedical applications, especially drug delivery, this type of material corrosion is desirable. Controlled release based on an eroding matrix of SiNWs allows diffusion of the drug from a degrading system with the carrier system itself being not necessarily inert. In our SiNW design and as it has been suggested by others, an active carrier system can sometimes be a part of an additional treatment in terms of contribution to the healing of the surrounding environment tissue (271). Another attractive advantage to Si degrading in an aqueous solution is that problems related to the removal of the material down the road after use can potentially be avoided (259).

Sequential and targeted drug delivery could be beneficial when managing a variety of drugs, such as anti-inflammatory agents, antibiotics, chemotherapeutic drugs, steroids, hormones and vaccines (271-272). Penicillin and streptomycin, common pharmaceutical antibiotics that reduce the formation of bacteria and biofilms and fights infection, was used as a model drug for our system. With this more advanced drug delivery system, improvements in delivery efficiency and localization may also provide a solution for reducing dosage (273). Typically, antibiotics are given in large doses up to 1gram/day. But, controlled release, from an implant for instance, would help minimize toxic side effects, drug waste, and further compliance problems that might occur due to prolonged oral antibiotic therapy (274-275). As well, the steady release rate from SiNW arrays could provide the right amount of drug to continuously meet the “therapeutic window” between the typical systematic

drug extremes of over dosages with adverse effects and large amounts of wasted drug, and under dosages or being sub-therapeutic.

Factors, such as adsorption properties (interactions between drug and matrix), pore size, pore connectivity, pore geometry and matrix reactions with surrounding media (dissolution properties) are just a few of the aspects to take into account when designing a controlled drug delivery system. We have also explored the option of tailoring the SiNW arrays for changing the drug profiles over time. By increasing the as-fabricated diameters of the nanowires near the top region by sputter coating with an additional $\sim 100\text{-}200$ nm dimension Si film added on the SiNW tips, an altered nanowire geometry was obtained which enabled us to alter the release rate. A 45° tilted, oblique-incident RF sputtering was carried out under a typical base pressure with the substrate continuously rotated (at a speed of 65 rpm) for improved thickness uniformity. The difference in the nanostructure reservoir geometry with increased bottle-necking SiNW diameters near the nanowire top slowed down the drug release rate by $\sim 25\%$ (Data not shown). Additional Si sputter deposition near the nanowire tip can further reduce the release rate to the extent of ultimately completely blocking the top entrance for a near-zero release rate. In this fashion we were able to control the morphology, dimension, and interface of the SiNW arrays by adding a Si coating and tailoring the release kinetics. The Si coating introduces a necking geometry and possibly surface capping of a portion of the reactive bonds from the electroless etching (EE) process, slowing the dissolution process

while the overall reservoir volume was decreased only slightly because of preferential deposition of Si near the nanowire tip.

In terms of bio-toxicity, it has been established that there is a relatively low toxicity of porous Si in various cellular and live animal systems (276-279). However, one of the desired properties of a drug delivery system is the ability to generate a fully controlled release profile free of cellular adhesion, surface macrophages, or foreign body reactions that would eventually lead to biofouling, impedance, blocking of the pores, and dysfunction of the drug delivery system in general (280-282). In order to test the cellular attachment to the SiNW surface an initial cell adhesion assay was performed, displayed in **Fig.5.2-4**. It was found that a very exciting phenomenon that the SiNWs have the ability to prevent cell adhesion and spreading. As shown in the comparative SEM micrographs of cell adhesion, **Fig.5.2-4(a)**, it is quite obvious that the cells cannot attach or spread on the SiNW arrays. The cells on the SiNW arrays appear balled up and do not begin spreading. In fact, the number of adhered cells decreases in the first 24 hrs of incubation because the cells simply cannot adhere to the surface. This could most probably be due to the extremely high surface energy of the SiNW surface. As mentioned previously, the surface contact angle is 0° indicating super-hydrophilicity. This is in agreement with earlier research showing that cells do not adhere or spread on high energy, super-hydrophilic surfaces (283-284). In addition, the nanowire tip contact surface area of approximately 20-40 nm diameter circle, and assuming that approximately one-half of the Si nanowires are too short to touch the cell because of the extremely uneven surface, it is

reasonable that the cells have a hard time attaching to the overall, very small area of available surface. In fact, the spreading of the cells is dramatically reduced on SiNW surfaces compared to control Si as shown in **Fig.5.2-5**. High magnification SEM images reveal that the cells cannot stick to the surface but instead tend to ball up and are sticking to each other, typically meaning that they do not “like” the surface environment. The overall cell spreading area on the SiNW surface was measured and found to be reduced by a significant factor of ~25 compared to the flat control Si surface. Another interesting observation to be noted is the low amount of protein adsorption that was observed on the SiNW surfaces when compared to Si controls. When bovine serum albumin (BSA) was incubated with the samples, there was a reduction of protein adhesion by approximately ~25% (Data not shown). This may also have some implications for the increased difficulty of cells adhesion as insufficient protein/integrin contact would reduce cell adhesion. This lack of protein and cell adhesion in the initial *in vitro* incubation indicates the reduced probability for biofouling and dysfunction due to foreign body reaction cells and proteins *in vivo*, which has significantly positive implications in the use of SiNWs for a more reliable, non-biofouling drug delivery system.

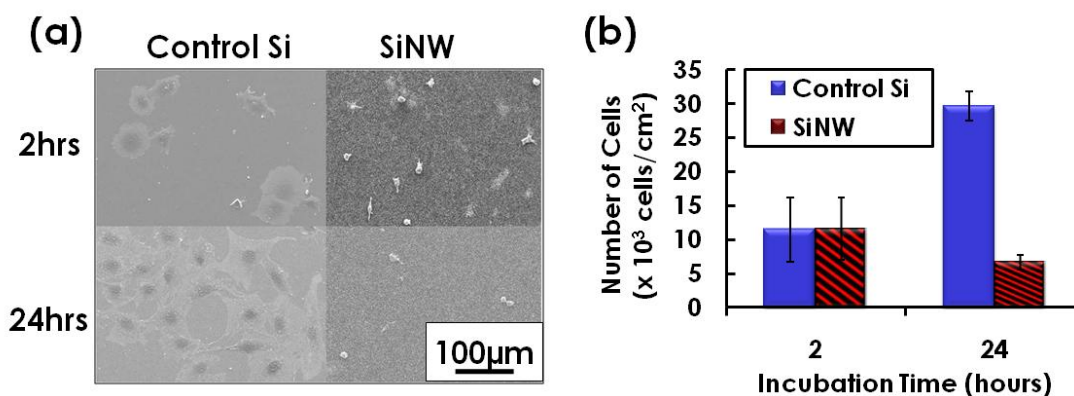


Figure 5.2-4. Cell adhesion assay. **(a)** SEM micrographs showing cell (lighter in color) adhesion on control Si and SiNW surfaces after 2 and 24 hours of incubation. The cells on the SiNW surface are unable to spread and adhere compared to cells on the control Si. **(b)** Quantitative graph depicting the number of adhered cells vs. incubation time. The cell number decreases with time on the SiNW surface.

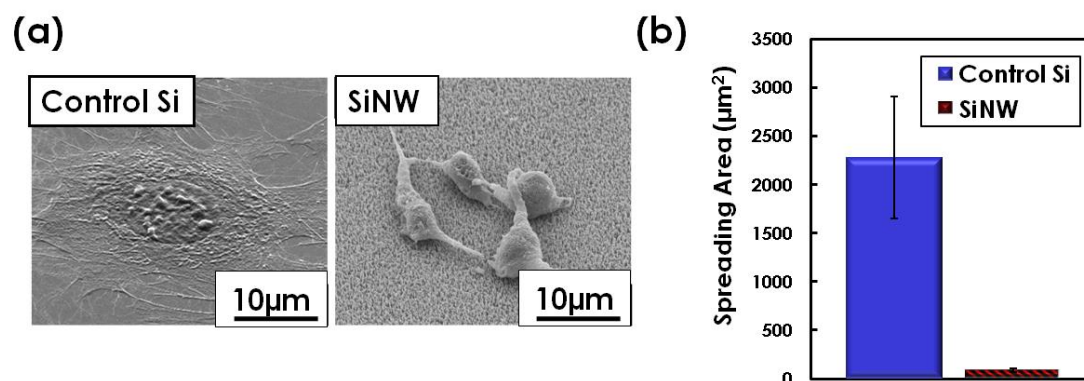


Figure 5.2-5. Cell spreading assay. **(a)** High magnification SEM micrographs showing cell spreading on control Si and SiNW surfaces after 24 hours of incubation. The cells on the SiNW surface are unable to spread and seemingly adhere to each other rather than the surface. **(b)** Quantitative graph depicting the spreading area of cells using Image J software on the Si vs. SiNW surfaces. Cells simply do not spread on SiNW surfaces.

In summary, it was shown that SiNW carriers can maintain a drug release level and longevity for 42 days, and also report on interesting behavior of the SiNWs preventing cell/protein adhesion. This type of nanowire based drug

release technology may have potential to make significant clinical and industrial impacts. Several interesting therapeutic implications based on the SiNW array drug release are envisioned:

- i. a sustained and tailored drug delivery profile for a desired duration of treatment
- ii. a favorable template for appropriate biodegradation, and
- iii. an initial anti-biofouling interface preventing or minimizing undesirable cell/protein adhesion for various implants, drug delivery vehicles or bio-sensors/bio-actuators.

The SiNW type controlled release approaches may also be useful for other slow release devices of other drugs, chemicals, pesticides, fertilizers, and/or fragrances(285).

5.3 Anodized Alumina Oxide (AAO) Nanotubes: A New Nano-Platform for Drug Release

Anodic aluminum oxide (AAO) has become one of the most popular self-ordered periodic, porous templates. In general the highly developed, superior ordering of nanopores in AAO templates is obtained by using a two-step anodization process (286), a rather simple processing method. The AAO porous structure can be uniquely altered based on processing parameters and both porous and tubular shapes can be achieved and tailored with pore diameters between 5nm-10 μ m and film thicknesses (vertical height) reaching over 100 μ m (287).

5.3.1 AAO Nanotube Formation Mechanism

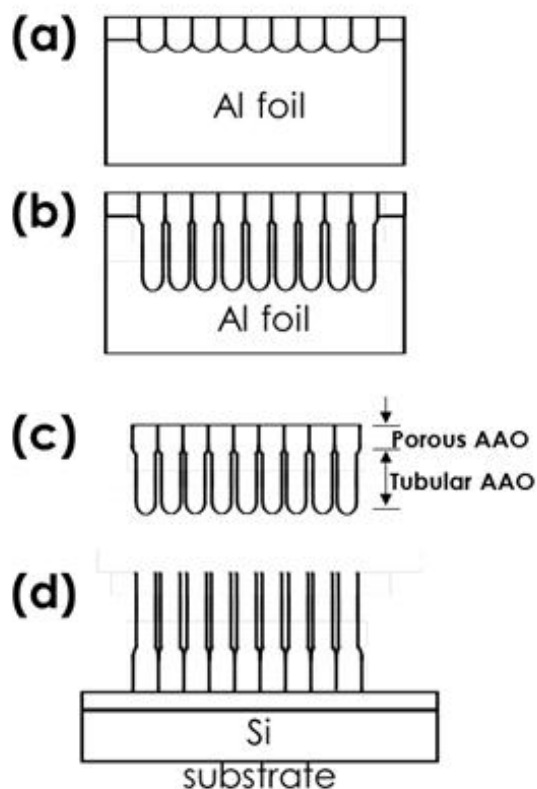


Figure 5.3-1. Schematic illustration of nanotubular AAO fabrication. **(a)** mild anodization to form porous AAO layer in 0.3M sulfuric acid **(b)** hard anodization to form tubular AAO layer for a few ten minutes **(c)** selective aluminum removal in mixed CuCl_2 and HCl solution **(d)** attaching of porous AAO layer-side to Si substrate with adhesive and pore widening for a few minutes in 5wt% phosphoric acid.

The mechanism for nanostructural formation of AAO nanotubes is portrayed in **Fig. 5.3-1**. A similar process of Ti anodization is described in Section 2.2.2. In this case of hard anodization, the barrier layer exposed after the Al substrate removal contains a significant depth of recessed voids, which were formed at between-cell triple junctions. The nanotube tip is etched away and the tubular geometry is realized.

5.3.2 AAO Nanotube Drug Carriers

AAO has been of great interest due to its outstanding material properties, including electrical insulation, optical transparency and chemical stability, and most recently because of its biologically inert and biologically compatibility properties (287). In terms of biological applications, the characteristic periodic porous films of AAO has been used for encapsulating enzymes (288), implant surface coatings on Ti alloys for bone in growth (289-290), membranes for hemodialysis (291), cardiovascular stent applications (292-293), biofiltration (294), and drug delivery (295-296).

Owing to porous AAO's ability to mimic the dimensions and nanoporous structural components of natural bone and the prospect of housing genes or drugs for therapeutic treatments within the pores (296), AAO films can be seen as promising coatings for medical, particularly orthopaedic, implants. Research on biomedical applications of both porous alumina, and along the same lines nanotubular titania (TiO_2) (121, 134, 297-301), has increased tremendously in the past few years. Titania in the form of nanotubular structures has been recently recognized for its impact in the future of nanomedicine (302).

In this work, the focus is on hard-anodized AAO which is recently of great interest because of its advantageous characteristics, i.e. (i) AAO film grows rapidly (ii) it is well ordered and (iii) there is a wide range of novel AAO nanostructures that can be obtained during the hard anodized AAO region which are yet to be discovered (303-306). For example, Zhao et al. reported a special AAO structure grown under constant current anodization, which is

represented by a six-membered ring symmetry around the center pores with a regular hexagonal pore arrangement.

While nanopore configuration has been studied greatly for potential biomedical and drug delivery type applications, the nanotube configuration has a unique feature in that the 10 – 20 nm spacing between neighboring nanotubes allow a continuous supply of body fluids, nutrients and proteins underneath adhered and growing cells that could potentially aide in tissue development and biointegration. In contrast, simple nanopored configuration gets covered with adhered cells thus blocking the supply of body fluid. This has been already been described for the case of TiO_2 nanotubes with significantly enhanced osteoblast (bone cell) and bone tissue growth, and applies to other adhered cells on the nanotubes, such as endothelial cells or chondrocytes.

To imitate the TiO_2 nanotube geometry, we have microstructurally altered traditional porous AAO to give rise to a unique nanotube morphology to determine its use in biological applications. It is projected that we can utilize the nanotubes as “nanodepots” for advanced drug delivery therapeutics. AAO nanotubes are an example of a multidisciplinary approach for combining nanotechnology, biomedical engineering, and controlled drug delivery where antibiotics, growth factors, etc. are appropriately needed as well as proper biointegration (osseointegration for example) is desired (296). The nanotube geometry, as compared to the nanopore geometry with comparable pore diameter, provides ~2X more surface area, which could be utilized to provide

more active spots and functional conjugation locations for increased adsorption or biomolecules, proteins, enzymes, and drug molecules.

The use of AAO nanotubes is a new clinical approach not only for orthopedics, but also for the treatment of several other drug eluting implants which ideally would release for longer periods of time, on the order of days, weeks, even months. The aim of this study is to elucidate the complex phenomenon of drug release from AAO nanotube drug carriers. Detailed, characterized release curves, accumulation plots, and kinetics were revealed for the antibiotic drug, amoxicillin. This type of geometrical structure of AAO is novel in itself, but also the time scale of prolonged release of amoxicillin from the AAO nanostructure is incredibly long, on the order of weeks, with a possibility of extending the drug release to much longer periods.

5.3.2.1 Experimental

Aluminum Pretreatment

0.5mm thick Al foil purchased from Alfar aesar (99.99%) was used as a starting material. The Al foil was successively degreased with isopropyl alcohol and acetone for 5 minutes with ultrasonication followed by a D.I. water rinse and a nitrogen gas blow. Then, the organic-free Al foil was slightly etched in 1M NaOH aqueous solution to remove any surface contamination before the electropolishing process. Conducting Cu tape was used as the electrode pathway to the Al foil and selected portions of the Al foil, such as the edges or backsides, were protected by a lacquer (Microshield) in order to make it resistant to the electrolyte used. A mirror-shiny Al foil was obtained after

electropolishing in a mixed solution of HClO_4 and Ethanol (1:4(v/v)) at 5°C under 20V for ten minutes with a Pt counter electrode. For a control sample, electropolished Al was used in the experiments. For the experimental nanotube samples anodization was conducted.

Anodization

Prior to hard anodization, mild-anodized porous AAO (~800nm thick layer) was formed under 25V in 0.3M sulfuric acid and then the electrolyte concentration was changed from 0.3M to 0.06M. Next, anodization voltage was increased (at a rate of ~0.5V/s) from 25V to 35V in order to inhibit local AAO film thickening due to localized high current concentration which otherwise would lead to inhomogeneous oxide film growth or even dielectric breakdown during hard anodization. Power supply (Agilent; E3612A) was connected to digital multimeter (Keithley; 2100) to monitor voltage-current evolution during anodization.

AAO post-treatment

After the anodization process, the top side of AAO layer was attached to Si substrate with adhesive glue for handling purpose. Then, Al substrate was selectively removed with a mixed HCl and CuCl_2 solution for ten minutes when the reaction ends. Any residual Cu debris adhered to the bottom of the AAO barrier layer was removed by placing in nitric acid for a few seconds and washed in D.I. water immediately after. The AAO barrier layer was then removed by 5wt% phosphoric acid for ten minutes to two hours depending upon barrier layer thickness of the as-grown AAO and observed under Scanning Electron

Microscope (SEM; Phillips XL30 ESEM). All samples were cut into identical size pieces (1x1cm²) and autoclaved before use as drug carriers.

Porosity and Surface Area Calculation

In order to characterize the AAO nanotube film structure, a porosity and surface area calculation was carried out. A theoretical porosity can be computed geometrically based on structural parameters such as pore size and inter pore distance assuming ideal hexagonal arrangement. The following equation, **Eq.5.3-1**, was used to approximate the porosity, P , of the samples,

$$P = \frac{2\pi}{\sqrt{3}} \left(\frac{r}{D_{int}} \right)^2 \quad (\text{Eq.5.2-1})$$

where r is the inner pore radius and D_{int} is the inter pore distance. To determine the surface area of the samples with an area of 1cm², first the nanotube density was established based on SEM images by counting the number of nanotubes per field for a given area. The nanotube density, N , was $\sim 2.19 \times 10^{10}$ (nanotubes/cm²). The following equation, **Eq. 5.2-2**, was used to approximate the surface area, SA , of the samples

$$SA = (2\pi(R^2 - r^2) + 2\pi h(R + r)) * N \quad (\text{Eq. 5.2-2})$$

where R is the outer tube radius, r is the inner pore radius, and h is the height or length of the film. This equation is based on the surface area of a tube multiplied

by the number of tubes in the sample area. This is a theoretical estimation. It may be that the voids located around the center pore are not completely void the length of the tube; nonetheless SA is dramatically increased based on the introduction of tubes on the surface.

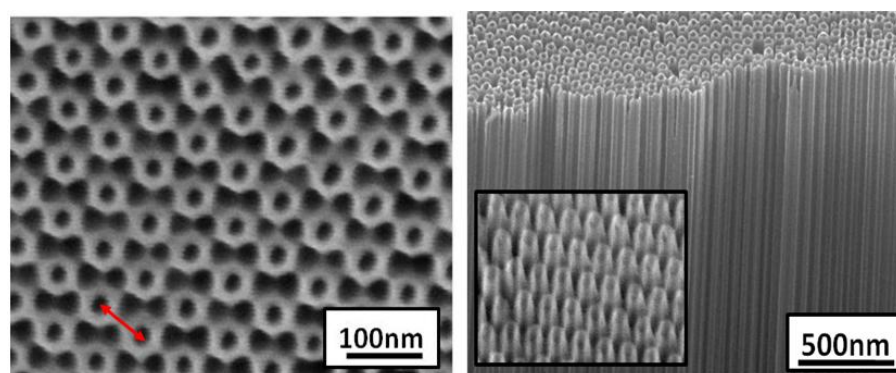
Antibiotic Loading, Release, and Collection

Insertion of liquid into AAO nanotubes is not always easy as the surface tension of the liquid has to be overcome. At room temperature (25°C), the nanotube samples were placed in a vacuum ($\sim 10^{-4}$ torr) for approximately 5-10 minutes to rid nanopores of any trapped air. Approximately 1ml of 1% amoxicillin (Sigma) in phosphate buffered solution (PBS) pH 7.4 (Invitrogen) was loaded onto each sample placed individually in separate wells of a 12-well plate (Nunc). To ensure dissolution of the amoxicillin prior to loading, a few microliters of 1N HCl was added until the solution became clear. The samples were incubated overnight under vacuum to allow sufficient time for the drug to fully penetrate into the nanotube structure. The drug-loaded nanotube samples were washed 3X with ice cold PBS (to restrict diffusion from the reservoir). Next, the samples were individually placed in new wells (Nunc, 12-wells) incubated in a humidified 95% air/5% v/v CO₂ incubator at 37°C in 1ml fresh simulated body fluid (PBS was used in this study). The solution was collected at hourly time points initially (hours 1-6) and daily time points thereafter (up to day 35 or 5 weeks) and 1ml fresh PBS was added after each collection. Drug concentration was determined by measuring the absorbance of the fluid using a UV-VIS spectrophotometer at $\lambda=230\text{nm}$ (Biomate_3, Thermo Electron, Madison, WI). The assay was calibrated

by use of PBS blanks and a standard curve was determined up to 2mg/ml amoxicillin. Three replicates per experimental sample for each time point were measured and the average values $\pm$ standard error (SE) was graphed to obtain release profiles, release rate, accumulation, and release kinetics.

5.3.2.2 Results and Discussion

The vertically aligned, periodic AAO nanotube structure used as a drug carrier is illustrated in the scanning electron microscopy (SEM) images in **Fig. 5.3-2 (top row)**. In contrast to conventional AAO nanopore structures, AAO nanotube unit cells are separated from each other while being loosely connected to each other, which is an interesting feature. For this nanotube sample, the nanotube center pore size ($\sim 20\text{nm}$) is practically the same as the size of the voids (spacing between adjacent nanotubes) surrounding the center pore. This makes the AAO nanotube structure favorable due to larger surface area in terms of loading drugs or catalyst chemicals into the AAO nanotubes. The equal size of the center pore and voids was achieved by the relatively low anodizing voltage (35V) which ensures both relatively small center pore size and interpore distance compared to the higher voltage evolution under the constant current anodization technique conducted by Zhao et al. The anodic current evolution during nanotube fabrication is given in **Fig.5.3-3**.



Nanotube Height (μm)	Pore size (nm)	Wall Thickness (nm)	Nanotube Center-to-Center Distance (nm)	Porosity (%)	Surface Area (cm^2)
38	20	15	73	25.1	1830

Figure 5.3-2. Upper left: Top-view SEM image of vertically aligned periodic AAO nanotubes. **Upper right:** Oblique view (inset=higher magnification image). The table describes the physical dimensions of the nanotubes.

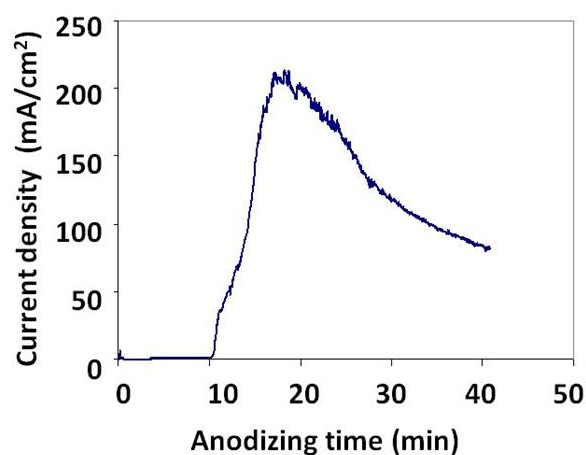


Figure 5.3-3. Representative anodic current evolution for AAO fabrication. Applied voltage was slowly increased from 25V to 35V and remained at 35V. Anodization was conducted for 30 minutes overall.

Based on the literature, this is the first study to utilize the AAO nanotube structure for applications in drug elution. The nanotube film shows a highly

ordered and uniform nanotube morphology and long-range order with nanotube height reaching $\sim 38\mu\text{m}$, which is the tallest used thus far in nanopore/nanotube ceramic alumina and titania drug elution studies. Physical details of the AAO nanotubes are portrayed in the chart shown in **Fig. 5.3-2 (bottom row)**. One of the advantages of the nanotube structure is the increased porosity ($\sim 25\%$) and the high surface-to-volume ratio. Here it is shown that the surface area is increased by three orders of a magnitude by introducing the nanostructures on the surface, where a $1 \times 1 \text{ cm}^2$ sample has perceivably 1830 cm^2 of surface area. The interstitial space between the tube walls and the inner pore walls aid in this calculation and is an advantage over a traditional pore structure. Another benefit to our AAO drug release system is that we can design the nanotubes to match a desired pore size, structural shape, available porosity, and surface area which can help tailor to specific implant needs and controlled release.

It has been suggested that during AAO fabrication, stress cracking and other residual defects due to the oxidation volume expansion (Al becoming Al_2O_3) may be present and these imperfections can leave charges on the surface, such as Al^{3+} and O^{2-} (307). For the purposes of drug loading, this may aid in electrostatic adsorption of the drug molecules and help concentrate the drug within the nanotube “depots” so to speak.

For this study, amoxicillin (AMX), a common pharmaceutical antibiotic, is used as a model drug in the following AAO drug elution studies. The size of the AMX molecule is $\sim 0.8 \text{ nm}$ (308), a reasonable size to enter and fill the 20 nm

diameter pores and interstitial spaces in between the nanotubes. Preoperative oral administration of AMX has been proven to reduce the risk of implant failure (309-310), and local delivery of AMX during orthopedic surgery reduced the infections associated with open fractures (311), compound limb fractures (312), and with osteoinductive and osteoconductive bone-graft substitutes (311). As well, local delivery of antibiotics was effective in reducing vascular infections from staphylococcal strains (313). It can therefore be hypothesized that localized AMX elution from both orthopedic and vascular implants would be highly advantageous. With the more advanced drug delivery, controlled release system such as AAO nanotubes on implants, would help make improvements in delivery efficiency and localization which may also provide a solution for reducing dosages and help minimize toxic side effects and drug waste. The nanotube shape also has its advantages over a porous structure because it provides an optimal surface shape to allow for cellular adhesion sites for better cell attachment and proliferation(314), aiding in surface integration and cellular locking (54).

In terms of controlled release, it was found that the AAO nanotubes were cable of carrying cargo molecules (AMX, a small drug molecule) and releasing them in a physiological environment of the simulated body fluid, phosphate buffered solution (PBS). There are several types of controlled release devices and the AAO nanotube system presented here can be considered a drug diffusion-controlled release, where the entrapped drug diffuses out of a matrix at a defined rate (315). An antibiotic release profile from the AAO nanostructures

filled with AMX was obtained for over 5 weeks (35 days), illustrated in **Fig. 5.3-4**. For a control experiment, electropolished aluminum, without a nanostructured surface, showed almost zero antibiotic release as expected (data not shown). This indicated that it was the nanotube design on the surface which created a reservoir for the AMX that was responsible for the drug release. **Fig. 5.3-4** shows the total amount of amoxicillin released as a function of time. A near steady release profile is achieved after the first week of release (after Day 7). The ideal release profile for most drugs would follow this type of a steady release rate so that the drug levels in the body remain constant while the drug is being administered (316). The drug elution from the AAO nanotubes accomplishes the primary objective of a controlled release device which is to provide a sustained release for long periods of time on the order of days, weeks, even months.

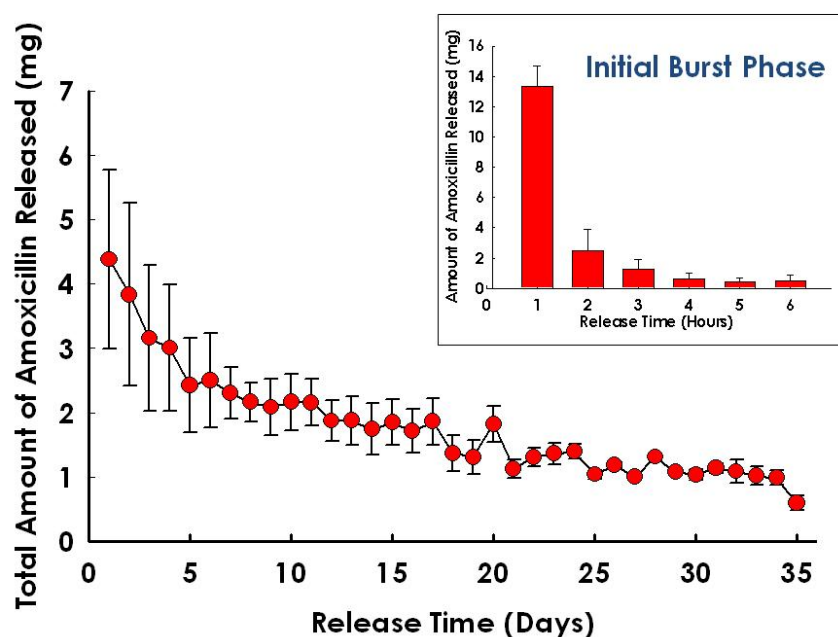


Figure 5.3-4. Absolute release rates of amoxicillin as a function of sampling time for the AAO nanotube drug carriers. The initial burst of drug from the surface is shown in the inset. The graphs show the mean $\pm$ SE ($n=3$).

In the inset graph of **Fig. 5.3-4**, which shows the initial release of drug from the nanotubes in the first 6 hours of release, the highest “burst effect” is in the first hour with $\sim 13\mu\text{g}$ of drug release. The “burst effect” is often seen as controversy as to whether this is due to near-surface entrapped drug or surface-absorbed drug (317). The initial burst and release of drug from the nanotubes may be related to several factors including (1) high relative top surface area (2) increased drug diffusivity through tube walls/channels and (3) high porosity. In addition, the specifics of the pore dimensions and their uniformity as well as subtle difference in physical form of the nanotubes may play a role at release during the initial short term release (first 7 days) before the steady elution (beyond 7 days in **Fig5.3-4**). At this stage in release, the drugs are being released from the top portion of the film where the so called “matrix surface” becomes a factor. After seven days, however, it is suggested that the drugs are traveling from a distance that is farther down in the matrix and less likely to be affected by the very top surface. Drug release from AAO $\sim 20\text{nm}$ and $\sim 40\text{nm}$ pore (not tube) structures with the same film thickness or height as the nanotubes studied in this report (data not shown) was also observed to have similar release kinetics, however it has been suggested that it is the height of the pore, not the pore size, that changes the diffusion characteristics (300). Varying film thickness should impart some of future studies, but this report focuses on the unique geometry and beneficial properties of the AAO nanotube structure.

To further characterize the AMX release from the AAO nanotubes, **Fig. 5.3-5 and 6** illustrate the accumulative release (showing daily and weekly

accumulation) and release rate per day over the 5 week elution study, respectively. A near steady release rate occurred over the course of the 5 weeks. This type of release would help maintain a drug level in a therapeutic window, avoiding the extremes of systematic drug over-dosages or under-dosages, eliminating the risks of adverse effects, drug waste, or being sub-therapeutic.

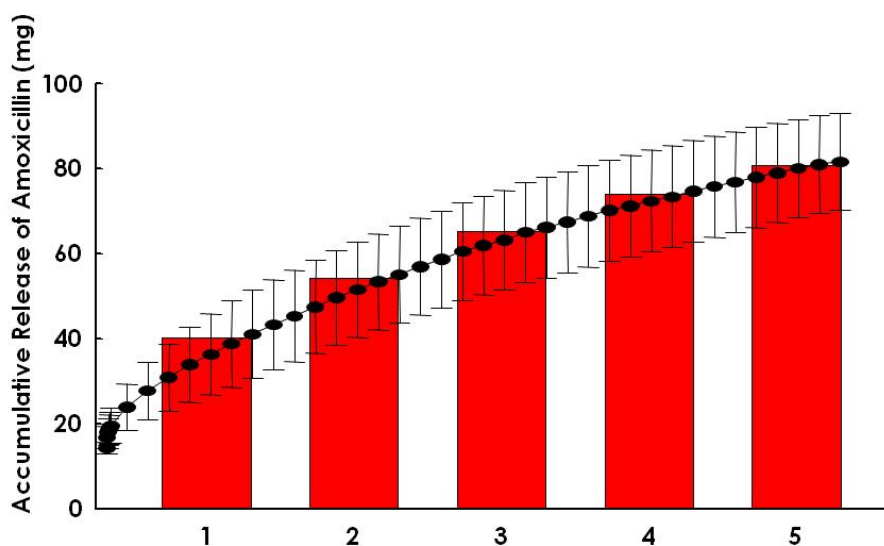


Figure. 5.3-5. Accumulative amoxicillin released as a function of time. The graph shows mean $\pm$ SE (n=3). The dotted line reveals the daily accumulation over time and the bars represent the average accumulation per week.

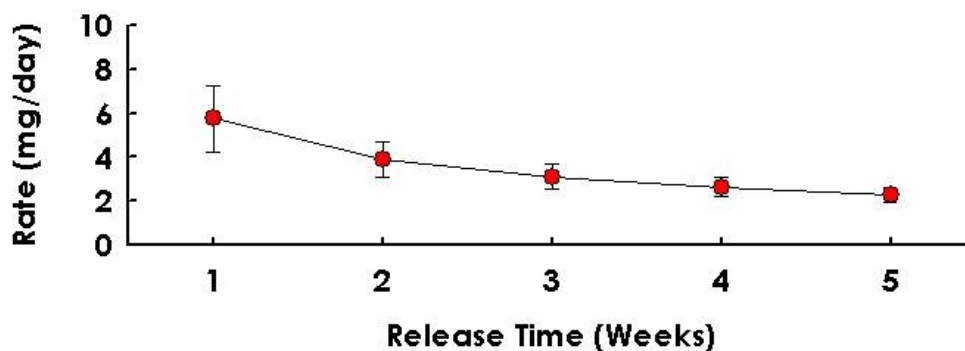


Figure 5.3-6. Release rate per day vs. time (normalized per weekly time point). The graph shows the mean $\pm$ SE (n=3).

When studying the drug release of molecules that have a size regime on the same scale as the matrix features, the basic principle of diffusion as a mixing process with solutes free to undergo Brownian motion in three dimensions may not necessarily apply, in at least on dimension, for the AAO nanotubes because the solute movement is physically constrained by the nanotube walls (318). The AAO nanotube geometry may impose a rate limiting condition due to the length of the nanotube walls, because the length dictates how far the solute molecules have to travel to be released from the reservoir. While it is not possible to draw significant conclusions without varying the wall height of the AAO nanotubes, this in part should form some future work.

Many studies have observed that the release rate of a drug dispersed in a solid matrix (with no erosion of the matrix occurring) is proportional to the square root of time, as predicted by the Higuchi model (319-321). It was determined that this was because the release rate is inversely proportional to the distance the drug must travel within the matrix to the matrix surface, since the diffusion distance increases with time, the release rate decreases with time (319). The Higuchi equation follows,

$$\frac{Q_t}{Q_\infty} = k\sqrt{t} \quad \text{(Eq. 5.3-1)}$$

where $\frac{Q_t}{Q_\infty}$ is the cumulative fractional release at time t and k is the release constant.

To identify the release rate mechanism and model the drug transport in the AAO nanotube system, the hypothesis was made that the release data

obtained could be fitted using **Eq. 5.3-1** and the results are given in **Fig. 5.3-7**, where the fractional AMX release was plotted versus the square root of time. A near perfect linear fit was observed, demonstrating that the drug kinetics approximately follow the square root of time relationship. The chart in **Fig. 5.3-7** describes the linear fit. The mechanism of release is most likely attributable to a novel constrained diffusion mechanism provided by the AAO nanotube walls.

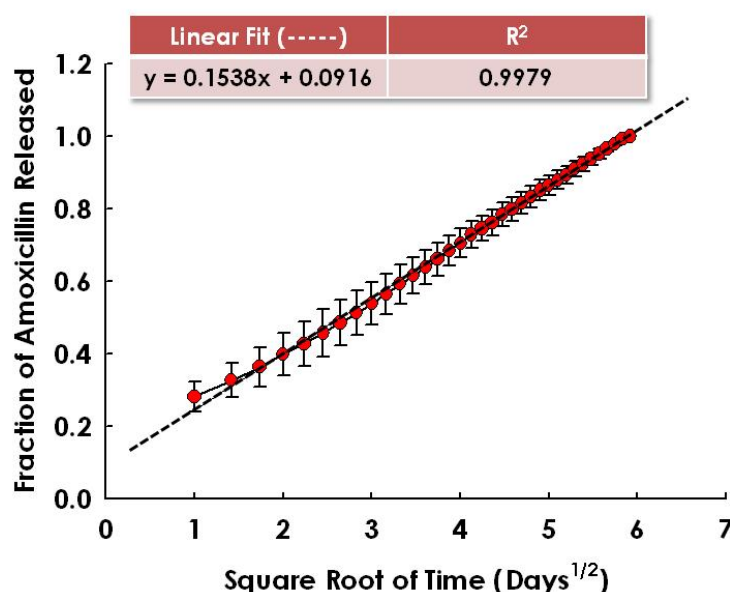


Figure 5.3-7. To assess the mechanism of drug release, a plot of fractional release vs. the square root of time was completed. A near perfect linear fit was observed and details are shown in the above table.

AAO films are simple to prepare and can be easily modified and structurally tailored. As well they are resistant to most physiologic and chemical reactions (bioinert), mechanically strong, and are considered biocompatible *in vitro* and *in vivo*. By utilizing AAO nanotubes as drug carriers, a variety of drugs can be loaded into the device reservoir in a range of physical states, including

solutions and crystalline or micronized suspensions (318). This flexibility with respect to encapsulated drugs provides options to substantially increase the load dose and duration of therapy, as well as stability of drugs that are unstable in certain biological fluids or different biochemical/acidic/alkaline environments. AAO films are structurally robust and will not swell or change its porosity under different pHs or temperatures (322). Thus, AAO nanotube drug carriers can be used to address the problems associated with conventional drug therapies such as limited drug solubility, poor biodistribution, lack of selectivity and unfavorable pharmacokinetics (296). Lastly, the potential for AAO nanotube arrays on implant surfaces will help mimic the complex geometries of natural tissue and will provide a porous template for the growth and maintenance of healthy cells and tissue (323), aiding in implant design as well as local delivery of therapeutics.

Next, the AAO nanotubes were investigated for the potential use of our alumina nanotube array structure as an implant surface coating by employing an *in vitro* cell adhesion assay. A fluorescein diacetate (FDA) viability marker was used to stain the cytoplasm of osteoblast cells cultured on the different surfaces show in **Fig. 5.3-8**.

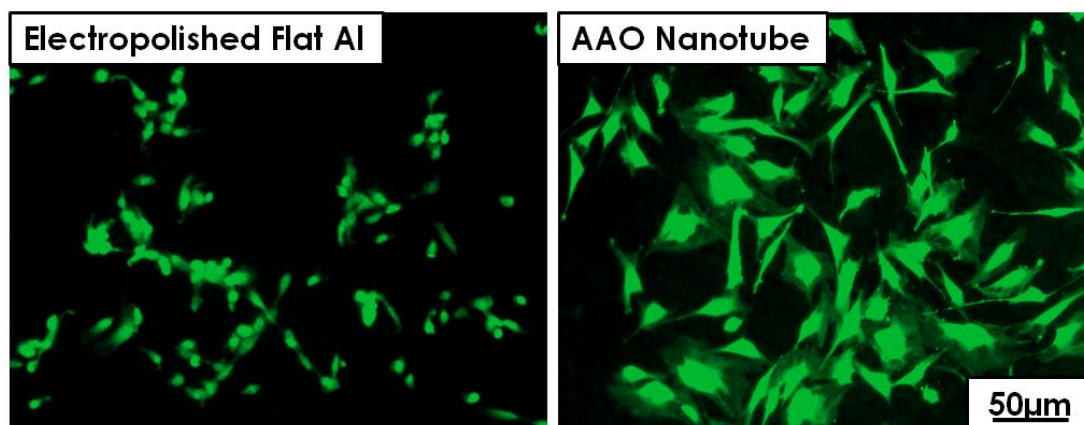


Figure 5.3-8. Osteoblast cell viability determined by FDA (live fluorescent staining, green color) after 24 hours of culture time on control electropolished flat Al (prior to anodization) vs. anodic aluminum oxide (AAO) nanotube surfaces.

The osteoblast cell adhesion after 24 hours of culture time is much more pronounced on the AAO nanotube substrate compared to a flat Al_2O_3 control substrate, and it is evident that the cell adhesion and spreading is significantly enhanced on the nanotube surface. These primary studies suggest that the AAO nanotube surface used as a possible orthopedic implant surface is an attractive template for improving the osteoblast response. The AAO nanotube surface, with a wide array of configurations, sizes, and shapes, can be useful for parallel experiments looking further into osteoblast response, functionality, and matrix production for improved osseointegration.

5.4 Conclusions

Controlled, sustainable release of therapeutic molecules was released from nanostructured reservoirs. These types of drug delivery devices have an enormous impact on medical technology, greatly improving the performance of

many existing drugs and enabling the use of entirely new therapies. Efforts to miniaturize drug delivery devices from the macroscale (>1 mm) to the microscale (100–0.1 μm) or nanoscale (100–1 nm) ultimately promise integrated systems that combine device technology with therapeutic molecules or combinations such as small molecules, nucleic acids, peptides and or proteins(273).

5.4.1 Considerations for Further Development

Research toward various drug release techniques has been based mostly on concepts of delayed drug release or a constant-rate release. Considering the nature of the human body with rhythmic biological cycles or with unpredictable/irregular needs to adjust body functions and to fight against aggravated illness, the assumption of delayed or constant rate drug release being optimal is not necessarily valid. There are still no, truly on-off switchable, “on-demand” drug delivery vehicles available which are simple, easy to use and inexpensive to benefit patients in several medical areas. Therein lies the opportunity to create “smart” drug delivery systems.

One aspect of implantable controlled delivery devices is designing the release of drugs at various dosages and for both intermittent and continuous delivery. It would be desirable to have the capability to operate for both short periods (on the order of days) and extended periods (on the order of years) as well as provide multiple compartments with multiple drugs/dosages. One type of design incorporates multiple sealed compartments, which are opened on demand to deliver a dose of a drug(280). The Jin lab has also proposed a nano-

reservoir array with latchable valves. Magnetically actuable, drug-delivery biomaterials approach is shown in **Fig.5.4-1(a)** open and **(b)** closed. The schematic illustration of the magnetically switchable and “latchable” drug delivery vehicles is proposed based on a nano-reservoir array. Each of the nano-reservoir cavity arrays (DUV (deep UV) lithographically fabricated) has an example dimension of e.g., ~160 nm dia x 600 nm deep, or 320 nm dia x 950 nm deep, **Fig. 5.4-1(c)**. As small as 100 nm dia. cavity arrays has been made at the Jin Lab. The top lid of the nano-reservoir cavity (valve) is made of special, square M-H loop magnetic materials with easily switchable coercivity for controlled opening (324-327), which are also stainless-steel-based and bio-compatible. The magnetic layer, either a ~10–100 μm thick, sputter deposited film or lamination bonded thin sheet, is magnetically processed to induce in-plane square M-H loop characteristics with “latchability”, and lithographically patterned into valves. The latchability enables selective, concurrent or sequential release of several drugs as well as a complete stop of release, with just a pulse magnetic field. There is no need to have continuous magnetic field, electrical current, or any other power requirements.

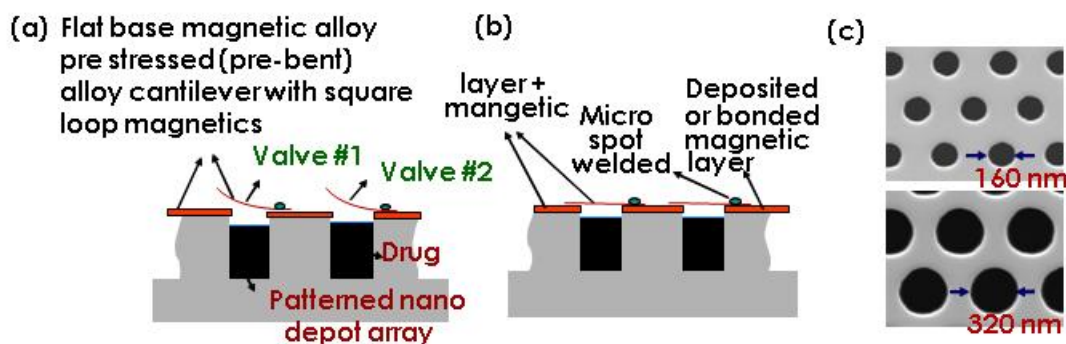


Figure 5.4-1. Nano-reservoir array with latchable valves.

In general, two main approaches are used to control the delivery of a drug to a target tissue: activation of molecular interactions using light, radiofrequency (RF) or ultrasound energy, and systems comprising materials with release kinetics that can be modified by an external stimulus(273). The revolutionary concept of “on demand” or programmable drug delivery methods offer an opportunity to create novel therapeutic devices.

Chapter 5, is in part a reprint of the material as it appears in Nano Letters Volume 9 (10), 2009, Page 3570 written by Karla S. Brammer, Chulmin Choi, Seunghan Oh, Christine J. Frandsen, Laura S. Connelly, Mariana C. Loya, Seong D. Kong, and Sungho Jin; and in the material submitted for publication in Advanced Engineering Materials written by Karla S. Brammer, Kunbae Noh, Chulmin Choi, Seung H. Kim, Christine J. Frandsen, and Sungho Jin. The dissertation author was the primary investigator and author of both these papers.

CHAPTER 6

6. Conclusions

In this work, the preparation of substrates with defined surface structures, chemistry, and material properties were systematically investigated for the role of the substrate topography on cell physiology. The surface topography had profound impacts on cell behaviour. Using this concept of controlled cell behaviour based on the physio-chemical impacts of the surface topography, even greater modulation of cells can be engineered for advanced tissue engineering and revolutionizing implant surfaces for host integration and enhanced bioactivity. The realization of the importance and advantages of the incorporation of biomolecules in surface nanostructures has also been reported in this work.

6.1 Contributions to Nanotechnology

Recent advances in nanostructured materials for medical applications have resulted from two complementary forces (328):

1. There is a natural evolution from the microscale to the nanoscale as new materials processing and characterization techniques.
2. Nanostructured materials provide the capability for specific interactions with proteins, DNA, viruses, and other nanoscale biological structures.

Highly specific interactions between these components and

nanostructured materials can provide unique biological functionalities not seen with conventional microstructured materials.

6.2 Contributions to Biomedicine

Nano-structures have recently been of great interest due to their high surface-to-volume ratio and the higher degree of biological plasticity compared to micro-structures. In terms of biomaterial development and implant technology, the cellular response can be affected by topographical circumstances. In the field of *in vitro* cell biology, there is a growing body of data that shows how cells respond positively to nanotopography (54, 68, 93, 117, 167, 329-330). It has been proven that cells sense and react to nanotopography, *in vitro* as well as *in vivo* by exhibiting changes in cell morphology, orientation, cytoskeletal organization, proliferation, signaling and gene expression.

The work here displays vast contributions to biomaterial and biomedicine research and technology areas with innovative ideas and experimental demonstration of new and enhanced materials and methods.

- I. *Orthopedic biomaterials* --- Using TiO₂ nanotube array surface biomaterials, significantly enhanced osteoblast adhesion, bone forming ability and bone mineralization was achieved. This discovery could lead to reduced bone loosening and lower incidents of revision surgery for millions of patients with orthopedic implants.
- II. *Contributed to the design, fabrication and testing of new cardiovascular stent implants having unique nanotube structures.* The TiO₂ nanotube structure significantly enhances endothelialization of stent surface which

is important to reducing the late stent thrombosis (LST), and may lead to all-metallic-stents that can eliminate polymer drug-eluting structures to prevent restenosis and heart attacks.

- III. *Demonstrated that the growth and functionality of cartilage-generating cells (chondrocytes) can be enhanced by using nanoscale substrates.*

For osteochondral cartilage repair, the use of a nanotube surface capable of i) osseointegration for the attachment to bone on one side, while the other side ii) accelerates cartilage generation, can be utilized as a dual material for cartilage repair of the knee.

- IV. *Enhanced stem cell differentiation with nano-geometry and surface effects only* --- It is demonstrated that the use of substrates with precise nanogeometries for manipulation of stem cell adhesion morphology could lead to elongation of stem cells and preferential guided differentiation of mesenchymal stem cells to bone cells. As well, manipulation of surface properties, such as hydrophobicity or surface chemistry, leads to cell aggregation and subsequent osteo-differentiation. This new surface-only-based approach of guided stem cell differentiation to specific lineage can advance stem cell therapeutics in a more practical and safer way by eliminating the use of toxic biochemicals to induce specific stem cell differentiations.
- V. *Anti-biofouling drug delivery* --- Using Si nanowire forests obtained by electroless chemical etching, it is demonstrated that undesirable cell adhesion and growth can be minimized (such as needed for protection

of implant surface and drug-delivery nozzle devices against scar tissue or protein biofouling). Also demonstrated was a sustained long-term slow drug release of antibiotics while providing desirably biodegradable characteristics for eventual removal of the drug delivery materials.

CHAPTER 7

7. References

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