INTRODUCTION
Chapter 1

INTRODUCTION

1.1. Introduction

Biomaterials are artificial or natural materials, used for fabricating structures or implants, to replace the lost or diseased biological structures, that would help to regain form and function. Use of biomaterials did not become practical until the advent of the aseptic surgical technique developed by Dr. J. Lister in the 1860s. The earliest successful implants, as well as a large fraction of modern implants, were in the skeletal system. Bone plates were introduced in the early 1900s to aid in long bone fracture. The global market for orthopedic implants generated total revenues of $12.5 billion in 2005, representing a compound annual growth rate (CAGR) of 10.4% for the five-year period spanning 2001-2005[www.marketresearch.com]. Looking forward, the global orthopedics market is forecast to continue its acceleration, with an anticipated CAGR of 8.4% for the period 2005-2010 which is expected to drive total revenues to $3.1 billion by the end of 2010. Valued at $2.48 billion in 2005, the European market for orthopedic implants is expected to be worth $3.78 billion in 2012 [1].

There are three major classes of metals used for developing orthopedic implants today: stainless steel (316L SS), cobalt chromium (Co-Cr) alloys and titanium (as alloys and commercially pure). Titanium and its alloys are widely used for orthopedic implant applications [2]. They have been extensively investigated and applied as implant materials due to their biocompatible nature resulting from a stable oxide layer that forms on the surface. Moreover, other desirable properties such as the relatively low modulus, good fatigue strength, formability, machinability, and biocompatibility of titanium facilitate its wide application in the biomedical field. The stability and corrosion resistance of titanium is provided
by the naturally occurring titanium dioxide layer present on the surface of the metal. This thin titanium oxide layer protects the metal from further oxidation [3].

Both 316L SS and Co–Cr alloys possess much higher modulus than bone, leading to an insufficient stress transfer to bone, leading to bone resorption (Stress shielding) and the loosening of implants after a few years of implantation. However, the low modulus of Ti and its alloys (55-110 GPa) compared to 316L SS (210 GPa) and Co-Cr alloys (240 GPa), endows Ti with mechanical properties that make it useful for implant applications. Although the strength of titanium alloys is similar to that of 316L SS, its density is ~55% lower than steel, yielding a very high specific strength (strength per density) in the range of 20-25 kgf/mm²/g/cm³ making titanium and its alloys an important implant material. Mechanical properties of metal implants employed in the field of biomedical devices are listed in Table 1.1.

Table 1.1: Mechanical properties of metallic biomaterials

<table>
<thead>
<tr>
<th>Material</th>
<th>Young's Modulus, E (GPa)</th>
<th>Yield Strength, $\sigma_y$ (MPa)</th>
<th>Tensile Strength, $\sigma_{UTS}$ (MPa)</th>
<th>Fatigue Limit, $\sigma_{fml}$ (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless steel</td>
<td>190</td>
<td>221–1,213</td>
<td>586–1,351</td>
<td>241–820</td>
</tr>
<tr>
<td>Cobalt-chromium (Co–Cr) alloys</td>
<td>210–253</td>
<td>448–1,606</td>
<td>655–1,896</td>
<td>207–950</td>
</tr>
<tr>
<td>Titanium (Ti)</td>
<td>110</td>
<td>485</td>
<td>760</td>
<td>300</td>
</tr>
<tr>
<td>Ti-6Al-4V</td>
<td>116</td>
<td>896–1,034</td>
<td>965–1,103</td>
<td>620</td>
</tr>
<tr>
<td>Cortical bone</td>
<td>15–30</td>
<td>30–70</td>
<td>70–150</td>
<td></td>
</tr>
</tbody>
</table>


1.2 Implant failure

Loosening of implants is one of the major complications associated with implant surgery [4]. Failure of implants after surgery occurs due to various factors such as wear/corrosion, fibrous encapsulation, inflammation, low fracture toughness/low fatigue strength, and mismatch in modulus of elasticity between bone and implant [Fig.1.1] [5].
Revision surgery, which is the only alternative to surmount these issues, is very expensive and cause pain for the patient. In addition to that, the success rate of such revision surgeries is very small. If fixation is not sufficient, loosening and osteolysis of the implant can occur. Fig 1.2 depicts schematic representation of stable and unstable fixation of implants to the bone tissue. This medical situation can be prevailed over only with a rapid bone formation that can help to fill deficient bone and fix the implant firmly to the adjacent bone. To achieve this, the implant material surface must be capable of recruiting bone forming cells (osteoblast) to colonize, thereby initiating bone formation. It is estimated that ~ 90% population above the age group of 40 suffer from degenerative disorders such as arthritis. It has been recently reported that the number of total hip and knee replacement surgeries would surpass 174 and 673% respectively by 2030 [6]. Due to these ever-increasing human needs, active research on long lasting biomaterial implants is booming.

Figure 1.1: Various causes for implant failure leading to revision surgery.
1.2.1 Implant failure – a remedy?

In implants made of titanium, the normal manufacturing steps usually lead to an oxidized, contaminated surface layer that is often stressed and plastically deformed, non-uniform and rather poorly defined. Such native surfaces are clearly not appropriate for biomedical applications and certain surface treatment procedures are mandatory to enhance its performance. An appropriate surface modification technique not only would retain the bulk attributes of titanium, but also improve its specific surface properties.

The success of any orthopedic and dental implantation procedure is based on the creation of a strong mechanical interface between the surface of implant materials and the bone tissue, without any fibrous tissue intervention. Hence the key target of current implant related research is to realize a mechanically well-bonded implant in a short period. This can be accomplished by an appropriate surface engineering of the biomaterial used for implant applications. Considerable efforts have been made in the recent past to optimize the surface topography of titanium implants for bone-contact applications, focusing mainly on engineering
surface structures to enable reduced healing times and accelerate integration with the host tissue. It is well known that micrometer scale roughness has an influence on cellular behavior. However, looking at the adhesion sites of cells, which are in nanometer range, it is clear that these very small components of a cell are influenced by nanoscale rather than microscale features. Over the past two decades, nanomaterials have attracted enormous amounts of interest from government, private enterprises and academic researchers. The major rationale for developing nanostructured materials for implant applications stems from the fact that many cell types are naturally adapted to function within a complex nanotopographical environment in vivo [7]. In addition, since bone is composed of constituent nanostructures such as collagen, hydroxyapatite and other matrix proteins, it is clear that instead of formulating surfaces with micron roughness, emphasis should be placed on techniques that create nanometer roughness. Fig. 1.3 illustrates the mechanism by which nanomaterials influence improved bone regeneration. Various studies have highlighted the significance of nanotextured surfaces on metals, polymers and ceramics in altering cellular response [8-9], thus emphasizing the role of nanosurface modification in increasing the efficacy of orthopedic implants.

![Figure 1.3: Schematic illustration of the mechanism by which nanomaterials influence improved bone regeneration. Compared to conventional material nanomodified material surface adsorb more proteins molecules followed by enhanced cell adhesion, proliferation and matrix production (improved bone regeneration).](image)

1.3. Surface modification techniques – An overview

Surface modification of biomaterials is broadly classified under two approaches with the aim of accelerated bone healing and enhanced bone bonding
to the implant [10-11]. The first approach is to incorporate inorganic phases viz; calcium phosphate on to biomaterial surfaces, which stimulate bone formation and thereby enhance biochemical interlocking between bone and the surface of the materials [12]. Biochemical surface modification is also included in this approach by incorporating organic molecules such as proteins/peptides on to the surface in order to stimulate specific cell responses [13-18].

The second approach for improving bone-implant interface is modifying the surface architecture or surface topography. The major rationale behind this approach is that a rough surface can increase bone anchorage and improve biomechanical interlocking between the bone and the implanted material [12]. Furthermore, roughness increases the surface area as well as surface energy of the material, which in turn enhances matrix protein adsorption, cell adhesion, proliferation and finally improves osteointegration [19].

There are multitudes of surface modification techniques which are categorized as:

- mechanical
- physical
- chemical and
- electrochemical methods

1.3.1 Mechanical methods

Mechanical processing is mainly intended to obtain specific surface topographies and roughness, remove surface contamination, and/or improve adhesion in subsequent bonding steps. Mechanical surface modification uses methods such as machining [20-21], grinding, polishing [22] and blasting [23-24]. It involves physical treatment, shaping or removal of the materials surface. The first osseointegrated implant developed by the machining of a titanium implant possessed periodic microgrooves on its surface [25]. Grit blasting has been performed by the deposition of silica (sandblasting), alumina or hydroxyapatite on the surface of implant materials in order to improve their surface functionality [26-28]. Various works on mechanically surface modified metallic implants report the influence of such modifications in cellular adhesion and proliferation [26-29].
1.3.2 Physical modification

i. Physical vapor deposition:

In the surface modification process by physical vapor deposition (PVD), a thin uniform film gets deposited on the biomaterial surface through resistive, laser beam or electron beam heating of coating materials which are in various forms (atoms, molecules or ions, generated from a target). The PVD method consists of evaporation [30], sputtering [31] and ion plating [32]. Although this is a common methodology adopted for thin film deposition, literature reports on the use of PVD for surface modification of implants are rare.

ii. Thermal spraying:

Thermal spraying (flame spraying or plasma spraying) is a process in which materials are thermally melted into liquid droplets and are energetically sprayed by means of a high temperature flame or a plasma jet on to the surface of the material on which the individual particles stick and condense. Plasma spray uses an electrical arc to melt the material and spray on to the surface and can be done under atmospheric (APS) as well as vacuum conditions (VPS) [2]. Several researchers have reported the use of plasma spraying technique to create biocompatible surfaces of titanium [33-34].

iii. Ion implantation and deposition

Ion beam processing is a process in which energetic ions such as oxygen, nitrogen, carbon, metal etc are introduced on the surface of a material through bombardment [2]. The use of this technique in altering the material surface topography as well as functionality has been recently utilized in biomedical arena [35, 36, 37, 38]. Raimondo et al have recently reported enhanced osteoblast and endothelial cell functions on surface modified titanium as well as polyethylene using electron beam deposition [36].

iv. Lithography

Lithography is a very sophisticated technique by which a biomaterial is coated with a film prior to the creation of desired features. The film is usually a polymer that is sensitive to a particular type of energy applied. Polymers sensitive to light or to electrons can be used. Depending on the sensitivity of the polymer (also called the resist), lithographical techniques are categorized as photolithography (light-sensitive resist) or electron beam lithography (electron-
sensitive resist) [39]. While photolithography helps to generate surface nanofeatures of ~ 300 nm, submicroscopic features down to 10 nm can be created with electron beam lithography [40].

Lithographic techniques have been demonstrated for its use in creating well aligned, ordered surface topographies on polymeric substrates with improved cell attachment and proliferation on nanostructured surfaces [9, 41, 42].

1.3.3 Chemical methods

The most popular and efficient ways to modify surfaces on the nanoscale involve direct chemical modifications with acids and oxidants. Chemical treatments are attractive for large-scale manufacturing because they are simple and provide efficient and uniform access to all surfaces, even on multifaceted devices with complex 3D shapes such as dental screws and cardiovascular stents. Although chemical treatments have yielded a variety of micro-textured implants with improved clinical outcomes, the cellular response on nanofeatured materials demonstrated in various laboratories has intensified the application of chemical treatments for nanostructuring biometals as well as other surfaces [2, 43]. Chemical methods include chemical treatment, biochemical modification, electrochemical treatment, chemical vapor deposition and hydrothermal modification.

i. Chemical treatment

Chemical treatment of titanium and its alloys are mainly based on chemical reactions occurring at interface between titanium and a solution. Chemical treatment involves acid and alkali treatment.

a. Acid treatment

Acid treatment is frequently used to remove oxide and contamination to obtain clean and uniform surface finishes. During pre-treatment of titanium, a combination of acids is commonly used [44, 45]. According to Wen et al, using a two step chemical treatment of acid followed by alkali, the bioactivity of Ti alloys was found to improve [46,47]. Here, a solution composed of 10-30 vol% of HNO₃ and 1-3 vol% of HF in distilled water has been recommended to be a standard solution for acid pre-treatment. Acid etching generally leads to a thin surface oxide layer (<10nm), which otherwise takes hundreds of days to form when exposed to air [48]. The oxide is predominantly TiO₂, but residues from the
etching solution are frequently observed, particularly chemicals containing fluorine. In addition, it is known that hydrogen incorporation below the oxide layer also occurs during acid treatment [49].

b. Alkali treatment

Improvement of bioactivity by alkali treatment was introduced by Kim et al [50]. In this method the material is first immersed in a 5-10M NaOH or KOH solution for 24 hours followed by rinsing with distilled water and ultrasonic cleaning for 5min. The specimens are then dried in an oven at 40°C for 24 hours and finally heated to around 600-800 °C for 1 hour [50, 51]. Because of the strong tendency of titanium to oxidize, the heat treatment is performed at a pressure of $10^{-4}$ to $10^{-5}$ Torr. A porous surface is formed after the heat treatment.

The use of both the above treatment protocols have demonstrated use in preparing bioactive substrates for improving deposition of calcium phosphate, promoting the formation of nanostructured metallic implants [50, 52,53, 54].

ii. Sol-gel coating

Sol-gel process is one of the widely used methods of depositing thin ceramic coatings on implant material surfaces. This method provides better control of chemical composition and microstructure of homogenous films. The major advantage of this method is its low temperature processing. Many coatings such as titanium oxide, calcium phosphate and TiO$_2$–CaP composite have been reported on titanium and its alloys for medical applications [2]. It is believed that titania coated by means of sol-gel technique is rich in Ti-OH group, which then induces calcium phosphate formation and thereby bone formation [55]. Calcium phosphate deposition by mean of sol-gel process commonly uses a solution mixture of phenyl dichlorophosphine in acetone and calcium nitrate for surface modifying titanium and its alloys for orthopedic applications [56,57].

iii. Chemical vapor deposition

Chemical vapor deposition (CVD) is a process involving the reaction between chemicals in gaseous phase and the surface of materials, resulting in the formation of a non-volatile compound on the surface. CVD is generally used in many industries to produce organic and inorganic coating on various materials. Deposition of a continuous diamond film of titanium carbide on titanium alloys surface by combustion assisted CVD was reported by Baek et al [58]. Diamond-
like carbon (DLC) is a metastable, amorphous carbon deposited on titanium via CVD also has been suggested as an important material for biomedical applications [32, 59, 60, 61].

iv. Hydrothermal method

Hydrothermal synthesis can be defined as a method of synthesis of single crystals that depends on the solubility of minerals in hot water under high pressure [62]. The crystal growth is performed in an apparatus consisting of a steel pressure vessel called autoclave, in which a nutrient is supplied along with water. A large number of compounds belonging to practically all classes have been synthesized under hydrothermal conditions: elements, simple and complex oxides, tungstates, molybdates, carbonates, silicates, germanates, etc. Hydrothermal synthesis is commonly used to grow synthetic quartz, gems and other single crystals with commercial value [63, 64, 65].

Hydrothermal method, due to its simplicity and cost effectiveness, has been utilized by various researchers for surface modification of metallic implants, for improving their biocompatibility [66, 67, 68, 69].

1.3.4 Biochemical modification

Biochemical modification of biomaterials utilizes biological and biochemical knowledge on cellular function, adhesion, differentiation and remodeling. The objective of modification is to induce specific cell and tissue response by means of surface immobilized peptides, proteins or growth factors. Although biochemical modification improves the biocompatibility through surface functionalization, bulk properties of the material remain unaltered [70]. Biochemical modification of titanium has been reported using silanization, photochemistry, self assembled monolayers, protein resistance and protein immobilization [71, 72, 73, 74, 75]. Protein resistance and immobilization of bone morphogenic proteins on titanium alloy has been found to enhance bioactivity [75, 76].

1.3.5 Electrochemical method (Anodic oxidation)

Anodic oxidation encompasses electrode reactions in combination with electric field driven metal and oxygen ion diffusion leading to the formation of an oxide film on the anode surface. It is a well established method to produce different types of protective oxide films on metals and thereby increase the
corrosion resistance capacity of the material. Different diluted acids (H$_2$SO$_4$, H$_3$PO$_4$, acetic acid, HF and others) can be used as electrolytes in the process. The structural and chemical properties of anodic oxides can be varied by changing the process conditions such as anode potential, electrolyte type, composition, temperature and current.

In the case of titanium metal, titanium and oxygen ions formed during the redox reactions are driven through the oxide by an externally applied electric field resulting in the formation of anatase/rutile phase of titania. The method has been reported as an efficient way for nanosurface modification of metals [77, 78, 79, 80]. Grimes and co-workers have investigated in detail the influence of various processing conditions in creating long self-standing nanotube arrays [77, 78, 79, 81]. Yang et al indicated that anodic oxidation in H$_2$SO$_4$ solution followed by heat treatment was an effective method to prepare bioactive titanium [82]. Several researchers have thereafter studied in detail the influence of such electrochemically modified nanostructures created on metals such as titanium and tantalum [83] in enhancing the osteoblast cell response for orthopedic implant applications [80, 84, 85, 86, 87, 88].

1.3.6 Self assembly

Self assembly is a biomimetic surface modification strategy to develop synthetic materials that resemble ECM [89]. In this, the molecules and supramolecular aggregates arrange themselves to form an ordered structure through weak and non-covalent bonds [90]. Hartgerink et al, has demonstrated the formation of a peptide amphiphile nanofibre network designed to mimic collagen protein, and mineralized with hydroxyapatite to recreate the nanoscale structure of bone, which provided a milieu for in vitro bone formation [91]. Kisiday et al also demonstrated the formation of self assemble peptide hydrogel for cartilage repair [92]. The technique has been also used for the surface modification of metals. Recently, a multilayer coating of chitosan (Chi) and heparin (Hep) was obtained on NaOH treated titanium substrate by means of self assembly [93]. They reported that the Hep-Chi coated titanium improved primary osteoblast adhesion, proliferation as well as differentiation, thus proving the feasibility of the self-assembly process in providing a biocompatible substrate through surface modification.
1.3.7 Electrospinning

Electrospinning uses an electric field for the controlled deposition of nanofibers made of synthetic, natural or combination of both materials on a substrate [94]. This is a widely used method for the design and fabrication of 3D fibrous scaffolds that mimic the natural tissue, mainly for tissue engineering applications. The developed fibers are usually in the range of 100nm to microns, and can mimic the extracellular environment in vivo. The materials generally used to form such fibres include synthetic biodegradable polymers such as poly-L-lactic acid, poly(ε-caprolactone), poly(glycolic acid), etc [95, 96]. In addition, neutral polymers such as collagen, silk, DNA, etc can also used for electrospinning. Recently this method has also been demonstrated for its use in preparing electrospun nanofibrous TiO₂ network on titanium metal surface for orthopedic implant applications [97,98].

1.4. Surface modification for orthopedic implant applications

1.4.1 Relevance of protein adsorption

Previously, several researchers have reported that surface topography, roughness, wettability and chemical composition may influence the protein adsorption on titanium surface and its subsequent cell behavior [24, 99, 100]. When an implant material is introduced into the body, prior to osteoblast cell (or other cells) adhesion, a dynamic process of adsorption, desorption and exchange of proteins from body fluids such as bone marrow, blood, and other tissues takes place within milliseconds [101, 102, 103, 104]. In this manner, it has been observed that proteins that initially adsorb onto the surface of implants control subsequent cell adhesion. For this reason, many investigators are now paying close attention to manipulating initial protein adsorption events by altering the surface properties of implant [105,106, 107].

Various studies have shown that nanotopography and surface energy influence the type, quantity and conformation of adsorbed protein, and controls cellular adhesion to the surface [24, 108,109, 110, 111, 112, 113]. The adsorbed molecules adopt conformations that are structurally distinct from their native fully unfolded state [114, 115]. Earlier studies showed that maximum vitronectin adsorption was on hydrophilic surfaces of high surface roughness [24, 116]. And
the active site of vitronectin (RGD sequence) exposed on nanophase ceramics, compared to conventional ceramics [58, 104]. Similar reports were revealed by Woo and colleagues that three dimensional nanofibrous poly (L-Lactic Acid) scaffolds specifically adsorb higher amount of vitronectin and fibronectin proteins compared to a solid pore wall of same material [117]. Accessibility of specific aminoacid sequences of adsorbed proteins such as vitronectin, fibronectin and laminin may either enhance or inhibit cellular adhesion and growth. Further studies using surface enhanced Raman spectroscopy showed changes in the conformation of vitronectin adsorbed on nanophase alumina [118]. Pre-requisite for such controlled surface is evident from the studies showing many techniques having tendency to alter surface topography, surface chemistry, surface charge or surface energy [119, 120].

1.4.2 In vitro and in vivo cellular response on surface modified implants

Substantial efforts have been done in the recent past to design implant surfaces in such a way that they control surface-protein interactions for better cellular adhesion. Surface treatments frequently used to modify titanium include polishing, grinding, machining, etc., resulting in the formation of inhomogenous microscale surface features [121, 122, 123, 124, 125, 126]. It was reported that such micrometer scale roughness influenced cellular behavior considerably [103, 127, 128, 129, 130]. Studies using osteoblast-like cells (SaOS-2) have concluded that even though cellular adhesion and proliferation was significantly higher on smooth surfaces, extracellular matrix production was abundant on rough surfaces [131]. Cell morphology studies using human osteoblast cells revealed a flattened fibroblast-like morphology on smooth titanium, while on rough, sandblasted surfaces, a three dimensional growth was induced [132]. Huang et al, investigated the initial adhesion of osteoblast cells (U-2OS) on mechanically polished titanium with roughness in the range of 30nm to 1.15μm and found that Ti with roughness (Ra) 150 nm provide an optimal cell adhesion behavior with respect of other rougher or smoother samples [133].

Morra et al studied the role of biochemical surface modification of titanium by collagen immobilization (by means of plasma deposition and acrylic acid grafting followed by collagen coupling) and found that collagen immobilized
titanium was safe in terms of cytotoxicity and in vivo biocompatibility [134]. Photo coupling of fibronectin onto titanium metal surface also enhanced keratinocyte cell adhesion and spreading, with a low thrombogenicity compared to pure Ti surfaces [135].

Cells are naturally adapted to grow on nanostructured environment in vivo [130, 136] and all biological events occur at the nanoscale regime [103, 137]. Specifically, ECM of bone in which osteoblasts cells interact consists of hydroxyapatite (with dimensions 20-40nm length), arranged within the collagen matrix (triple helix 300nm in length, 0.5nm in width, and periodicity of 67nm) [138]. A few studies carried out using corrugated surfaces report that cells can sense and react to surface topographical features upto 5-10 nm range [139, 140]. Hence, it is clear that instead of formulating surfaces with micron roughness, emphasis should be placed on techniques that create nanometer roughness, thus highlighting the role nanophase materials can play in universally increasing the efficacy of orthopedic implants.

One of the major studies highlighting the importance of nanometer roughness was conducted by Price and colleagues using nanofiber based polymer materials, revealing rapid bone cell function compared to the conventional carbon fiber materials [141]. Further, cytocompatibility studies using carbon nanofibers by the same group showed an increased osteoblast adhesion on compacts of carbon fibers with individual fibers in nanometer regime [142]. Another study by Elias et al, also showed enhanced long term functions of osteoblasts cultured on nanometer diameter carbon fibers [143].

Surface modification by chemical treatment is one of the most popular and efficient techniques to fabricate nanofeatures on biomaterial surfaces. One of the reports by Lim and Donahue showed that protein adsorption, cell proliferation and gene expression can be regulated by chemically modified surface properties of biocompatible materials [144].

TiO₂ nanotubes developed by anodization proved to be another effective method for surface modification. There are several previous reports demonstrating that TiO₂ nanotubes can improve osteoblast adhesion, proliferation and function
Other studies also suggested the applicability of nanotubes in vascular applications [86, 145, 146].

With the help of advanced micro-electronics fabrication technology, viz., electron-beam lithography, engineers were able to fabricate well-defined and ordered nanostructures down to a possible lateral feature size in the 10–20 nm scale range [147]. This enabled biologists to look at the effect of nanofeatures with sizes similar to those that surround a cell in vivo, such as the 66 nm repeat unit of collagen. However, several conflicting variations in cell response to regularly patterned nanosurfaces have been reported, such as enhanced osteoblast function in some cases [148] and invariant responses or even a significant reduction in cell proliferation in some other cases [149]. Cai et al., also recently studied how micro and nanoscale surface features on metallic titanium influenced protein adsorption and cell growth. They could not find any major differences in cell proliferation as well as viability on the different surfaces studied [150]. Thus, nanotopography is reported to modulate cell adhesion and proliferation both positively and negatively. However, no conclusions can be derived solely based on in vitro investigations alone, thus emphasizing the need for in vivo studies on any of the surface modified structures.

Cellular interaction to surface structures is a complex phenomenon, influenced by several parameters, with no proven experimental conclusions or empirical theories suggesting an ideal nanosurface topography/chemistry for better cell response. The nanofeatures on any surface can be arranged in either an organized or a random manner that mainly depends on the method adopted for modification. The organized nanofeatures such as grooves or pits are largely created by optical methods that cannot be directly translated to implants of complex shapes. Studies on lithographically patterned nanosurfaces have shown that a periodic pattern of 400 nm dots enhanced osteoblast differentiation of human mesenchymal stem cells (hMSCs), but not when cultured on 150 and 600nm dot patterns [151]. Similarly, periodic, vertically arranged nanotube arrays, with less than 30nm spacing and varying pore diameters (< 100nm) enhanced osteoblast cell functions [152] as well as endothelial cell proliferation without vascular smooth muscle proliferation [146]. Dalby and co-workers studied the
behavior of human osteoblast cells on nanopitted surfaces and found reduced cell spreading, suggesting these ordered arrays to be effective in this regard. [153]. Similarly Dalby et al in a detailed investigation on patterned polymeric surfaces with disordered arrangement of dots in square arrays, having a displacement of 50 nm between dots, showed enhanced osteoblast differentiation of hMSCs compared to an ordered substrate [41,42].

Since the adhesion sites of cells to the ECM or to the substratum are in nanometer range, it is clear that these very small components of a cell are influenced by nanoscale rather than microscale features [102]. Clustering of integrins is necessary for the formation of focal adhesion points, which is a prerequisite for cell spreading [154, 155, 156]. Arnold et al, reported that a separation of >73 nm between integrins will reduce cell spreading [157]. Earlier studies using 120nm diameter, 300nm pitch pits in hexagonal arrangement reduced cell adhesion of both mesenchymal [41,42] and fibroblast cells [42,147]. Mandonca et al recently showed that osteoblast specific gene expression on titanium implant can be enhanced due to nanoscale surface features [158]. Detailed investigation of the mechanism behind enhanced cellular response prompted a series of studies that probed the influence of cellular response towards nanotopography mediated signal transduction and mechanotransduction [147,159, 160].

There are various reports revealing the effect of surface modification on in vivo bone formation [43, 161, 162, 163, 164]. In vivo studies carried out by Aita et al using ultraviolet functionalized titanium in rat model showed an accelerated establishment of implant fixation four times than on conventional titanium [165]. An enhanced in vivo bone formation was observed on nanohydroxyapatite coated tantalum substrate compared to uncoated or conventional micron size HA coated tantalum [166, 167]. Similar observations were found for other nanostructures including alumina, zinc oxide and titania [167,168, 169]. Recently, Sitharaman et al reported an in vivo study of single walled carbon nanotubes (SWCNT) - polymer nanocomposites using rabbit model, wherein it was reported that CNTs induced 300% greater bone volume than all other experimental groups and control polymer at four weeks [170]. All these studies provide proof of the concept that
nanostructuring of substrates is an effective approach towards improving bone integration.

1.5. Aim and Research Hypothesis

The conventional methods of surface modification such as mechanical, physical, sol-gel, plasma spraying, lithography, etc., although are well established in literature for a variety of applications, have not yet been adopted for their use in implant manufacturing. Furthermore, researchers have not carefully investigated how surface topography alterations for a material with similar surface chemistry, influences cellular behavior. Hence, the current demand is for the development of simple, yet versatile processing methods that enable nanostructuring of implant materials having identical surface chemistry, enabling translational applications.

Thus, the aim of the present thesis work is to develop ‘cell-favored’ non-periodic vertically aligned nanofeatures on implant surfaces by means of a cost-effective and simple processing technique that enables the creation of distinctly different nanosurface morphologies with similar surface chemistry.

1.5.1 Specific objectives of the study

- To develop and optimize a cost-effective and simple hydrothermal method for the fabrication of TiO₂ nanostructures on Ti metal surface
- Nanomodification of Ti metal surface using conventional methods such as mechanical polishing and electrochemical modification (anodization)
- Compare the influence of different nanostructures developed through different modification methods viz., mechanical polishing, anodization, and hydrothermal modification on cellular response
- *In vitro* evaluation of long term cellular functions of primary osteoblast cells on different nanomodified Ti through biochemical as well as gene expression studies
- *In vitro* evaluation of influence of nanomodification on changes in cytoskeletal arrangement and early signal transduction events of primary osteoblast cells
• To evaluate the *in vivo* osteointegration capability of nanomodified Ti metal using a rat model

• Analyze the *in vivo* inflammatory response evoked by nanomodified titanium implants after surgical implantation into rat femur condyle.

**Major research questions** that are addressed in this thesis:

• What are the hydrothermal conditions needed for obtaining vertically arranged non-periodic TiO₂ nanostructures of different shapes and dimensions on metallic Ti?

• Can these nanostructures cause significant differences in osteoblast cell response (proliferation, differentiation and gene expression) compared to vertically arranged periodic nanotubular structures fabricated by anodization as well as the unmodified Ti implants?

• How effectively can these nanostructures improve the *in vivo* bone bonding capability of implants?
1.5.2 Research hypothesis

Surface modification of conventional titanium implants by hydrothermal method would be a unique way to fabricate vertically arranged non-periodic nanostructures having different shapes and dimensions. These differences in surface nanotopography can alter the cellular response, which potentially would influence bone integration.
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Introduction


Amrita Centre for Nanosciences and Molecular Medicine

Introduction


