NANOSCALE MODIFICATION OF BIOMATERIALS TO REDUCE INFECTION AND OPTIMIZE CELL GROWTH AND PROLIFERATION

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Hypothesis and specific aims

**Hypothesis 1:** The surface properties of nanomaterials, specifically the surface roughness, can be controlled to promote cell growth and a linear correlation can be established between the two.

**Specific aims:** Examine nanostructure-tissue interactions by using XanoMatrix cell culture scaffolds made from cellulose acetate and PET.

1. Evaluate the surface characteristics of the scaffold.
2. Study the activity of various cell lines on the scaffolds.
3. Correlate the cell performance to the surface characteristics of the scaffolds, especially surface roughness.

This research has been discussed in Part-B of this thesis in chapters 1 and 2.

**Hypothesis 2:** Nanotopography on the surface of orthopedic implants can help reduce infection without the use of antibiotics and can also be used to control inflammation for faster wound healing.

**Specific aims:**

**Specific aim 1:** Establish nanophase hydroxyapatite coatings on titanium surfaces using electrophoretic deposition and evaluate performance against samples coated with plasma spray deposition technique.

1. Synthesize and characterize hydroxyapatite nanoparticles.
2. Coat titanium surfaces using electrophoretic deposition.
3. Evaluate surface characteristics of coated materials.
6. Correlate the performance of bacteria and cells to the surface characteristics of the materials, especially surface roughness.

**Specific aim 2:** Establish composite coatings on titanium surfaces using nanophase hydroxyapatite, nanophase ceria and nanophase selenium and evaluate their biological performance.

1. Synthesize and characterize hydroxyapatite nanoparticles, ceria nanoparticles and selenium nanoparticles.
2. Coat titanium surfaces with nanophase hydroxyapatite using electrophoretic deposition and then deposit ceria and selenium nanoparticles on top.
3. Evaluate surface characteristics of coated materials.
6. Correlate the performance of bacteria and cells to the surface characteristics of the materials, especially surface roughness.

This research has been discussed in Part-A, chapters 1, 2, 3 and 4.

**Part A-Surface coatings on orthopedic implants**

**Abstract**

Reducing bacterial density on titanium implant surfaces has been a major concern because of the increasing number of nosocomial infections. Controlling the inflammatory response post-implantation has also been an important issue for medical devices due to the detrimental effects of excessive chronic inflammation on device performance. It has recently been demonstrated that manipulating medical device surface properties including chemistry, roughness and wettability can control both infection and inflammation. Here, we synthesized nanophase (that is, materials with one dimension in the nanoscale) hydroxyapatite coatings on titanium to reduce bacterial adhesion and inflammatory responses (as measured by macrophage functions) and compared such results to bare titanium and plasma sprayed hydroxyapatite titanium coated surfaces used clinically today. This approach is a pharmaceutical-free approach to inhibit infection and inflammation due to the detrimental side effects of any drug released in the body. Here, nanophase hydroxyapatite was synthesized in size ranges from 110-170 nm and was subsequently coated onto titanium samples using electrophoretic deposition. Results indicated that smaller nanoscale hydroxyapatite features on titanium surfaces alone decreased bacterial attachment in the presence of gram negative (P. aeruginosa), gram positive (S. aureus) and ampicillin resistant gram-negative (E. coli) bacteria, altered the surface energy subsequently affecting protein adsorption and were able to control inflammatory responses by reducing
macrophage growth; properties which should lead to their further investigation for improved medical applications.

**Introduction**

A large population of the world is affected by a number of orthopedic problems. According to the American Association of Orthopedic Surgeons (AAOS) between the years 2009 and 2012, there has been an 83.72% increase in total hip and knee replacement procedures[1]. Despite the high success rates of these surgeries, about 20% of these implants fail due to infection within 10-20 years [2]. In the pathogenesis of infection around implants, the initial adhesion of bacteria onto biomaterial surfaces is a critical first step. There is a race for the surface between healthy cells and bacteria. An important strategy in the reduction of orthopedic infections is to develop implant materials that prevent initial bacteria adhesion onto implant surfaces [3]. Implanted devices may be colonized by bacteria at the time of surgery or via a hematogenous route from a distant source. The most significant factor in the development of device-related infections appears to be the skill of the surgical team; prosthetic hips have been reported to become infected in less than 0.2% of cases, but in as many as 4% of cases in less proficient conditions [4]. Generally, large and complex medical devices that require long and complicated surgery for their placement are at high risk of bacterial infection [4]. When infection occurs, it can be life threatening. Device-related infections may occur almost immediately post-surgery or may be very slow to develop, with overt symptoms occurring months, or even years, after the device is implanted.

Most medical device infections are currently treated by the use of antibiotics such as amoxicillin, erythromycin etc [1]. This strategy targets free-floating planktonic bacteria and fails to target the
biofilm forming bacteria that are more sessile. The threat of antibiotic resistance is also real because of the increasing occurrence of antibiotic resistant strains of bacteria, especially MRSA. The CDC pointed out 72,444 incidences of invasive MRSA infections in the US in the year 2016 itself.

Nanotechnology is a promising alternative to overcome the problems faced in the traditional administration of antibiotics, proteins, peptides, and new drugs in the discovery pipeline. Many of these drugs used to fight infections have been shown to be poorly soluble in aqueous and organic media and show erratic absorption and poor bioavailability. Nanoparticles can be used to increase drug efficacy and decrease toxicity by controlling biodistribution, improving intracellular penetration, facilitating absorption through the mucosa, and improving protection against degradation. However, strategies that avoid drugs completely, if effective, could provide the best approaches to avoid drugs completely. Implant materials that prevent initial bacterial adhesion are important tools for fighting infections. [3] Bacterial growth and adhesion can be controlled by altering various surface properties like porosity, roughness, hydrophobicity, hydrophilicity, the use of a functional group, etc without drugs. Several antibiotics (like vancomycin) have been incorporated into coatings on Ti surfaces, but issues of optimal incorporation and controlled release have yet to be dealt with [1]. One major restriction on the incorporation of antibiotics in coatings is the use of high temperature processes like plasma spraying, which denatures the coating [5-7]. Furthermore, the loading capacity and the release kinetics of antibiotics are restricted by their physical adsorption onto substrates and their use could trigger antibiotic resistance which is a problem yet to be solved [8, 9].

Coatings that reduce bacterial attachment are produced by modifying the implant surface to have structures that alter the surface energy and also provide mechanical cues, which disrupt the
bacterial membrane [10]. Many studies have indicated that surface roughness and hydrophobicity are primarily responsible for controlling bacterial attachment and, since nanostructures give us the ability to do this, nanostructured implant surfaces are far better at reducing orthopedic infections than conventionally structured implants. Thus, by incorporating nanofeatures onto implant surfaces, we can reduce orthopedic infections by up to 5 times as compared to plasma sprayed implants which create conventional, micron structured features [11, 12] [13].

Surface coatings of different sizes and geometries to reduce infection and inflammation without using drugs can be achieved by various methods, one of which is Electrophoretic Deposition (EPD). EPD is a method that can be used to form coatings on metal surfaces. It involves the colloidal suspension of solid particles in a liquid electrolyte under an electric field and then the subsequent migration and deposition of these particles onto the electrode surface leading to the formation of a dense, solid film [14]. It has been found to be an efficient technique to make ceramic coatings from powder suspensions and it is an easy process for obtaining nanostructured deposits from colloidal solutions. The other advantages of EPD are that it is less time consuming, less expensive, leads to uniform coatings, and can be implemented on complex shapes [15, 16].

The culminating aim of part of this research is to modify the surface of titanium by coating it with nanoscale hydroxyapatite, nanoscale ceria and nanoscale selenium (all materials which may reduce bacterial growth) using electrophoretic deposition with a DC current. It also imparts anti-inflammatory, and bone induction responses – all from the same material without the use of pharmaceutical drugs which have detrimental side effects in the body.

**Literature Review**
The concepts and principles relevant to investigating the effectiveness of surface modified titanium for orthopedic tissue engineering applications are discussed here. First, because the aim of surface modified titanium is to mimic the structure and function of bone, a review of the current features of orthopedic implants will be presented, followed by current methods of tissue engineering to control infection and inflammation. The final section presents a review of selenium, ceria (novel chemistries) and their applications in reducing bacterial infection, as this project focuses in large part on creating composite coatings with hydroxyapatite and these materials to reduce bacterial infection and inflammation.

**Early interaction of implants with the body**

When an implant is inserted into the body, it triggers a wound healing response (extracellular matrix production and vasculature) (Figure 1). There is a complex cascade of events which takes place at the bone-implant interface and it has been investigated extensively. This is often termed “the foreign body response” to implanted materials [17, 18]. The cellular action required for healthy tissue regeneration may be compromised through this inflammatory reaction [19], thus hindering natural wound healing and the long-term success of implantable biomedical devices such as intra-osseous, transcutaneous and percutaneous implants [20, 21] and stent grafts [22, 23]. Hence it is crucial to try and get a favorable physiological response at the implant site for optimum wound healing.
Figure 1: Schematic of two fates of intra-osseous transcutaneous biomaterials after implantation, either leading to tissue/biomaterial integration or biomaterial rejection via fibrous encapsulation (©Barbara Symie Smith, 2012) [17].

The scale of body components has been studied extensively and it has been seen that, even though bone and soft tissue cells are a few micrometers in size, most crucial interactions in the body take place at the nanoscale level [24-27]. Therefore, a thorough understanding of the effect of nanotopographical cues, at a morphology similar to that of natural tissue, with altered cellular functionality is essential for determining how nano-biomaterials can direct tissue/biomaterial integration, thereby affecting the foreign body and wound healing responses.

Since the natural hierarchical scale of the body shows that most interactions take place at the nanoscale, the effect of nanotopographical cues on cell adhesion and proliferation, inflammation
and infection has been widely investigated [28-31]. Nanoscale features on implant surfaces may offer subsequent increased protein adsorption and cell adhesion [32, 33]. However, cells are not the first responders to implanted biomedical devices. Immediately following implantation, biomaterials initiate a complex cascade of events [34]. (Figure 2)

Figure 2: Schematic of the complex cascade of events following biomaterial implantation (©Barbara Symie Smith, 2012) [17].
As soon as the implant is inserted into the body, proteins on the surface and undergo a series of conformational changes that further dictate the adhesion and activation of platelets on the surface. Platelet activation further affects leukocyte recruitment to the surface and also the formation of a fibrin clot for wound healing. The inflammatory cell infiltration subsequently leads to acute and chronic inflammation, promoting additional cellular activation (monocyte differentiation into macrophages), apoptosis and intercellular communication (cytokine/chemokine). Intercellular communication takes place through the release of cytokines and chemokines such as TNF-α, IL-1, IL-7 etc. This cascade of events perpetuates additional cellular infiltration and activation (lymphocytes and fibroblasts) initiating granulocyte recruitment. The formation of foreign body giant cells (FBGC) potentially leads to fibrous encapsulation in a time period of around 3 weeks [17]. Hence, it is essential to control and optimize the foreign body response to cells to promote faster integration of implants.

**Titanium for implants**

Titanium (Ti) is one of the most widely used implant materials due to its superior specific strength, light weight, and corrosion resistance as a result of the formation of a protective titanium dioxide (TiO) layer [35, 36]. Other important factors contributing to the widespread use of titanium in biomedical implants include their impressive mechanical and biocompatible properties [36, 37], non-toxicity [38, 39] and ease of process ability [40]. Titanium has been used in a variety of clinical devices including joint prostheses [41], hard and soft tissue grafts [42], dental [43, 44] and craniofacial implants [45-47] and cardiovascular stents [48, 49]. Since their surfaces are constantly exposed to blood, there are some serious concerns that arise with regards to biomaterial integration [50, 51]. Although titanium and titanium-based alloys are among the
better choices for implantable biomedical devices, to date, all long-term implanted biomaterials have the potential of initiating physiological events in the form of inflammation, infection, thrombosis and fibrosis [52, 53]; potentially leading to complete implant failure. Hence, it is important to characterize the events that could sequentially lead to implant failure or beneficial implant integration [54].

**Infection associated with titanium implants**

Implant associated infections can start as soon as the implant is inserted into the body or during pre- and post-operative times. The implant surface is susceptible to infection mainly because of compromised immune capability at the implant/tissue interface. The initial adhesion of bacteria to the implant is an important event and dictates the progression of the infection of the implant. This adhesion is guided by several mechanical and biochemical cues and occurs in several phases [3]. The first phase includes adhesion of the bacteria on the surface based on mechanical and topographical features like surface energy, surface area, roughness, hydrophobicity, hydrophilicity, etc. The second phase includes biochemical and cellular interactions with the surface and strengthening of the bacterial adhesion via the release of several adhesins. In many cases, there is release of an extracellular polysaccharide matrix by the bacterial cells, which protects the bacteria from the immune response and causes growth of a biofilm, which is sustained by the nutrients trapped in this layer. [55] The biofilm protects adherent bacteria from the host defense system and bactericidal agents via several proposed mechanisms [12]. Since bacteria in a biofilm are difficult to combat with antibiotics because of antibiotic resistance, many techniques are being investigated to eradicate them another way. One way of doing this is preventing the initial adhesion of bacteria on the implant surface by using
techniques like coatings loaded with antibiotics that offer the advantage of local and timely release, thus increasing efficiency. The antibiotic-HA-coatings exhibit significant improvement in infection prophylaxis compared with standard HA coatings in vivo [1], but some problems still exist. The antibiotics such as vancomycin, ampicillin etc cannot be incorporated into the calcium phosphate coatings during its formation because of extremely high processing temperatures encountered in plasma spraying. Moreover, physical adsorption of these drugs onto calcium phosphates limits the loaded amount and release characteristics.

Regarding the risk of antibiotic resistance associated with the application of antibiotics-containing coatings, antimicrobial agents such as chlorhexidine, chloroxylenol, and poly(hexamethylenebiguanide)[56-60] may be good alternatives to include in a coating [57]. On account of a lower risk of drug resistance, such antimicrobial agents may be applied in vivo for a relatively long period of time. However, several reports have pointed out that the antimicrobial agents may cause cell damage [61]. Because of all the systemic and potential toxic effects of these antimicrobial strategies, a better solution would be to introduce nanoscale modifications on the surface of titanium implants to alter surface characteristics that reduce initial adhesion of bacteria.

**Nanomaterials in the body**

The natural hierarchy of scale in the human body proceeds from the nanoscale to the macometer range (Figure 3).
Figure 3: Schematic of the natural hierarchy of biological components in the human bone, showing multiple size scales (©Barbara Symie Smith, 2012) [1].

The interaction of micro-scale cells with their environment occurs through a number of nano-topographical and biochemical cues. Thus, material surfaces with biochemical [62-65] or topographical [66-68] modifications similar to that of the natural in vivo environment have been shown to elicit cell-specific functionality, enabled through biomimetic cues. Peptide modified [69, 70] and growth factor functionalized [71, 72] surfaces have shown increased control of cellular function. Various biomaterial surfaces such as nanoparticles, nanofibers [73, 74], nanopores, nanowires, nanostructured hydrogels and nanotube arrays have been fabricated and extensively studied. The goal of these nanostructures is to provide nanoscale cues for a variety of cell types [75]. A few examples are the control of macrophage response by hydrophilic carbon nanostructures as demonstrated by Khang et al [76], altering alumina nanocrystalline phases to reduce macrophage adhesion and proliferation via surface roughness control through nanometer surface features [77] and using carbon nanotubes impregnated with subventricular progenitor cells to promote recovery from stroke as demonstrated by Moon et al.[78]
Controlling the bone implant interface

In the search for methods to improve bone tissue response to titanium implants, much attention has been directed towards the potential use of chemical surface modification. Since the amount and type of products released from a metal implant in the body could affect bone tissue response, electrochemical characteristics of titanium might be very useful to estimate its functional performance. Ca-implanted Ti specimens show more active electrochemical behavior than non-implanted specimens, and easier formation of apatite with a good Ca/P ratio in the range of 1.38 to 1.60, revealing its potential as a bioactive material [79].

Different approaches are being used in an effort to obtain a desirable bone–implant interface. The ideal implant should present a surface conducive to inducing osseointegration, regardless of implantation site, bone quantity, bone quality, etc. As Kasemo and Lausmaa [80], among others, have described, biological tissues interact with mainly the outermost atomic layers of an implant. Although secondary and other by-product reactions will occur, the ‘primary interaction zone’ is generally about 0.1–1 nm. Consequently, much effort is being devoted to methods of modifying surfaces of existing biomaterials to achieve desired biological responses. As described by Ito et al [81], with respect to polymers, the approaches can be classified as physicochemical, morphological, or biochemical.

Physicochemical methods

Surface energy, surface charge, and surface composition are among the physicochemical characteristics that have been altered with the aim of improving the bone–implant interface.
Glow discharge has been used to increase surface free energy in order to increase tissue adhesion [82]. Considering the role of electrostatic interactions in many biological events, charged surfaces have been proposed as being conducive to tissue integration [83]. Calcium phosphate coatings have been extensively investigated because of their chemical similarity to bone mineral [83]. Each approach, however, has drawbacks. Increased surface energy does not selectively increase the adhesion of particular cells or tissues, and it has not been shown to increase bone–implant interfacial strength [84]. Contradictory results with charged materials in bone have been reported; indeed both positively [85] and negatively [83] charged surfaces were observed to promote bone formation. Although short-term clinical results have been encouraging [86, 87], dissolution of coatings as well as cracking and their separation from metallic substrates remain concerns [88, 89].

**Morphological methods**

Alterations in surface morphology and roughness have been used to influence cell and tissue responses to implants. Porous coatings were developed with the rationale that, because of mechanical interlocking, bone ingrowth would increase fixation and stability of the implant. Data from retrieval studies of orthopedic implants, however, indicate that only a relatively small portion of the available pore volume is filled with bone [90-92]. In addition to providing mechanical interlocking, surfaces with grooves can induce `contact guidance', whereby the direction of cell movement is affected by the morphology of the substrate [93]. This phenomenon has applications in preventing epithelial down-growth on dental implants and directing bone formation along particular regions of an implant. Mineral deposits in bone cell cultures can also be altered by surfaces with pits and grooves [94].
**Biochemical methods**

Biochemical methods of surface modification offer an alternative or adjunct to physicochemical and morphological methods. Biochemical surface modification endeavors utilize the current understanding of the biology and biochemistry of cellular function and differentiation. Much has been learned about the mechanisms by which cells adhere to substrates [95], and major advances have been made in understanding the role of biomolecules in regulating differentiation and remodeling of cells and tissues, respectively [96]. The goal of biochemical surface modification is to immobilize proteins, enzymes, or peptides on biomaterials for the purpose of inducing specific cell and tissue responses or, in other words, to control the tissue–implant interface with molecules delivered directly to the interface.

Although there are several reports of biochemical surface modification for modulating tissue responses to cardiovascular materials [97-100], this approach has received comparatively little, but increasing, consideration for orthopedic and dental applications [101-104]. This methodology has great potential for controlling initial bone–implant interactions. In contrast to calcium phosphate coatings, biochemical surface modification utilizes critical organic components of bone to affect tissue response. This includes the immobilization of enzymes, proteins and peptides on the biomaterial surface, for example, increase in bone growth by use of BMP coated surfaces [105].
Chapter 1

Hydroxyapatite and Calcium Phosphate Coatings

Titanium implants have been coated with HA and CaP for several decades using a variety of techniques like thermal spraying [106], dip coating [107], sol-gel process [108], sputter coating [109], plasma spraying [110], dynamic mixing [111], pulsed laser ablation [112], hot isostatic pressing [113] and electrophoretic deposition [114]. Biological fixation of implants is an important process and it includes integration of the bone tissue with the implant by overgrowth or ingrowth, without the use of bone cement. [115] Although HA and CaP are brittle and cannot be used for load bearing applications, coating of titanium implants with these allows for the rapid integration of these surfaces with bone. Coating implants this way can also replace the use of PMMA bone cement around these implants [116]. Following the implantation process, the release of CaP into the peri-implant region causes the saturation of body fluids resulting in the precipitation of apatite onto the implant surface forming a matrix rich with endogenous proteins. This helps in recruitment of osteogenic cells to the surface, thus helping in faster bone healing [117]. These coatings are also an effective way of making the implant surface more osteoconductive in nature without altering the bulk mechanical properties of the material and hence compromising on mechanical strength. Moreover, the Ha/CaP coatings take part in bone remodeling. The osteoblastic activity associated with HA degradation post-implantation causes the replacement of HA with autologous bone, much like the natural sequence of bone healing.[105]
The FDA and ISO [118] have certain prescribed conditions for HA coatings as shown in this table:

Table 1: HA coating requirements [118]

<table>
<thead>
<tr>
<th>Property</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness</td>
<td>Not specific</td>
</tr>
<tr>
<td>Crystallinity</td>
<td>62% minimum</td>
</tr>
<tr>
<td>Phase purity</td>
<td>95% minimum</td>
</tr>
<tr>
<td>Ca/P ratio</td>
<td>1.67–1.76</td>
</tr>
<tr>
<td>Density</td>
<td>2.98 g/cm³</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>&lt;50 ppm</td>
</tr>
<tr>
<td>Tensile strength</td>
<td>&gt;50.8 MPa</td>
</tr>
<tr>
<td>Shear strength</td>
<td>&gt;22 MPa</td>
</tr>
<tr>
<td>Abrasion</td>
<td>Not specific</td>
</tr>
</tbody>
</table>

Currently, plasma spraying is one of the most popular techniques for coating titanium implants and, for the purpose of this research, we compared it to electrophoretic deposition (EPD).

**Plasma Spraying vs. Electrophoretic Deposition**

Plasma spraying is a technique in which HA particles are injected into a plasma torch at high temperature and then projected onto a surface where they condense and fuse together to form a
The substrate being coated is usually roughened beforehand for mechanical retention of the coating and the thickness of the coating achieved can vary from a few micrometers to a few millimeters\[119\]. However, this method has a few disadvantages like the inability to coat small implants and complex shapes uniformly, high residual stress at the substrate/coating interface, the drastic variation in the crystallinity and composition of the initial CaP/HA powder and possible delamination of the coating from the implant surface \[119\]. Because of all these reasons, the clinical use of plasma sprayed HA implants is limited and other coating techniques are being investigated.

One such technique is electrophoretic deposition. Electrophoretic deposition (EPD) is a technique in which charged powder particles in a suspension or dispersion are deposited onto a conductive surface of the opposite charge on the application of an external DC voltage. It has advantages of short formation time, a simple apparatus, little restriction of the shape of substrate, no requirement for binder burnout as the green coating contains few or no organics. Compared to other advanced shaping techniques, the EPD process is extremely versatile since it can be modified easily for a specific application. For example, deposition can be made on flat, cylindrical or any other shaped substrate with only a minor change in electrode design and positioning. In particular, despite being a wet process, EPD offers easy control of the thickness and morphology of a deposited film through simple adjustment of the deposition time and applied potential \[120\].

**Experimental**

**Material synthesis and characterization**
Hydroxyapatite coatings were performed on titanium meshes (purchased from Alpha Aesar (Catalog no.7440-32-6)) by electrophoretic deposition (EPD) with a DC current at 151 V for a minute each in accordance with similar methodology as described in previous work [121]. Here, nanoscale hydroxyapatite was synthesized using a wet chemical synthesis process to produce 4 different particle sizes ranging from 110-170 nm, using Ca(NO$_3$)$_2$.4H$_2$O, KH$_2$PO$_4$, distilled water and ammonia in an acid digestion vessel with hydrothermal treatment followed by drying in the oven. The particle size was controlled by varying the reaction time (1.5-12 min) and the precipitation time (1.25-10 min) for the hydroxyapatite particles. Shorter reaction times and precipitation times produce smaller particle sizes. Following material synthesis, the particle size of the hydroxyapatite powders was determined using Transmission Electron Microscopy (TEM) (JEM 1010, JEOL) at a 80 keV accelerating voltage. X-ray diffraction (XRD) (PANalytical Philips Xray Diffractometer, X’Pert Pro) was used to confirm the crystalline structure of the particles.

After coating, samples were characterized under Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM) and contact angle analysis to confirm surface roughness and wettability of the coatings, all according to standard procedures [122]. Following material characterization, the samples were cleaned by washing with 70% ethanol for 5 minutes and then sterilized under UV light overnight. They were washed with PBS three times prior to seeding the cells on the surface.

**Macrophage adhesion and proliferation assay**

Macrophages purchased from ATCC (RAW 264.7 (ATCC® TIB-71™)) were cultured using EMEM (ATCC® 30-2003™) supplemented with 10% FBS (ATCC® SCRR-30-2020™) and a 1% penicillin-streptomycin solution (ATCC® 30-2300™). Cell adhesion and proliferation were determined using the MTS assay after 1, 3, 5 and 7 days of culture. The cells were seeded at
15,000 cells/cm² for the adhesion assay and at 3500 cells/cm² for the proliferation assay. The MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, G3581 Promega) reagent (1:5 ratio with cell culture media) was added to each well and incubated for 3 hours on the day of the measurement. At the end of incubation, a color change from pink to dark brown was observed, and absorbance from each well was measured by a SpectraMax M3(MT05412) at 490nm.

**Bacterial culture and adhesion**

Bacterial assays were conducted using *Staphylococcus aureus* (ATCC® 29740™), *Pseudomonas aeruginosa* (ATCC® 39324™) and an ampicillin resistant strain of *E.coli* (BIO-RAD Strain HB101 K-12 #166-0408 and pGLO Plasmid #166-0405). 0.03% tryptic soy broth (TSB) (Sigma Aldrich, Cat # 22092) and agar (Sigma-Aldrich, Cat # A1296) were used as the media for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. LB-agar containing 100µg/mL of ampicillin was used as the medium for *E. coli*. For each experiment, a single colony from the inoculation plate was used. A small amount of bacteria was taken from the stock culture, streaked onto an agar plate, and then used as the stock plate for further experiments. A single colony was picked from the TSB agar-plate, added to 5 mL of 3% TSB and incubated at 37°C in humidified conditions under a 5% carbon dioxide atmosphere for 16 hours. Samples were then placed in a 24 well non-tissue culture plate followed by a cleaning procedure. 10⁶ cells/mL were then exposed to the material surface and were incubated for 16 hours at 37°C. The bacteria solution was removed and the samples were rinsed twice with PBS while pipetting. Following this, the samples were transferred into 5 mL of PBS and sonicated for 5 min. Then, samples were transferred to 5 mL of fresh PBS buffer and sonicated for an additional 10 minutes. 10 mL of the bacterial suspension was diluted to create subsequent dilutions (10⁻³ and 10⁻⁴). Following this, 0.1 mL of each of the 10⁻³ and 10⁻⁴ dilutions were plated and incubated for 16 hours. The
number of bacterial colonies formed on each sample was counted and, using these values, the number of bacteria/mL was determined.

**Confocal and fluorescence microscopy**
To further verify the data collected by adhesion and proliferation assays, confocal microscopy was used to visualize the surface after growing the three types of bacteria mentioned above on the surfaces for 16 hours of incubation, fixing the cells using glutaraldehyde followed by successive dehydration using 50%, 70%, 90% and 100% ethanol and then staining with 2 µm Syto 9 dye. For the macrophages, the same fixation procedure was followed after growing cells on the samples for 1 day and then staining with 20 nm Syto 9 dye. The macrophages were visualized using fluorescence microscopy.

**Effect of protein adsorption on bacterial adhesion and macrophage adhesion and proliferation**
To study the effect of protein adsorption on these surfaces, they were incubated with 1%, 5% and 10% FBS and then the same procedure was used to perform bacterial adhesion and proliferation assays on all the samples.

**Statistics**
All cell and bacterial culture experiments were conducted in triplicate and differences between means were determined using analysis of variance (ANOVA) followed by Student t-tests.

**Results**

**Material surface characterization**
**TEM**
Figure 4 shows that the hydroxyapatite nanoparticles obtained were in a range of sizes varying from 110 to 170 nm. The particle size was varied using different reaction times. The particles were also analyzed for a distribution ratio.
Figure 4: TEM images of (a) 110±2 nm, (b) 130±5 nm, (c) 150±8 nm and (d) 170±8 nm hydroxyapatite powders. Scale bar=100 nm.

**XRD**

The phases formed in the crystalline nanophase hydroxyapatite were investigated using XRD analysis as shown in Figure 5. The XRD pattern of the synthesized nano HA powder was compared with JCPDS#9-432, which confirmed the presence of HA along with some amount of calcium phosphate (JCPDS#21-839) [15, 123]. The ratio of calcium to phosphate (Ca:P around 1.67) in the coating mimics the composition of bone which may lead to enhanced osseointegration as shown in our prior work[121].
Figure 5: XRD images of the (a) 170 nm, (b) 150 nm, (c) 130 nm and (d) 110 nm hydroxyapatite (HA) powders reveal the presence of crystalline nanometer sized HA phases, which were found to be consistent with the phases listed in the ICDD database.

SEM
The surface features of the samples were visualized using SEM as depicted in Figure 6. The EPD coated samples showed enhanced nanoscale features as compared to the plasma sprayed samples, which showed agglomeration of the micron sized HA particles due to the high heat involved in the coating process.
**Figure 6:** SEM images of the Ti surfaces coated with (a) 170 nm, (b) 150 nm, (c) 130 nm, and (d) 110 nm hydroxyapatite powders as well as (e) plasma sprayed micron sized hydroxyapatite onto Ti and (f) plain Ti. Scale bar-1 micrometer.

**AFM**

The surface morphology of the samples was analyzed by using AFM and the root mean square (RMS) surface roughness for each sample was measured as shown in Figure 7. The roughness value for the plasma sprayed samples was found to be 34.6 nm and it was 102.6 nm, 110 nm, 116 nm and 122 nm for the samples coated with 170 nm, 150 nm, 130 nm and 110 nm HA, respectively.
Figure 7: AFM images of the Ti surfaces coated with (a) 170 nm (RMS-102.6 nm), (b) 150 nm (RMS-110nm), (c) 130 nm (RMS-116nm) and (d) 110 nm (RMS-122nm) hydroxyapatite powders and (e) plasma sprayed micron sized hydroxyapatite onto Ti (RMS-34.6nm). (f) For the graph depicting roughness values, [(a) – (e), in order], data= mean ± standard error of the mean; N=3.

Contact angles
Contact angle analysis was used to test the hydrophilicity/hydrophobicity of the samples as shown in Figure 8. The samples coated with nanophase HA using EPD showed complete wetting of the surface whereas the plasma sprayed samples and the plain titanium samples were comparatively hydrophobic with contact angles of 48.8 and 79.0 degrees, respectively. Moreover as described by the Wenzel equation[33], adding surface roughness will enhance the surface wettability caused by the chemical composition of a surface.
**Figure 8:** Contact angle images of the (a) 110 nm HA coated onto Ti by EPD showing complete wetting; (b) 130 nm HA coated onto Ti by EPD showing complete wetting; (c) 150 nm HA coated onto Ti by EPD showing complete wetting; (d) 170 nm HA coated onto Ti showing complete wetting; (e) Micron sized HA coated onto Ti by plasma spray (48.8 degrees) and (f) plain Ti (79.0 degrees).

**Bacterial adhesion on nanophase HA**

To understand the effect of the various sizes of HA nanoparticles on bacterial growth, the samples were tested in the presence of three types of bacteria (S. aureus, P. aureginosa and Ampicillin resistant E. coli). Figures 9, 10 and 11 depict the growth of bacteria on the surfaces of the electrophoretically coated samples as compared to the plasma sprayed samples and the plain titanium after 16 hours of incubation. It is clear from the graphs that samples coated with 110 nm HA particles by EPD showed a significant decrease in bacterial activity as compared to the plasma sprayed samples and the plain titanium. There was almost 5 times more bacteria on the plasma sprayed samples as compared to the 110 nm samples and 2 times more bacteria on the plain Ti as compared to the 110 nm samples. Also, there was 2 times more bacteria on the 170 nm samples as compared to the 110 nm samples. For further verification of results, the EPD coated sample that performed the best i.e. the 110 nm was selected and seeded with S. aureus, P.aureginosa and Ampicillin resistant E.coli and incubated for 16 hours. The cells were then fixed on the surface, dyed and observed using confocal microscopy. Plain titanium samples as well as the samples coated with hydroxyapatite using plasma spray technique were used as control. As depicted in Figure 11, the 110 nm sample showed significantly less bacterial growth compared to the other two samples for all three types of bacteria. Thus, bacterial growth was
successfully reduced with the reduction in the size of the HA particles coated on the surface of titanium (without using antibiotics), making the surface more rough and hydrophilic in nature.

**Figure 9:** *S. aureus* colony forming units/ml on nano-sized HA samples (110, 130, 150, and 170 nm), plain titanium and plasma sprayed samples after 16 hours of incubation. Data are expressed as the mean ± standard error of the mean; N=3; *P <0.01 compared with plasma-sprayed-deposited hydroxyapatite on Ti; **P <0.01 compared with Ti (control); ***P <0.01 compared with samples coated with 170 nm hydroxyapatite.
Figure 10: *P. aureginosa* colony forming units/ml on nano-sized HA samples (110, 130, 150, and 170 nm), plain titanium and plasma sprayed samples after 16 hours of incubation. Data are expressed as the mean ± standard error of the mean; N=3; *P <0.01 compared with plasma-sprayed-deposited hydroxyapatite on Ti; **P <0.01 compared with Ti (control)
**Figure 11:** Ampicillin resistant *E. coli* colony-forming units/ml on nano-sized HA samples (110 nm), plain titanium and plasma sprayed samples after 16 hours of incubation. Data are expressed as the mean ± standard error of the mean; N = 3; *P <0.01 compared with Ti (control); **P <0.01 compared with plasma-sprayed-deposited hydroxyapatite on Ti.

**Macrophage growth and proliferation**
MTS assays were performed to obtain the rate of macrophage adhesion and proliferation over 1, 3 and 5 days on the samples coated with HA nanoparticles by EPD as compared to the samples coated with micron sized HA by plasma spray methods as shown in Figure 12. At 4 hours, there was approximately a 20% difference in cell adhesion between plain Ti and the 110 nm HA samples. After 1 day, there was an approximate 20% difference in cell viability in between plain Ti and the 110 nm HA surface and 15% difference between the plasma sprayed and 110 nm HA samples. After 3 days, there was almost a 10% difference in cell viability between the 150 nm and 110 nm HA samples and, after 5 days, the difference between 170 nm and 110 nm HA
samples was almost 15%. The obtained results were further verified using fluorescence microscopy as shown in Figure 13 where less macrophage density can be clearly seen on the 110 nm sample as compared to the plain titanium and the titanium coated with HA using plasma spray technique. So, with just the reduction in the size of the HA particles coated on these surfaces (without using pharmaceutical agents), one can down regulate the attachment and growth of macrophages, leading to a controlled inflammatory response and, hence, faster wound healing. Critically, the same approach (reducing the size of HA when coated on titanium) reduced bacteria and inflammatory cell responses. This is in contrast to a pharmaceutical-based approach in which different drugs would be needed to inhibit bacteria and limit inflammatory cell responses.

**Figure 12:** Macrophage adhesion and proliferation on nano-sized HA samples (110, 130, 150, and 170 nm), plain titanium and plasma sprayed samples after 4 hours, 1, 3 and 5 days. Data are expressed as the mean ± standard error of the mean; N=3; *P <0.01 as compared to plain Ti.
**P <0.01 as compared to the surface coated with plasma sprayed HA. ***P <0.01 as compared to surface coated with 170 nm HA.
**Figure 13:** Confocal and fluorescent microscopy image showing bacterial and macrophage density on Plain Ti, Plasma Sprayed HA on Ti and Ti coated with 110 nm HA by EPD respectively. (a), (b), (c) represent *S.aureus*; (d), (e), (f) represent *P.aureginosa*; (g), (h), (i) represent Ampicillin resistant *E.coli*. The scale bar for these images is 20 µm. (j), (k), (l) represent macrophage density on day 1 of culture. The scale bar for these images is 60 µm.

**Effect of protein adsorption on bacterial adhesion and macrophage adhesion and proliferation**

To study the effect of protein adsorption on bacterial adhesion and macrophage adhesion and proliferation, the samples were incubated with media (DMEM+ 1% penicillin and streptomycin) and 10%, 5% and 1% of FBS respectively. The aforementioned bacterial adhesion and macrophage adhesion and proliferation assays were then performed again on these samples. As shown in Figure 14, there was lesser adhesion and proliferation of cells with reduced levels of FBS in the media but the original trend observed in Figure 12 still continued. With bacteria, lesser bacterial adhesion was seen on the samples that had been incubated with greater amount of FBS containing media as depicted in Figure 15 and 16.
Figure 14: Macrophage adhesion and proliferation on nano-sized HA samples (110, 130, 150, and 170 nm), plain titanium and plasma sprayed samples after 4 hours, 1, 3 and 5 days. Data are expressed as the mean ± standard error of the mean; N = 3.
Figure 15: *S. aureus* colony forming units/ml on plain titanium, plasma sprayed titanium and 110 nm HA on titanium coated using EPD after 1 hour of incubation in 1%, 5% and 10% FBS and 16 hours of bacterial incubation. Data are expressed as the mean ± standard error of the mean;

N = 3; *P* <0.01 compared with plasma-sprayed-deposited hydroxyapatite on Ti, incubated in 1% FBS; **P** <0.01 compared with Ti (control), incubated in 1% FBS; ***P** <0.01 compared with samples coated with 110 nm hydroxyapatite, incubated in 1% FBS; ****P** <0.01 compared with samples coated with 110 nm hydroxyapatite, incubated in 5% FBS.
Figure 16: *P. aureginosa* colony forming units/ml on plain titanium, plasma sprayed titanium and 110 nm HA on titanium coated using EPD after 1 hour of incubation in 1%, 5% and 10% FBS and 16 hours of bacterial incubation. Data are expressed as the mean ± standard error of the mean; N = 3; *P* <0.01 compared with plasma-sprayed-deposited hydroxyapatite on Ti, incubated in 1% FBS; **P** <0.01 compared with Ti (control), incubated in 1% FBS; ***P*** <0.01 compared with samples coated with 110 nm hydroxyapatite, incubated in 1% FBS; ****P*** <0.01 compared with samples coated with 110 nm hydroxyapatite, incubated in 5% FBS.

**Surface roughness, bacterial adhesion and cell growth**

As discussed earlier, the insertion of an implant into the human body is followed immediately by the adsorption of proteins onto the surface. One of the most important parameters in controlling this adsorption is control of surface features like surface roughness and surface energy and thus by controlling these features, we can guide the adhesion of select proteins onto the implant.
surface. To that effect, we measured the surface free energy and the root mean square roughness of the coated surfaces and fitted it to a line using the equation provided by Khang et al:[77]

\[ \text{Es(RMSeff)} = \rho \times \text{RMSEff} + \text{Eo,s} \]

In this formula, Eo,s (ground surface energy) is a material property determined purely by chemical interactions between the surface and the interacting liquid for negligible surface roughness (not a function of nanoscale topography), and \( \rho \) is a coupling constant, which determines the extent of the influence of nanoscale topography on the surface energy of material. Line fitting the present experimental results gave, \( \text{Es} = -0.9064 \times \text{RMS} + 124.21 \) (R2 = 0.9757), where \( \rho = -0.9064 \) and \( \text{Eo,s} = 124.21 \).

**Figure 17:** The effect of the change of surface feature size i.e RMS(nm) on the surface free energy(mJ/m\(^2\))
The surface roughness was also correlated to the bacterial adhesion and the cell growth as depicted in the graphs below. A linear correlation was established in both cases leading to the hypothesis that increased surface roughness would lead to reduced bacterial adhesion and also reduced macrophage adhesion and proliferation.

**Figure 18:** Graph depicting correlation between surface roughness and number of bacterial colonies on the surface.
Figure 19: Graph depicting correlation between surface roughness and macrophage growth on days 1, 3 and 5 respectively.

Discussion

HA has generated a great amount of interest as an advanced orthopedic and dental implant material as it elicits a favorable biological response and forms a bond with surrounding tissues. It has been suggested that surface engineering can be used to promote implant biocompatibility [124]. One way to change surface properties of HA is to formulate nanophase HA which is inherently osteoconductive. Properties of nanocrystalline HA provide chemical and structural stability and therefore confer the advantage of long-term shape integrity. Finally, nano HA is mechanically robust and can support and protect bone tissue growth during the implantation procedure and within the body [125, 126]. Furthermore, from a literature review, it is known that, to create surfaces that effectively inhibit bacteria adhesion and growth, a hydrophilic, highly
hydrated, non-charged surface may help. Furthermore, the morphology of a surface should be nano-structured to reduce bacteria adhesiveness due to changes in surface energy and alterations in bacteria membrane tension inhibiting their adhesion. As established, the smaller the size of the nanofeature and/or nanoparticle, the greater the bactericidal activity (especially for sizes <30 nm). The shape and chemical composition of the nanoparticles can further decrease bacteria adhesion and growth [55].

According to the discussion above, it was hypothesized that implant surfaces coated with nanophase HA by EPD would display greater anti-bacterial properties. Indeed, it was found that, with a reduction in the size of the HA nanoparticles, the roughness of the surface increased at the nanoscale and hydrophilicity increased, leading to reduced bacterial adhesion.

As mentioned, insertion of an implant of any type within the body including insertion into bone evokes an inflammatory, and (usually) limited, foreign body reaction [17, 127]. During the use of an orthopedic implant, wear particles and other byproducts are generated from the bearing surfaces of joint replacements, and non-articulating implant surfaces that impinge or fret (e.g., screws in a plate for fracture fixation or spinal stabilization). Depending on the anatomical location, the number and characteristics of the wear byproducts and the host’s ability to degrade, isolate or detoxify the particles, these wear byproducts may be benign or harmful. A localized foreign body and chronic inflammatory reaction may occur, resulting in bone destruction, called osteolysis [128, 129].

Cell adhesive serum proteins, such as fibronectin, albumin and vitronectin play a critical role in cell adhesion to an artificial material, as the first thing that happens when an implant is inserted into the body is adsorption of serum proteins onto the surface. Hence, the effect of protein adsorption on bacterial adhesion and macrophage adhesion and proliferation was also
studied. It was seen that bacterial adhesion decreased with increased amounts of protein adsorbed on the surface. The original trend of lesser bacterial adhesion with reduced size of the HA nanoparticles still continued. Also, there was lesser macrophage adhesion and proliferation with reduced amounts of FBS in the growth medium but the original trend was still maintained.

The chronic inflammatory response to orthopedic implants and their wear debris has been mainly attributed to cytokines, chemokines and pro-inflammatory molecules released by macrophages, among other immune cells. Hence, a useful strategy for long-term stabilization of these implants might be the mitigation of macrophages at the implant site. With this study, it was established for the first time that a reduction in the size of the HA nanoparticles alone could help us establish coatings that were highly hydrophilic and nano-rough in nature and down regulate macrophage density on these surfaces. Coupled with prior studies by our research showing increased osteoblast (or bone forming cell) functions on these same substrates [19], this new data could help reduce a prolonged inflammatory period, thus, leading to faster wound healing after implantation.
Chapter 2

Composite coatings

Introduction

With the rise of antibacterial resistance and the failure of conventional approaches like drug releasing coatings and antibiotics to tackle this problem completely; scientists have been seeking inspiration from nature and exploring the potential of nanostructured surfaces. This can also be termed as a smart surface design wherein the physical barrier against bacterial adhesion by the nanostructures (as demonstrated in chapter 1) can be combined with a chemical defense mechanism as well. Nanocomposite ceramics offer exciting potential in this area. Just by a reduction in size with no change in substance; these materials can provide an adequate microstructural design that exhibits properties like greater reactivity, durability, wear resistance and so on.

This sort of physicochemical approach can be employed while developing a coating for orthopedic implants by the use of nanoscale hydroxyapatite particles combined with other nanoscale particles like cerium oxide and selenium oxide that have demonstrated antibacterial properties in the recent years.

Composite coatings using cerium oxide nanoparticles

Cerium oxide nanoparticles (CeO$_2$ NPs) are among the most widely used rare earth compounds finding applications in industrial and commercial products. Cerium oxide nanoparticles (or nanoceria) have enormous potential as antioxidant and radioprotective agents for cancer applications [130-134]. Of particular interest due to the acidic tumor environment, it has been reported by several groups that nanoceria’s antioxidant properties change with pH, due to its ability to switch from Ce$^{3+}$ to Ce$^{4+}$ [132, 135]. Optimal antioxidant properties have been reported
for nanoceria at physiological pH values, whereas, at slightly acidic (more tumor-like) pH values, it acts as an oxidant. This property of nanoceria may be utilized for cytoprotection towards healthy cells and be a selective killing agent to cancer cells where the tumor pH is lower than physiological values.

Another remarkable feature of nanoceria is its ability to be a free radical scavenger, which allows for its use as a therapeutic agent to fight cancer and other diseases due to its reduction of reactive oxygen species (ROS). In cancer, it has been widely reported that ROS can drive both initial cancer development and progression. In addition, by causing damage to DNA, ROS production also affects enzymes, which normally would combat such free radical production [136, 137].

Only four reports are available on the antibacterial activity of CeO$_2$ nanoparticles. Thill et al. and Pelletier et al. have reported the antimicrobial activity of CeO$_2$ nanoparticles against *Escherichia coli*, whereas Fang et al. showed the antibacterial activity against *Nitrosomonas europaea* [138-141]. Kuang et al. compared the toxicity of bulk CeO$_2$ against the NPs counterpart and found that the NP form of CeO$_2$ is more toxic to *E. coli* than the bulk form [141]. In addition, the knowledge of how the toxicity or cell viability changes with changes in the physical and chemical environment is absent. It is known that a change in the physical and chemical environment can significantly influence the toxicity of NPs. Thill et al. and Pelletier et al. provide some insight on the effect of pH, growth media, and particle size and concentration of NPs on the antibacterial properties of CeO$_2$ nanoparticles [141].

**Composite coatings using selenium nanoparticles**

Selenium (Se) is an essential trace element for human health, which is also an antioxidant that can remove free radicals in vitro. It improves the activity of seleno-enzymes and glutathione peroxidase, which can prevent free radicals from damaging cells and tissues in vivo. [142, 143]
The intervention of Se is also advantageous for reducing the risk of incidences in all forms of cancers, especially liver, prostate, colorectal, and lung cancers.[144] Se compounds have been used in nutrition and medicine for a long time. Topical antifungal medications that contain 2.5% Se sulfide have been recommended for treating tinea versicolor, which is caused by the Malassezia globosa fungus.[145] Compared with Se compounds, the elemental Se in a nano size [i.e., Se nanoparticles (SeNPs)] has become the new research target, because it is found to possess excellent bioavailability, low toxicity, and contribute to a wide spectrum of health-promoting as well as disease prevention and treatment activities. Hence, the use of both of these materials as composite coatings with nano HA should be investigated.

The successful reduction in bacterial adhesion and inflammation achieved with the reduced size of the hydroxyapatite nanoparticles motivated us to combine the mechanical barrier offered to the bacteria with other nanoparticles that demonstrated potential anti-bacterial activities to further enhance this effect and act as a stronger front against the many strains of bacteria encountered in a nosocomial setting. The main objective of this project is the development of a novel optimum coating for the surface of titanium which incorporates both nanophase hydroxyapatite for osseointegration and also other chemistries (nanophase ceria, nanophase selenium,) that offer increased resistance to bacterial adhesion and a controlled inflammatory response. The hypothesis is to be able to develop a composite coating with these chemistries on the surface of titanium implants via EPD and thus promote faster wound healing and increase implant lifetime; all without the use of any externally delivered antibiotics.

**Experimental methods**

**Synthesis of cerium oxide nanoparticles**
Cerium oxide nanoparticles were synthesized by a wet chemical synthesis process using cerium nitrate hexahydrate \((\text{Ce(NO}_3\text{)}_3\cdot6\text{H}_2\text{O})\) and sodium hydroxide (\(\text{NaOH}\)) as precursors. After these solutions were stirred for an hour, they were allowed to react for 24 hours at 150° C. The final product was then uniformly powdered and used for further studies.

**Synthesis of chitosan coated selenium nanoparticles**

Chitosan coated selenium nanoparticles were synthesized by dissolving 3.2 mg of chitosan in 10 ml 1% acetic acid. The chitosan solution was added dropwise to 10ml of 20mM sodium selenite solution under constant magnetic stirring and allowed to react for 10 minutes. Ascorbic acid (120mM) was then added dropwise to the mixture and the reaction was allowed to further proceed for 30 minutes. The final solution was then centrifuged at 10,000 rpm for 10 minutes and the resulting pellet was retrieved and redispersed with vigorous magnetic stirring in DI water.

**Coating of titanium surfaces**

The synthesized nanoparticles were applied in suspension to titanium surfaces using a combination of electrophoretic deposition and dip coating. The hydroxyapatite nanoparticles (1g) were formed into a colloidal suspension with 2-propanol (20 ml) as the main solvent and DMF (2ml) as the additive that provides better crack resistance. Prior to coating, the mixture was stirred for 15 minutes and sonicated for 15 minutes to provide a stable suspension. Coatings were performed using a DC voltage supply at 150 V for a minute each, using titanium as both the anode and the cathode. After coating, the samples were allowed to dry overnight and sintered for an hour in a furnace at 500 degrees Celsius. The samples were cooled, the ceria and selenium nanoparticle synthesis processes, described above, were carried out on top of these surfaces enabling a composite coating with hydroxyapatite and ceria and hydroxyapatite and selenium
respectively. The samples were further allowed to dry overnight before being subjected to characterization, cell and bacterial assays.

**Material characterization**

**DLS**

Dynamic light scattering (DLS) (Zetasizer, NanoZS, Malvern Instruments) experiments were performed to analyze the particle size and distribution of nanoparticles as shown in figure 20.

**Figure 20:** Graphs depicting the size distribution of ceria and selenium nanoparticles when suspended in DI water. The effective diameter of the ceria nanoparticles was found to be around 60.5 nm and the selenium nanoparticles were around a 221 nm respectively.

**SEM and EDAX**
The surface features of the samples were visualized using SEM as depicted in figure 21. Samples coated with HA depicted a mesh of rod like nano-features on the sample surface whereas the samples coated with HA-Ceria showed more agglomerated micron scale topography. The samples coated with HA-Se had a uniform distribution of spherical nanoparticles covering the surface of the sample. The chemical composition of the coatings was confirmed using EDAX analysis. Characteristic peaks were observed for Ca, P, Se, Ce, O, Ti and so on confirmed the presence of HA, Ha-Ceria and HA-Se coatings respectively.
Figure 21: SEM images and EDAX analysis of the Ti surfaces coated with (a) HA, (b) HA-Ceria and (c) HA-Se nanoparticles. Scale bar-1 micrometer.

**Bacterial adhesion**

To analyze how different composite coatings performed against different strains of bacteria, the samples were tested in presence of two types of bacteria (*S. aureus* and *P. aureginosa*). Figures 19 and 20 depict the growth of bacteria on the surfaces of the HA-Se and HA-Ceria coated samples as compared to the HA coated samples and the plain titanium after 16 hours of incubation. It is clear from the graphs that samples coated with HA-Se showed a significant decrease in bacterial activity as compared to the HA-Ceria samples, the HA samples and the plain titanium. For the *P. aureginosa* strain, there were almost 5 times more bacteria on the plain Ti samples as compared to the HA-Se samples and 2 times more bacteria on the HA-Ceria samples as compared to the HA-Se samples. Also, there was 4 times more bacteria on the HA-Ceria samples as compared to the HA samples but there was still fewer bacteria than the plain Ti itself.
**Figure 22:** *P. aureginosa* colony forming units/ml on plain Ti and Ti coated with nanoscale HA, HA-Se and HA-Ceria after 16 hours of incubation. Data are expressed as the mean ± standard error of the mean; N = 3; *P* <0.05 as compared to plain Ti.

For the *S.aureus* strain, there were almost 9 times more bacteria on the plain Ti samples as compared to the HA-Se samples and slightly more bacteria on the HA-Ceria samples as compared to the HA-Se samples. Also, there were 2 times more bacteria on the HA-Ceria samples as compared to the HA samples but there were still fewer bacteria than the plain Ti itself.
Figure 23: *S. aureus* colony forming units/ml on plain Ti and Ti coated with nanoscale HA, HA-Se and HA-Ceria after 16 hours of incubation. Data are expressed as the mean ± standard error of the mean; N = 3; *P* <0.05 as compared to plain Ti.

Macrophage growth and proliferation

MTS assays were performed to obtain the rate of macrophage adhesion and proliferation over 1, 5 and 7 days on the samples coated with HA-Se, HA-Ceria and HA nanoparticles as compared to the unocated plain Ti samples as shown in figure 21. At 1 day, there was approximately a 70% difference in cell adhesion between plain Ti and the HA-Se samples and a 63% difference in cell adhesion between the plain Ti and the HA-Ceria samples with the HA-Ceria samples showing greater adhesion. On day 5, there was an approximate 75% difference in cell viability in between plain Ti and the HA-Se surface and 6% difference between the plain Ti and HA-Ceria samples with plain Ti showing greater adhesion and proliferation of cells. On day 7, there was almost a 77% difference in cell viability between the plain Ti and HA-Se samples and an 18% difference between the plain Ti and HA-Ceria samples with the plain Ti showing greater cell adhesion and proliferation.
**Figure 24:** Macrophage adhesion and proliferation on nano-sized HA samples, plain titanium, HA-Se and HA-Ceria after 1, 5 and 7 days. Data are expressed as the mean ± standard error of the mean; \( N = 3 \), \(* P < 0.05\) as compared to plain Ti.

**Discussion**

Millions of medical devices are used each year, and, in spite of many advances in biomaterials, a proportion of each type of device becomes colonized by bacteria [146]. Topical devices (e.g., contact lenses) are colonized by bacteria as soon as they are placed on tissue surfaces; transcutaneous devices (e.g., vascular catheters) are progressively colonized by skin microorganisms; and even surgically implanted devices regularly become the foci of infection[146]. Implanted devices may be colonized by bacteria at the time of surgery or via a hematogenous route from a distant source. The most significant factor in the development of device-related infections appears to be the skill of the surgical team; prosthetic hips have been reported to
become infected in less than 0.2% of cases, but in as many as 4% of cases in less proficient facilities. Generally, large and complex medical devices that require long and complicated surgery for their placement are at high risk of bacterial infection [4]. However, when infection occurs, it can be life threatening.

Device-related infections may occur almost immediately post-surgery or may be very slow to develop, with overt symptoms occurring months or even years after the device is implanted. At the cellular level, implant associated infections include bacterial adhesion to a biomaterial surface and start as a competition for integration of the material into the host tissue or the bacteria into the material. For a successful implant, material integration occurs prior to bacterial adhesion thus preventing colonization of the implant surface. A time period of 6 hours post implantation is usually considered the decisive period, during which all preventative measures are taken to reduce bacterial adhesion. Over this time period, the implant surface is particularly susceptible to colonization and there is a possibility for biofilm formation. Infectious bacteria can be traced to several sources including the ambient atmosphere of the operating room, surgical equipment, clothing worn by medical professionals, resident bacteria on the patient’s skin, and bacteria already in the body. Although sterilization and the use of aseptic techniques greatly reduces the levels of bacteria found in hospital settings, pathogenic microorganisms are still found at the site of approximately 90% of all implants.

During biofilm formation, bacteria secrete an exopolysaccharide layer that retains nutrients and protects the microorganisms from the immune response. With the protective polysaccharide coating and sequestered nutrients, bacteria in biofilms exhibit extreme resistance to antibiotics [4, 147]. In some cases, it has been found that killing bacteria in a biofilm requires roughly 1000
times the antibiotic dose necessary to achieve the same results in a suspension of cells. The tremendous resistance of biofilms to conventional antibiotic therapy has prompted a great deal of research on synthetic surfaces and coatings that resist bacterial colonization [86, 146]. Coatings have been developed that reduce bacterial adhesion by altering the physicochemical properties of the substrate so that conditioning films do not form and/or bacteria-substrate interactions are not favorable. These coatings are referred to as “passive” and include surfaces modified with poly(ethylene glycol), poly(ethylene oxide) brushes, and hydrophilic polyurethanes, as examples. Unfortunately, the effectiveness of passive coatings for reducing bacterial adhesion is limited and varies greatly depending on bacterial species. The physicochemical properties of the surface (coating) can be masked by an adsorbed conditioning film, thereby diminishing their effectiveness [3, 4, 148]. A recent alternative approach to reducing bacterial adhesion is based on coatings that actively release antibacterial agents [147]. Such “active” coatings have been designed to release high initial fluxes of antibacterial agents during the critical short-term post-implantation period (several hours) to inhibit the initial adhesion of bacteria. Continued release beyond this short-term period is desirable because of protective fibrous capsule formation and tissue integration over a longer time period (weeks to months). Active strategies have shown to prevent biofilm formation both in vitro and in vivo.

The currently used strategies have several side effects like local inflammatory response, limited chemical stability and limited release of control kinetics. Nanomaterials have shown tremendous promise as potential bone tissue engineering materials. Nanostructured surfaces have been exploited as coatings for orthopedic implants in recent years. In many cases, nanoscale topography has shown to reduce bacterial adhesion and also to cause reduced inflammation [12, 148]. Their combination of a physical barrier to bacteria along with a chemical barrier are
interesting to explore. The aforementioned study showed that, for the first time, composite coatings of hydroxyapatite nanoparticles with ceria nanoparticles and hydroxyapatite nanoparticles with selenium nanoparticles demonstrated lesser bacterial adhesion for both gram positive and gram negative bacteria as compared to plain Ti samples. Moreover, the samples with hydroxyapatite and selenium nanocomposite coatings showed a bacterial reduction greater than seen with just plain hydroxyapatite.

Also, lesser macrophage growth and proliferation were seen on the samples coated with nanophase hydroxyapatite and selenium as compared to composite coatings of hydroxyapatite nanoparticles with ceria nanoparticles, nanophase hydroxyapatite coatings and plain Ti.

Chapter 3

Titanium dioxide coatings for orthopedic implants

Introduction
Currently, antibiotics are the only common therapy for treating medical-device-related infections. However, this treatment predominantly targets free-floating planktonic bacteria and does not effectively interrupt sessile bacteria formation in a biofilm. The efficacy of antibiotics toward treating bacterial infections is decreasing based on the rise of multiple antibiotic-resistant strains of bacteria including Staphylococcus aureus [40-41].

Nanotechnology is a promising alternative to overcome the problems faced in traditional administration of proteins, peptides, and new drugs found in the discovery pipeline. Many of these drugs used to fight infections have been shown to be poorly soluble in aqueous and organic media and show erratic absorption and poor bioavailability. Nanoparticles can be used to increase efficacy and decrease toxicity by controlling biodistribution, improving intracellular penetration, facilitating absorption through mucosa, and improving protection against degradation. An important strategy in the reduction of orthopedic infections is to develop implant materials that prevent initial bacteria adhesion onto implant surfaces [3]. Different material surface related properties, such as surface morphometry (porosity and roughness) and physico-chemical properties (surface energy, hydrophilicity, hydrophobicity, and functional groups) have an important role in controlling bacterial adhesion and growth. The biocompatibility of titanium is determined by the properties of the surface oxide layer in terms of its structure, morphology and composition [106]. Various physical and chemical treatments of the Ti surface have been proposed with the aim of enhancing osseointegration and improving initial stability. Approaches that have been beneficial to the biological performance of the implants include increasing surface roughness, and oxidation to form thicker or otherwise modified TiO$_2$ layers on the surface [149]. Various antibiotics (such as vancomycin) and others have been loaded into coatings on titanium implants [1]. Although a significant improvement has
been achieved with antibiotic loaded coatings, there are still many limitations regarding antibiotic incorporation and their controlled release. For example, antibiotics cannot be incorporated into most ceramic coatings during their formation because of the extremely high temperatures encountered in traditional coating processes like plasma spraying [5-7]. Moreover, the physical adsorption of antibiotics and other biomolecules on coatings restricts their loading capacity and release kinetics [8, 9]. Most importantly, any strategy that involves antibiotics will likely contribute to the generation of antibiotic resistant bacteria, which is a growing health problem for which we have no solution.

Bacteria adhesion-resistant implant coatings are produced by using physical structures on implants since such physical structures can change implant surface energy to minimize bacteria attachment. Bacteria have also been reported to respond to mechanical cues as implant topographical features of the right size and geometry may mechanically distort bacteria membranes to inhibit their adhesion and growth [10]. Multiple linear regression analyses have indicated that surface roughness and hydrophobicity are the main determinants for bacterial adherence. In fact, nanophase coated prostheses can also reduce bacterial adhesion more than conventionally surfaced prostheses [11]. Hence, altering surface roughness and energy by switching these surface features from conventional micron sized to nano sizes may decrease bacterial adhesion as established in previous studies [12]. Accordingly, novel nanostructured coatings significantly reduce up to five times bacterial surface colonization when compared to commercial state-of-the-art vacuum plasma sprayed coatings [13].

Titanium dioxide (TiO$_2$) generally exists in three primary phases (anatase, rutile, and brookite) with different sizes of crystal cells in each case [1]. Recently, TiO$_2$ has been utilized widely for the preparation of different types of nanomaterials, including nanoparticles, nanorods, nanowires,
nanotubes and mesoporous and nanoporous TiO$_2$ containing materials, due to its eminent photocatalytic abilities [2]. Regardless of scale, TiO$_2$ maintains its photocatalytic abilities. In addition, nanoscale TiO$_2$ has a surface reactivity that fosters its interactions with biological molecules, such as phosphorylated proteins and peptides [3], as well as some nonspecific binding with DNA [4].

Electrophoretic deposition (EPD) is a technique in which charged powder particles in a suspension or dispersion deposited onto a conductive surface of the opposite charge on the application of an external DC voltage. It has advantages of short formation time, simple apparatus setup, little restriction of the shape of substrate, and no requirement for binder burnout as the green coating contains few or no organics. Compared to other advanced shaping techniques, the EPD process is very versatile since it can be modified easily for a specific application. For example, deposition can be made on flat, cylindrical or any other shaped substrate with only minor change in electrode design and positioning. In particular, despite being a wet process, EPD offers easy control of the thickness and morphology of a deposited film through simple adjustment of the deposition time and applied potential [120]. The following study made the use of electrophoretic deposition to treat Ti-6Al-4V with a layer of titanium dioxide (TiO$_2$) to modify the surface by creating a nanotopography that prevents bacterial adhesion and proliferation while promoting osseointegration.

**Materials and methods**

**Nano treatment – Electrophoretic processing, EPD**

Using ethanol as the electrophoretic medium, the EPD cell was established using titanium as the anode and the titanium sample as the cathode. A potential difference of 40-80V for a minute per sample was used to obtain a uniform topography. The treated samples were dried overnight and
sintered in a furnace to finish the process. The processing parameters were adjusted to produce the two distinct surface nano-topographies for Ti-120 and Ti-160.

**Surface characterization**
After the nano treatment, the samples were characterized under scanning electron microscopy (SEM) to visualize the nanoscale topography [122]. The nano surfaces were also characterized by atomic force microscopy (AFM) to provide scale and quantitative topography measurements. Following material characterization, the samples were cleaned by washing with 70% ethanol for 5 minutes and then were sterilized under UV light overnight. They were washed with sterile PBS three times prior to seeding the cells on the surface.

**Bacterial assays**
Bacterial assays were conducted using 3 strains of bacteria: *Staphylococcus aureus* (ATCC® 29740™), *Pseudomonas aeruginosa* (ATCC® 39324™) and an Ampicillin resistant strain of *E. coli* (BIO-RAD Strain HB101 K-12 #166-0408 and pGLO Plasmid #166-0405). 0.03% tryptic soy broth (TSB) (Sigma Aldrich, Cat # 22092) and agar (Sigma-Aldrich, Cat # A1296) were used as the media. A single colony from the inoculation plate was used for each experiment. A small amount of bacteria was taken from the stock culture, streaked onto an agar plate, and then used as the stock plate for further experiments. One colony from the TSB agar-plate was added to 5 mL of 3% TSB and incubated at 37°C in humidified conditions under a 5% carbon dioxide atmosphere for 16 hours. 10⁶ cells/mL were seeded onto the material surface and incubated for 16 hours at 37°C. The supernatant was removed and the samples were rinsed twice with sterile PBS followed by sonication for 5 minutes in PBS. The supernatant was once more replaced with fresh PBS and the samples were sonicated for an additional 10 minutes. The final bacterial
suspension was diluted to create subsequent dilutions (10^{-3} and 10^{-4}). Following this, 0.1 mL of each of the 10^{-3} and 10^{-4} dilutions was plated and incubated for 16 hours. The number of bacterial colonies formed on each sample was counted and, using these values, the number of bacteria/mL was determined.

**Cell culture**

Osteoblasts from ATCC (catalog number C12720, population number 1-3) were grown in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin (HyClone; Thermo Fisher Scientific, Waltham, MA, USA). The samples were sterilized with 70% ethanol for 20 minutes and then rinsed thrice with phosphate-buffered saline (PBS). The cells were then seeded onto the sterilized samples. An MTS assay was used to determine cell adhesion and proliferation after 1, 3 and 5 days. The cells were seeded at 5,000 cells/cm^2 for the adhesion and proliferation assays and the medium was changed every other day. The MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, G3581 Promega) reagent (1:5 ratio with cell culture media) was added to each well and incubated for 3 hours on the day of the measurement. Absorbance from each well was measured by a SpectraMax M3(MT05412) at 490nm and a color change from pink to dark brown was seen.

**Statistical analysis**

Each experiment was completed in triplicate with new bacteria, osteoblasts, media and samples. A $P$-value of <0.01 was deemed to be statistically significant.
Results

Surface characterization
The surface of the EPD coated samples showed enhanced nanoscale features as depicted in figure 22 which is an SEM image of the Ti-120 and Ti-160 treated and untreated samples. The number following Ti in the nomenclature represents the atomic force microscopy root-mean-square (RMS) roughness value in nm. The untreated Ti control was much smoother at the nanoscale, possessing RMS values of 40 nm at this 2 by 2 micron AFM scan.

Figure 25: SEM images of the: a) untreated titanium, b) Ti-120, and c) Ti-160 as well as AFM of d) untreated titanium, e) Ti-120, and f) Ti-160 illustrating the nanoscale surface topography.
**Bacterial adhesion and growth**
The bacterial assays conducted demonstrated that there was a significant decrease in bacterial adhesion across all three strains of bacteria on the nanotextured Ti. There was a 95.6% decrease (a 1 log reduction) for *S. aureus*, a 90.2% decrease (a 1 log reduction) for *P. aeruginosa*, and an 81.1% decrease (close to a 1 log reduction), for ampicillin resistant *E. coli* for the Ti-160 surfaces. For Ti-120, there was an 86.8% reduction in *S. aureus*, 82.1% reduction in *P. aeruginosa*, and 48.6% reduction in ampicillin resistant *E. coli*. The graphical results are depicted in figures 23, 24 and 25. Graphs were plotted to show the number of bacteria versus the RMS roughness of the treated and control samples and it was seen that there was an almost linear relationship with $R^2$ values of 0.93797, 0.93855 and 0.99574 for *S. aureus*, *P. aeruginosa* and ampicillin resistant *E. coli*, respectively (figure 26). This demonstrates that the nanoscale roughness created was the primary factor resulting in decrease bacteria density on the nanotextured samples.

**Osteoblast proliferation**
Figure 27 illustrates the improved osteoblast proliferation achieved on the samples treated with Ti-120 versus untreated Ti. Combined with the results mentioned above, the results indicated that the Ti-120 surface topography led to decreased bacterial colonization with increased osteoblast proliferation. Importantly, such results were achieved by only changing the raw surface roughness values at the nanoscale and not using antibiotics or growth factors.
Figure 26: *S. aureus* colony forming units/ml on plain titanium and treated titanium. Data are expressed as the mean ± standard error of the mean; N=3; *P <0.01 compared with Ti-120, **P <0.01 compared with Ti-120 and Ti-160.

Figure 27: *P. aeruginosa* colony forming units/ml on plain titanium and treated titanium. Data are expressed as the mean ± standard error of the mean; N = 3; *P <0.01 compared with Ti-120, **P <0.01 compared with Ti-120 and Ti-160.
**Figure 28:** Ampicillin resistant *E. coli* colony forming units/ml on plain titanium and treated titanium. Data are expressed as the mean ± standard error of the mean; N = 3; *P < 0.01 compared with Ti-120, **P < 0.01 compared with Ti-120 and Ti-160.
Figure 29: The root mean square (RMS) roughness (as obtained by AFM) of the surface treated samples plotted against the number of bacterial colonies for all three strains of bacteria after 16 hours of culture.
Figure 30: Osteoblast adhesion and proliferation on Ti-120, Ti-160, and untreated titanium surfaces. Data are expressed as the mean ± standard error of the mean. * P<0.01 compared with Ti-120 on days 1 and 3, ** P<0.01 compared with Ti-120 and Ti-160 on days 1 and 3.
Discussion

Since the natural hierarchy of scale of the body shows that most interactions take place at the nanoscale level, the effect of nanotopographical cues on cell adhesion and proliferation, inflammation, and infection has been widely investigated [28-31]. Nanoscale features on the implant surface offer increased cell adhesion and protein adsorption due to changes in surface energy [32, 33]. The interaction of micro-scale cells with their environment occurs through a number of nano topographical and biochemical cues. Thus, material surfaces with biochemical [62-65] or topographical [66-68] modifications similar to that of the natural in vivo environment have been shown to elicit cell-specific functionality, enabled through biomimetic cues. Peptide modified [69, 70] and growth factor functionalized [71, 72] surfaces have shown increased cellular function. Various biomaterial surfaces such as nanoparticles, nanofibers [73, 74], nanopores, nanowires nanostructured hydrogels, and nanotube arrays have been fabricated and extensively studied. The goal of these nanostructures is to provide nano-scale cues for a variety of cell types [75]. Different approaches are being used in an effort to obtain the desired bone–implant interface. The ideal implant should present a surface conducive to osseointegration, regardless of implantation site, bone quantity, bone quality, etc. As Kasemo and Lausmaa [80], among others, have described, biological tissues interact mainly with the outermost atomic layers of an implant. Although secondary and other by-product reactions will occur, the ‘primary interaction zone’ is generally about 0.1–1 nm. Consequently, much effort is being devoted to methods of modifying surfaces of biomaterials to achieve desired biological responses.
The emergent antimicrobial properties of nanotextured surfaces reduce bacterial adhesion and proliferation. Materials such as ZnO, titanium dioxide (TiO$_2$), polymers, and carbon nanotubes have been good examples of this. TiO$_2$ is a naturally occurring oxide formed on titanium surfaces [43]. Nanotechnology serves as a promising tool in tissue engineering as it allows the generation of surfaces that mimic the constituent properties of natural tissues. Altering the surface energy of implants can subsequently change protein adsorption, alter protein bioactivity and promote cell function while at the same time reducing bacterial adhesion and proliferation. This is now a popular concept in the industry and nanoparticles are being incorporated into paints, dyes, etc. Nanoparticles disrupt the bacterial cell membrane by penetrating it or causing free radical formation. This allows researchers to selectively target bacterial cells while maintaining healthy cell function.

**Conclusion**

Biofilm formation and bacterial infection of implants are complex issues in which many variables are involved. Bacteria that are present throughout the body can be treated by the host immune system and traditional antibiotic treatment. However, once the bacteria have colonized and form a biofilm, that then causes the course of treatment to become more challenging since biofilms are not very responsive to traditional antimicrobial treatments. *S. aureus* is the most common bacterium present in clinical infections and biofilms on medical devices [42] where preventing the adhesion and colonization of bacteria can be a tremendous benefit to reducing infections and facilitating the treatment of free floating planktonic bacteria not adherent to implant surfaces. Technology that can
create nanoscale surface roughness, like that presented here, can reduce bacteria colonization and, thus, may have a synergistic effect in treating infections. These promising results show that just by changing the surface topography of the implant surface by creating Ti-120 and Ti-160 nanotextured TiO$_2$ surfaces, one can significantly reduce bacterial adhesion and growth for both gram-positive and gram-negative bacteria. A reduction in bacteria adhesion and growth was also observed for antibiotic resistant bacteria, as well as inducing upregulation of osteoblast activity for Ti-160. Finally moving forward from these results, with the aid of a biological, mechanical, and mechanistic understanding of the coatings, we can develop optimum coatings that offer improved osteoblast adhesion and proliferation, while reducing bacterial colonization, all without the use of the antibiotics.
Chapter 4

Conclusions and future directions

Conclusions

Thus far this project has demonstrated that:

1. Electrophoretic deposition (EPD) is a better coating technique as compared to plasma spraying as it offers better control over coating thickness on complex shapes and is more cost efficient.

2. Electrophoretic deposition of nanophase hydroxyapatite onto titanium surfaces offers reduced bacterial adhesion and reduced macrophage adhesion and proliferation as compared to plasma sprayed hydroxyapatite on titanium surfaces.

3. The smaller the size of nanophase hydroxyapatite coated onto titanium using EPD, the lesser the macrophage adhesion and proliferation and also the lesser bacterial adhesion.

4. The effect of protein adsorption on bacterial adhesion and macrophage adhesion and proliferation was also studied. It was seen that bacterial adhesion decreased with increased amounts of protein adsorbed on the surface. The original trend of lesser bacterial adhesion with reduced size of the HA nanoparticles still continued. Also, there was less macrophage adhesion and proliferation with reduced amounts of FBS in the growth medium but the original trend was still maintained.

5. Composite coatings that combine nanoparticles with already demonstrated antibacterial properties like selenium and ceria with hydroxyapatite can offer an advanced physio-chemical defense mechanism against different strains of
bacteria; both gram positive and gram negative and should be investigated further for use in orthopedic implants.

6. Composite coatings with nanophase hydroxyapatite and selenium and nanophase hydroxyapatite and ceria also offer a more controlled inflammatory response as compared to plain Ti and Ti coated with nanoscale hydroxyapatite.

7. Ti-120 and Ti-160 nanotextured TiO$_2$ surfaces, one can significantly reduce bacterial adhesion and growth for both gram-positive and gram-negative bacteria.

8. The combined effect of lesser bacterial adhesion and mitigated inflammatory response could lead to a faster wound healing and osseointegration of the bone implant.

**Proposed future directions**

In the future, the following aims should be investigated:

1. **Mechanical testing:**

   The shear fatigue strength, the static shear strength and the static tensile strength of the surface coating shall be tested out using standard protocols. The adhesion and cohesion strength of the coating shall be tested using various tests like ASTM C633, ASTM F 104 and the ASTM F 1147. The ASTM C633 test method covers the determination of the degree of adhesion (bonding strength) of a coating to a substrate or the cohesion strength of the coating in a tension normal to the surface. It consists of coating one face of a substrate fixture, bonding this coating to the face of a loading fixture, and subjecting this assembly of coating and fixtures to a tensile load normal to the plane of the coating. The ASTM F 1044 is a standard test method for the shear testing of calcium phosphate coatings and metallic
coatings. This test method covers shear testing of continuous calcium phosphate coatings and metallic coatings adhering to dense metal substrates at ambient temperatures. It assesses the degree of adhesion of coatings to substrates, or the internal cohesion of a coating in shear, parallel to the surface plane. The ASTM F 1147 test method is used for tension testing of calcium phosphate and metallic coatings and can provide information on the adhesion and cohesion strength of the coating under uniaxial tensile stress. The abrasion of a coating due to the insertion of the implant into bone during surgery and due to micromotion after surgery will be simulated in a test method which involves rubbing a block of hardened material against a flat test coupon consisting of the substrate and the modified surface under study. A range of different normal loads will be tested, the minimum load being the lowest load that produces a detectable loss in the modified surface and the maximum load being either high enough to remove at least 50% of the modified surface or being the lowest load that will cause significant plastic deformation/densification of the modified surface. Upto 5 different loads between the minimum and maximum loads shall be studied. Plastic deformation shall be verified with micrographs. All these test methods will help in quality control and design of an optimum coating via the process of elimination.

2. **Biological testing:**
All the coated surfaces that satisfy the criteria mentioned in the mechanical testing phase shall then be subjected to biological testing. Growth of osteoblasts shall be assessed on these surfaces, as these are the cells responsible for bone repair,
growth and development in the human body. Macrophage adhesion and proliferation shall be investigated over a number of days, as they are the primary cells that govern immune response. Bacterial response shall be investigated by growing both gram positive and gram negative species of bacteria on the coated surfaces. Ideally the optimum coating would show reduced bacterial adhesion, reduced macrophage adhesion and proliferation and increased osteoblast adhesion and proliferation.

3. **Studying protein adsorption on coated surface and the release of inflammatory cytokines:**

   The formation of an oxide layer on implant materials is fundamental to its biological properties, as it affects the surface energy of the material, resulting in a differential response in vivo. This is due to a change in the adsorption of serum components, which may include growth factors, cytokines or other macromolecules enhancing cellular attachment of proteins such as fibronectin. It may have different characteristics, depending on the actual composition of the implant material (e.g. pure titanium or alloy).

   The substrate and the surface topography, as well as the coating of an implant greatly affect the success rate of bone fixation, and four interrelated factors have been proposed which influence cell behavior: composition, surface energy, topography and surface roughness. It has been shown that a number of factors (TGF-β1, PGE2) are produced by cells as a result of exposure to implant surfaces, and the quantity in which they are released is dependent on the titanium surface, with an increased production elicited by cells attaching to rougher surfaces.
Most of these cells like osteoblasts, macrophages etc., are established cell lines that require the secretion of these specific cytokines to maintain a specific phenotype. If insufficient signals are provided by the substrate then a different phenotype is induced.

Protein adsorption on implant surface is the first event to occur after insertion of an implant into the body. This greatly influences the attachment and proliferation of bacteria and cells on the surface. Hence, to investigate the mechanism for reduced macrophage adhesion and proliferation and reduced bacterial adhesion, the release of inflammatory cytokines like TNF-α, IL-1, IL-6 and nitrite released shall be studied via the use of qPCR. Protein adsorption shall be determined using ELISA assay. Factors affecting protein adsorption such as as surface roughness, surface energy and wettability shall be linked together using the equation provided by Khang et al:[77]

\[ F(E_s) = (\kappa + \beta) E_s - \kappa E_{0,s}. \]

Here, \( \kappa = \alpha / \beta \), \( \alpha \) is a coupling constant of protein adsorption with roughness, \( \beta \) is a coupling constant of protein adsorption with surface energy and \( \rho \) is the coupling constant of surface energy with roughness, \( E_{0,s} \) is the ground surface energy (when \( r_{eff} = 0 \), determined just by chemical interaction) and \( F(E_s) \) is the total protein adsorption.

4. Establishing the mechanism of infection:

Bacterial adhesion to a material surface can be described as a two-phase process including an initial, instantaneous and reversible physical phase (phase one) and a time-dependent and irreversible molecular and cellular phase (phase two).
Bacterial adhesion usually occurs by attraction, adsorption and attachment of the bacteria to the surface. Bacteria move to or are moved to a material surface through the effects of physical forces, such as Brownian motion, van der Waals attraction forces, gravitational forces, surface electrostatic charge and hydrophobic interactions. The initial physicochemical interactions between substrate and bacteria, are effected by both specific forces that act over a wide range and non specific forces that act over a distance smaller than 5nm. The long range forces are responsible for transporting bacteria to the surface and upon closer contact the short range forces like hydrogen bonds, ionic bonds, hydrophobic interactions etc take over and are responsible for adhesion behavior. Hence it is important to study the surface topography of the implant surface carefully and establish what role all these forces play by obtaining experimental data like EDS, AFM, SEM, Zeta potential and contact angle measurements using various fluids with different viscosities.

After this initial phase of bacterial adhesion comes the molecular and cellular phase of bacterial adhesion where the bond between the bacteria and surface is strengthened by way of release of certain adhesins which adhere to host determinants such as glycolipids, proteoglycans, surface, and matrix proteins. Some strains of bacteria also form a biofilm layer after this step, which is protected by an exopolysaccharide layer which retains nutrients and protects the bacteria from the immune system. These adhesins will be observed using techniques like flow cytometry, SDS PAGE, etc by tagging the bacteria with fluorescent dyes to observe specific adhesins.
Several other environmental factors also influence bacterial adhesion like the local pH of the area, the presence and absence of serum proteins like fibronectin and albumin, presence or absence of antibiotics, etc. Certain pH values are more appropriate for bacterial adhesion as compared to others and to this effect, the adhesion of bacteria on the synthesized samples shall be studied under different pH conditions. Fibronectin causes an increase in the adhesion of some strains of bacteria like *S.aureus* whereas Albumin causes a decrease in adhesion of both gram positive and gram negative strains of bacteria.[150] Hence the effect of both these proteins on the adhesion of bacteria on synthesized samples shall be observed.

Since biofilm formation is a major cause of implant rejection, metabolic assays shall be conducted for quantification of bacterial viability in biofilms. These assays are indirect methods based on the detection of metabolic products produced by bacteria and have the advantage of being able to assess viability without sample manipulation since these assays generally do not require the removal of the biofilm from the adherent surface. Also, they are simple, fast and perfectly suitable for high-throughput quantification of biofilms grown in a microtiter plate. Some of these assays include colorimetric biomass (crystal violet), Syto 9, resazurin and fluorescein diacetate (FDA).

5. **Optimum coating:**

Finally with the aid of the biological, mechanical and mechanistic understanding of the newly developed composite coatings, coatings that offer improved osteoblast
adhesion and proliferation; reduced bacterial adhesion; reduced macrophage adhesion and proliferation and greater shear, tensile and loading strength shall be selected. Coatings that do not satisfy these criteria shall be eliminated.
Part B- ECM like scaffolds for improved in vitro cell culture

Chapter 1

Abstract

Clinical in vitro studies and basic research rely heavily on cell culture systems as their basic tools. The current conventional 2D systems are a poor representation of the actual physiological conditions in a living organism and this may seriously compromise the reliability or significance of data obtained from experiments, which use such 2D systems. Advances in materials chemistry, materials fabrication, processing technologies, and developmental biology have led to the design of 3D cell culture matrices that better mimic the geometry, chemistry, and signaling environment of the natural extracellular matrix. Therefore, we present here a comparative study on selected 3D and 2D cell culture substrates for NIH 3T3 fibroblast, human osteoblasts (bone forming cells), endothelial cells and mesenchymal stem cells. The 3D system used was a novel XanoMatrix nanofibrous scaffold mimicking the natural nanometer dimensions of natural tissues and traditional Corning and Falcon cell culture petri dishes were used as reference 2D culture systems.

Introduction

Tissue engineering is a multidisciplinary research field that has gained popularity over the past few years. It involves the repair/regeneration of diseased/damaged tissue to restore such tissue to partial or full functionality. [151] Tissue engineering scaffolds are often designed for a variety of functions and have functional properties that enable them to perform those functions. In general, all tissue engineering scaffolds must be porous;
bio-compatible; promote the attachment, migration proliferation and differentiation of cells in a natural way; promote the secretion of ECM proteins in a natural way; and degrade into non toxic materials that can be eliminated from the body safely. In addition to these characteristics, tissue engineering scaffolds must have a large volume fraction of interconnected pores to facilitate the migration of cells as well as diffusion of growth factors and nutrients. The pore size must be tailored according to specific cell morphologies, they must have sufficient mechanical integrity to be handled easily during surgery, and lastly they must degrade at a controllable rate so that the targeted tissue becomes fully formed via ECM deposition as the scaffold is fully resorbed.

Most tissues are 3D for proper cell functions and are responsible for providing cells with a specific growth environment; structure; water diffusion; as well as cytokine, growth factor and nutrient availability. [152] Therefore, in order to expedite the process of cell growth and proliferation for tissue growth and regeneration, it is necessary to develop a 3D scaffold for growing cells.[152-154] Also, if designed correctly, these scaffolds can help in the development of new vasculature [154, 155] and they could actively participate in the tissue regenerative process through the release of growth/differentiation factors, present in their structure.[156]

The next step after selecting an adequate biodegradable polymer is to develop or choose an adequate fabrication process. In order to do so, and to be sure that all the scaffold characteristics are fulfilled, the chosen processing technique should not adversely affect the biocompatibility and chemical properties of the implant and it should be consistent with regards to maintaining porosity, pore distribution, pore size and interconnectivity.
Furthermore, these results should be able to be repeated when more than one scaffold is produced from the same batch with the same processing technique. Thus, based on all this information, despite their popularity, it is clear that traditional cell culture substrates are poor replicates of the complex 3D in vivo complex extracellular matrix environment which allow cells to communicate with each other and their environment by responding to the release of cytokines to elicit specific responses. In traditional 2D cell culture systems, cells are often grown on a flat surface that lacks all these characteristics and are not true representatives of the natural in vivo system. The growth of a cellular monolayer is significantly different than a 3D cellular structure. This 2D confinement is far removed from the aforementioned 3D complexities of living tissue, yet such systems are widely used today across all aspects of in vitro assays. Such problems are exacerbated when conducting cell culture.

There are a variety of technologies available that enable the growth of cells in a 3D environment but these technologies have not necessarily been developed for performing general cell culture assays in day to day lab experiments. These include naturally occurring materials as well as products fabricated from naturally derived and synthetic polymers. Natural substrates (such as alginate, which is a seaweed-derived material), have been used to support cell growth in a number of ways including cell encapsulation. It is true that this technology enables the growth of cells in a 3D environment but this spherical mass is not necessarily very useful as the distribution of cells throughout this mass could be different with different rates of mass transfer. An alternative, and one of the most early successful approaches, has been the culture of cells on biodegradable polymers such as poly(glycolic acid), poly(lactic acid) and their copolymers poly(lactic-
co-glycolic acid).[162] Hydrogels are also common and have been successfully used to support ex vivo 3D stem cell growth for a variety of systems, such as bone, cartilage and nervous tissues. [163]

Through the years a series of processing techniques (such as solvent casting, phase inversion, fiber bonding, melt based technologies, high pressure based methods, freeze drying and rapid prototyping technologies) were developed with the aim of producing scaffolds with adequate properties for tissue engineering but all these processes have a lot of variables involved which makes them hard to monitor for consistency and also less viable economically. XanoShear™ is a novel method that is capable of creating nanofibers through a combination of shear force and polymer phase separation without the use of nozzles or spinnerets and is different from its counterpart electrospinning where the use of electric charge is made to draw individual fibers to a grounded surface.(Figure 28)

![Figure 31](image)

**Figure 31:** Basic schematic of the XanoShear™ process.

A polymer solvent, which is miscible with the medium but still precipitates in the medium is chosen and the bulk polymer is introduced into the viscous medium under shear. The ultra low facial tension between the polymer droplets and the medium gives
rise to thin individual fibers with a high degree of stretching and great surface area. The shear force causes the solvent to be highly stretched in parallel as the solvent diffuses out of the droplets leaving behind polymer fibers in a diameter range of 50 nm - 2 µm. This process can be used to draw nanofibers from a variety of different materials. Functional groups of interest can also be added to the material by simply adding them to the polymer solvent. Due to the above and the relatively unstudied application of nanofibers prepared this way for applications involving cell culture, the objective of the present study was to determine the functions of different cells on a biologically-inspired nanofibrous XanoMatrix compared to traditional tissue culture polystyrene dishes. The XanoMatrix tested here is made with biocompatible materials PET (Polyethylene terephthalate) and cellulose acetate (CA). Polyethylene terephthalate (PET), also known as Dacron, has been widely used as a prosthetic vascular graft and has shown excellent mechanical strength and good biocompatibility. [164] CA is an industrially important cellulose ester with good mechanical and wetting properties. CA nanofibers have also increasingly been used in tissue engineering. [165] Improving cell functions on tissue cell culture dishes to match that on natural materials, could lead to improved identification of medical devices and drug delivery materials as well as an understanding of disease prevention, diagnosis and treatment. XanoMatrix scaffolds offer the advantage of mimicking the natural stem cell environment while combining the advantage of nano-fibered tortuous beds and supports.

Moreover, in order to develop a tissue engineered product for bone implants, it is necessary to develop a scaffold out of a material that mimics the natural properties of bone and, thus, helps bone growth by providing appropriate nutrients and recruitment of
the right cells by releasing necessary cytokines and growth factors. [166] Up to now, several materials (such as metals, ceramics and polymers) from both natural or synthetic origins have been proposed as improved bone implants. However, all of these materials were first tested in vitro using non-biologically-inspired 2D cell culture systems and have current orthopedic implant lifetimes of less than 15 years. In addition, most metals and ceramics are not biodegradable. Ceramics have been a popular choice for medical devices since they can be from natural (e.g., coralline hydroxylapatite (HA)) origin or synthetic origin (such as synthetic HA or β-tricalcium phosphate (β-TCP)).[167] Their osteoconductive and osteoinductive nature makes them prime candidates for bone tissue substitutes as they promote faster bone growth and regeneration. This property has been highlighted in numerous research efforts [168, 169]. Even though these materials offer some great advantages, they have a few major drawbacks like poor mechanical stability, brittleness, and poor load bearing capacity. The activity of osteoclasts (bone resorbing cells) in vivo makes the degradation rate of ceramics difficult to predict and this offers another problem for their use because faster degradation compromises the mechanical stability of implants. In addition, during degradation of calcium phosphates, an increase in the local and systemic concentration of Ca and P ions may cause ion imbalances and cell death, as demonstrated by Adams et al.[170] As an alternative to the above referred to materials, biodegradable polymers have emerged an ideal material for bone tissue engineering since once bone forms and the polymer degrades, a completely natural environment is created. [154, 171]

The next step after selecting an adequate biodegradable polymer for orthopedic applications is to develop or choose an adequate processing technique. In order to do so,
and to be sure that all necessary scaffold properties are fulfilled, a polymer processing technique should not adversely affect the biocompatibility and chemical properties of the implant and should be consistent with regards to maintaining porosity, pore distribution, pore size and interconnectivity. [157] Furthermore, the polymer processing technique should consistently produce the same scaffold from batch to batch.

Thus, based on all this information, there is an essential need to identify i) a new 3D in vitro cell culture substrate that can provide for a more biologically-inspired matrix for determining cell function than what we use today and ii) to explore whether that same 3D scaffold may be suitable for orthopedic tissue engineering applications.

Due to the above and the relatively unstudied application of nanofibers as 3D cell culture scaffolds, improved bone tissue scaffolds and vascular graft substrates, the objective of the present study was to determine the functions of NIH 3T3 fibroblasts, osteoblasts, endothelial cells and mesenchymal stem cells on a biologically-inspired nanofibrous XanoMatrix compared to traditional tissue culture polystyrene dishes. XanoMatrix scaffolds can be easily placed in traditional 6-well, 48-well and 96-well formats as well as 10”x11” sheets for custom shapes and sizes.

**Materials**

The PET and cellulose acetate nano-fiber scaffolds (XanoMatrix) were fabricated by XanoShear technology as described above by Xanofi. Sterile 96 well cell culture dishes were obtained from Xanofi for all of the studies below. Sterile traditional plasma treated polystyrene cell culture dishes were obtained from Corning and Falcon for comparative analysis. Adhesion and proliferation studies were performed with osteoblasts and endothelial cells as described below.
Methods

Surface characterization
Contact angle analysis
The wettability of the samples was determined using the Pioneer contact angle 300 goniometer. The tests were performed using 3 different liquids each with different viscosities. The liquids used were distilled water, polyethylene glycol (PEG) and also 70% ethanol.
Figure 32: Contact angle images of the surfaces of: a) Corning (81.53 degrees, distilled water); b) Falcon (76.724 degrees, distilled water); c) XanoMatrix (121.593 degrees, distilled water); d) Corning (12.54 degrees, 70% ethanol); e) Falcon (10.42 degrees, 70% ethanol); f) XanoMatrix (0 degrees, 70% ethanol); g) Corning (21.7 degrees, PEG); h) Falcon (10.4484 degrees, PEG) and i) XanoMatrix (0 degrees, PEG). PEG – polyethylene glycol.
Scanning Electron Microscopy (SEM)

A Hitachi 4800-S SEM with a voltage of 5.0 kV and a magnification of 250 was used to visualize the surface of the samples.

Figure 33: SEM images of the surfaces of a) Corning; b) Falcon and c) XanoMatrix surfaces of the cell culture petridishes. Scale bar = 500 um
Figure 34: AFM images of the surfaces of a) Corning; b) Falcon and c) XanoMatrix surfaces of the cell culture petri dishes. Surface roughness is 27.84nm, 30.027 nm and 62.182 nm respectively.

Cell culture
Osteoblasts purchased from ATCC (catalog number C12720, population number 1-3) were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin (HyClone; Thermo Fisher Scientific, Waltham, MA, USA). Endothelial cells (RFPEC obtained from Dr. Eno Ebong’s lab at Northeastern
University) were cultured in Eagle’s Minimum Essential Medium (EMEM) (ATCC® 30-2003™) supplemented with 10% Fetal Bovine Serum (FBS) (ATCC® SCRR-30-2020™) and a 1% penicillin-streptomycin solution (ATCC® 30-2300™). NIH 3T3 fibroblasts were cultured using Eagle’s Minimum Essential Medium (EMEM) (ATCC® 30-2003™) supplemented with 10% Fetal Bovine Serum (FBS) (ATCC® SCRR-30-2020™) and a 1% penicillin-streptomycin solution (ATCC® 30-2300™). Mesenchymal stem cells purchased from ATCC (catalog number PCS-500-012™, population number 1-3) were cultured in Dulbecco’s Modified Eagle Medium supplemented with 17.5% fetal bovine serum (ATCC® 30-2300™) and 1% penicillin (HyClone; Thermo Fisher Scientific, Waltham, MA, USA). The samples were sterilized with 70% ethanol for 20 minutes then rinsed thrice with phosphate-buffered saline (PBS). MTS assay was used to determine cell adhesion and proliferation after 1, 7, 14 and 21 days. The cells were seeded at 5,000 cells/cm² and 3,500 cells/cm² for the adhesion assays and proliferation assays. For the proliferation assay, the media was changed every other day. The MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, G3581 Promega) reagent (1:5 ratio with cell culture media) was added to each well and incubated for 3 hours on the day of the measurement. Absorbance from each well was measured by a SpectraMax M3(MT05412) at 490nm and a colour change from pink to dark brown was seen.

Confocal Microscopy

The data gathered by the MTS assays was verified by using confocal microscopy after fixing the cells using glutaraldehyde followed by successive dehydration using 50%, 70%, 90% and 100% ethanol and then staining with 20 nm Syto 9 dye.
**Figure 35**: NIH 3T3 fibroblast adhesion and proliferation on Corning, Falcon and XanoMatrix cell culture petri dishes. after 4 hours, 1,7,14 and 21 days respectively. Data are expressed as the mean ± standard error of the mean; N=3; * p<0.01 as compared to Corning and Falcon petri dishes on the 7th day of culture. **p<0.01 as compared to the Corning and Falcon petri dishes on the 14th day of culture. ***p<0.01 as compared to Corning and Falcon petri dishes on the 21st day of culture. # p <0.01 as compared to XanoMatrix cell culture petridishes on 1st day of cell culture. ## p <0.01 as compared to XanoMatrix cell culture petridishes on 7th day of cell culture.
Figure 36: Confocal microscopy image of NIH 3T3 Fibroblast adhesion on day 7 on (a) XanoMatrix; (b) Falcon and (c) Corning surfaces. Scale bar-60 µm. Magnification 10X.

Figure 37: MSC cell adhesion and proliferation on Corning, Falcon and XanoMatrix cell culture petri dishes after 1, 7 and 14 days. Data are expressed as the mean ± standard error of the mean; N=4; * p<0.01 as compared to Corning and Falcon petri dishes on the 7th day of culture; **p<0.01 as compared to the Corning and Falcon petri dishes on the 14th day of culture; and # p <0.01 as compared to XanoMatrix cell culture petri dishes on 1st day of cell culture.
Figure 38: (a) (b) MSC cell density on XanoMatrix surfaces at 1 and 14 days, respectively; Scale bar = 60 µm.
Figure 39: Endothelial cell adhesion and proliferation on Corning, Falcon and XanoMatrix cell culture petri dishes after 1, 7 and 14 days. Data are expressed as the mean ± standard error of the mean; N=4; * p<0.01 as compared to Corning and Falcon petri dishes on the 7th day of culture; **p<0.01 as compared to the Corning and Falcon petri dishes on the 14th day of culture; and # p <0.01 as compared to XanoMatrix cell culture petri dishes on 1st day of cell culture.
Figure 40: Osteoblast adhesion and proliferation on Corning, Falcon and XanoMatrix cell culture petri dishes after 1, 7 and 14 days. Data are expressed as the mean ± standard error of the mean; N=3; * p<0.01 as compared to Corning and Falcon petri dishes on the 7th day of culture; **p<0.01 as compared to the Corning and Falcon petri dishes on the 14th day of culture; # p <0.01 as compared to XanoMatrix cell culture petri dishes on the 1st day of cell culture; and ## p <0.01 as compared to XanoMatrix cell culture petri dishes on the 7th day of cell culture.
Figure 41: (a) (b) (c) Endothelial cell density on XanoMatrix, Falcon and Corning surfaces at 7 days, respectively; (d) (e) (f) Osteoblast density on XanoMatrix, Falcon and Corning surfaces at 7 days, respectively. Scale bar = 60 μm.

ALP Assay
A BioAssay QuantiChrom™ Alkaline Phosphatase Assay Kit (DALP-250) was used for the colorimetric kinetic determination of serum alkaline phosphatase activity. After equilibrating reagents to room temperature, the Working Solution was prepared by mixing for each 96-well assay, 200 μL of the Assay Buffer, 5 μL of Mg Acetate (final 5 mM) and 2 μL of pNPP liquid substrate (10 mM). 200 μL of distilled water (H₂O) and 200 μL of a calibrator solution were transferred into separate wells of a clear bottom 96-well plate. 5 to 50 μL samples were carefully transferred into other wells. 150 to 195 μL the Working Solution was pipetted into sample wells. The final reaction volume in the
sample wells was 200 μL. The optical density was measured at 405nm (t = 0), and again after 4 min (t = 4 min) on a plate reader. ALP measurements were normalized to the reference plate of day 1 and expressed as the ALP fold induction.

![Figure 42](image)

**Figure 42:** ALP activity fold induction as observed on the Corning, Falcon and XanoMatrix surfaces after 14 days of culture

**Surface roughness and cell growth**

The insertion of a biomaterial into the human body is followed immediately by the adsorption of proteins onto the surface. One of the most important parameters in controlling this adsorption is control of surface features like surface roughness and surface energy and thus by controlling these features, we can guide the adhesion of select proteins onto the biomaterial surface. To that effect, we measured the surface free energy and the root mean square roughness of the 3 surfaces and fitted it to a line using the equation provided by Khang et al:[77]

\[ \text{Es(RMSeff)} = \rho \times \text{RMSeff} + E_{o,s} \]
In this formula, $E_{o,s}$ (ground surface energy) is a material property determined purely by chemical interactions between the surface and the interacting liquid for negligible surface roughness (not a function of nanoscale topography), and $\rho$ is a coupling constant, which determines the extent of the influence of nanoscale topography on the surface energy of material. Line fitting the present experimental results gave, $E_s = 0.9631 \times \text{RMS} - 20.643$ ($R^2 = 0.9622$), where $\rho=0.9631$ and $E_{o,s}=-20.643$.

**Figure 43**: Graph depicting correlation between surface roughness and the surface free energy of the materials.

The surface roughness was also correlated to the cell growth as depicted in the graph below. A linear correlation was established in leading to the hypothesis that increased surface roughness would lead to improved cell growth for these cell types.
Figure 44: Graph depicting the correlation between surface roughness and cell density for fibroblasts, endothelial cells, mesenchymal stem cells and osteoblasts respectively.

Results

In vitro, cells can behave very differently depending on the growth substrate employed. Conventional tissue culture is carried out on 2D surfaces without scope for cells to adopt natural morphologies or to communicate efficiently with their neighbours. This 2D confinement is far removed from the 3D complexities of living tissue. Engineering the cell culture microenvironment to create growth conditions that more accurately mimic the in vivo behaviour of cells is an essential step for improving predictive accuracy during pharmaceutical development/clinical trials.

As expected, the XanoMatrix cell culture well plates increased fibroblast, osteoblast, endothelial cell and stem cell adhesion and proliferation as compared to the Corning and Falcon cell culture petri dishes since they offered a more biologically-inspired 3 dimensional nano fiber structure or a structure that mimics the extracellular matrix in the
body. Also, the ALP assay indicated increased ALP activity by stem cells on the XanoMatrix surface compared to the other surfaces. There was almost 4 times more increase in ALP fold induction on the 14th day on the XanoMatrix samples as compared to the Corning and Falcon samples which proved that osteogenic differentiation was happening.

The XanoMatrix surface also had increased roughness at the nanoscale and hydrophobicity as compared to the other control surfaces used in this study. The interaction between fibroblasts and nanofibers polymers plays an important role in biotechnology and biomedical applications. Alteration in surface energy, as observed here, can change initial protein interactions, which will influence stem cell responses. Around the world, the increasing use of polymeric materials in these applications ranges from substrates for cell and tissue growth to vascular and other numerous prostheses. Materials for tissue and cell culture are not inert polymers without biological relevance, as it is known, that the cellular response is driven by polymer surface characteristics such as topography, hydrophobic/hydrophilic properties, and chemistry. [172] Improvement in biocompatibility due to surface modification is mainly based on incorporating new functional chemical groups, changes in polarization and surface free energy, as well as changes in topography. [173] The 3-dimensional growth of cells (as observed here) opens up the possibility of true 3D migration, invasion and nutrient exchange as seen in native tissues. Synthetic polymeric nanofibers are increasingly being used to imitate these structures for research related to tissue engineering, cancer, stem cell, high-throughput cell culture and regenerative medicine applications. Nanofibers have an advantage over traditional scaffolds and gels because of their lack of animal-derived products, batch-to-
batch consistency, ability to be shaped into various forms, and structural and size similarity to native tissue. Traditional electrospun nanofibers produce a flat, webbed matrix that on the surface mimics native extracellular matrices, but often does not allow deep penetration of cells into the structure. Porous or foamed plastic allows z-axis cellular growth, but still do not create a fibrous surface for the cells to grow more naturally and remodel. XanoMatrix scaffolds combine the advantages of nano-sized polymeric fibers with true, tortuous fiber beds and supports to create a robust, consistent and versatile growth platform that properly mimics native tissue.

**Conclusions**

This study shows that replacing the use of traditional tissue culture treated polystyrene petri dishes with more biologically-inspired XanoMatrix scaffolds provided a better environment for the adhesion and growth of a wide array of cells. In this manner, this study supports that cell culture dishes which mimic features of natural tissues should be continually studied for a wide range of application in which mimicking natural cellular functions are important.

**Chapter 2**

**Conclusions and future directions**

**Conclusions**

So far in this project, we’ve demonstrated that:
1. XanoMatrix surfaces offer a better 3 dimensional surface for the adhesion and proliferation of a wide array of cells like fibroblasts, endothelial cells, stem cells, osteoblasts etc, as compared to traditional 2 dimensional tissue culture polystyrene surfaces.

2. The XanoMatrix surfaces showed greater osteogenic differentiation in stem cells (measured by increase in ALP Activity) as compared to traditional 2 dimensional cell culture scaffolds, proving that these scaffolds could be used further in bone tissue engineering applications.

3. The basic cellulose acetate and PET composition of the nanofibrous scaffold offers a highly biocompatible scaffold that combines the advantages of nano-sized polymeric fibers with true, tortuous fiber beds and supports to create a robust, consistent and versatile growth platform that properly mimics native tissue.

**Proposed future directions**

In the future, the following experiments are proposed for this project:

1. **Cartilage regeneration studies:** Chondral and osteochondral defects affect a large number of people in which treatment options are currently limited. Current studies on chondrocyte functions are limited due to non-biologically inspired cell culture substrates. With its PET (poly ethylene terephthalate) and cellulose acetate composition, good mechanical properties and nanofibrous structure resembling an extracellular matrix, XanoMatrix offers an ideal surface for chondrocyte growth and proliferation. The XanoMatrix™ scaffolds also have greater hydrophobicity, 3 dimensional surface area and greater tensile strength, making them ideal
candidates for alternative treatment options for chondral and osteochondral defects as well as cell culture substrates to study chondrocyte functions.

Taking all these facts into mind, these scaffolds should be investigated for use in cartilage regeneration studies. In vitro studies that include cell culture and proliferation assays like the MTS assay, confocal microscopy, ALP assay etc should be conducted with chondrocytes.

2. **Surface characterization:** The cell adhesion process is most critical to tissue culture applications so the surface properties of these scaffolds should be well established. Surface energy often plays a major role in surface adsorption of cell adhesion promoting proteins and to understand it better dynamic contact angle studies should be conducted.

3. **Protein adsorption:** Cell adhesion takes place in two different stages. The first stage consists of the adsorption of a layer of proteins that selectively adhere onto the bio- material surface, and is completed in an interval from seconds up to a few minutes. The first stage is mainly mediated by surface properties but the second stage takes place in response to extracellular matrix proteins, membrane proteins and cytoskeletal proteins. Fibronectin, vitronectin and type I collagen are some of the most common proteins involved in the cell adhesion process and hence protein adsorption studies should be done with these.

4. **Mechanical testing:** The shear fatigue strength, the static shear strength and the static tensile strength of the scaffolds should be tested out using standard ASTM protocols. The mechanical properties of the scaffolds are usually measured by performing compression tests. For scaffolds with 85–95% porosities, stiffness
ranges between 100 and 150kPa, yield stresses range between 25 and 35kPa, and yield strains ranged between 15 and 60% as reported in literature. [154]
References


[163] Blackshaw SE, Arkison S, Cameron C, Davies JA. Promotion of regeneration and axon growth following injury in an invertebrate nervous system by the use of three-


