SURFACE ENGINEERING OF TITANIUM BASED METAL FOR CELL INTERACTION

by

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LIST OF ABBREVIATIONS

1-D	One dimensional
3-D	Three dimensional
α-MEM	Minimum Essential Medium
βME	B-mercaptoethanol
A	Anatase
AB	Alamar Blue
AFM	Atomic force microscope
Al	Aluminium
ALP	Alkaline phosphatase
AMDI	Advanced Medical and Dental Institute
ANOVA	One-way analysis of variance
APS	Ammonium persulfate
AR	Aspect ratio
ARS	Alizarin Red-S
ATP	adenosine triphosphate
BMSC	bone marrow stromal cells
BrdU	bromodeoxyurindine
BSA	bovine serum albumin
Ca	Calcium
CaCl	Calcium Chloride
CD	Cluster of diffentiation
CK	Cytokeratin
Cl	Chlorine
CFU	Colony-forming unit
CO_2	Carbon dioxide
CSF	Cerebral spinal fluid
DAB	3,3'-diaminobenzidine
dc	Direct current
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EC	endothelial cells

ECM	Extra cellular matrix
EDX	Energy dispersive X-ray spectroscopy
F	Fluoride
Fe	Ferum
Fe_3O_4	Ferum Oxide
FESEM	Field emission scanning electron microscope
FTIR	Fourier transform infrared spectroscopy
FWHM	Full width half maximum
H	Hydrogen
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HA	Hydroxyapatite
HCl	Hydrochloride acid
HF	Hydrofluoric acid
HGF	Human gingival fibroblasts
hMSC	human bone marrow stem cells
HNEpC	Human nasal epithelial cells
HRB	Hardness, Rockwell B Scale
HRC	Hardness, Rockwell C Scale
HSC	Hematopoietic stem cells
IL	Human interleukin receptor
ISCT	International Society of Cell Therapy
JCPDS	Joint Committee on Powder Diffraction Standards
K	Potassium
KCl	Potassium Chloride
Mg	Magnesium
Mo	Molibdenum
MSC	Mesencymal stem cells
MTS	(3 - (4, 5 – dimethylthiazol – 2 - yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
Na	Sodium
Na_2SO_4	Sodium sulphate
NaCl	Sodium Chloride

$NaHCO_3$	Sodium bicarbonate
NB	Nanobacteria
Nb	Niobium
NH_4F	Ammonium fluoride
NPC	Neural progenitor cells
O	Oxygen
O_2	Oxide
OCPI	Osteoblastic precursor cell line
P	Phosphorus
PAEKs	Polyaryletherketones
PI	Propidium iodide
PMS	phenazine methosulfate
R	Rutile
ROS	Radical Oxidative Stress
SBF	Simulated body fluid
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Scanning electron microscope
Ta	Tantalum
TEM	Transmission electron microscope
Ti	Titanium
TiO_2	Titanium dioxide
TNF	Tumor necrosis factor
USM	Universiti Sains Malaysia
V	Vanadium
VSMC	vascular smooth muscle cells
WHO	World Health Organization
XRD	X-ray diffraction
Zr	Zirconium

LIST OF SYMBOLS

cm	centimeter
E_{corr}	Potential where current density increases with increasing potential
f	Mass fraction percent
g	gram
G	Geometric surface area factor
h	hour
I_{corr}	Corrosion current density
I_{pass}	Passive current density
k	constant
kDa	kilodalton
keV	Kiloelectron volt
L	Nanotube length
M	Molar
mA	Miliampere
MPa	Mega Pascal
min	Minute
nm	Nanometer
R_a	Average roughness
V	Voltage
w	Wall thickness
wt%	Weight percent
$Ø_i$	Inner diameter
$Ø_o$	Outer diameter
λ	Wavelength
β	FWHM in radiam
θ	Angle position of plane peak in radian
μm	Micrometer
°C	Degree Celsius

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4. Micrograph Competition – "μ-Size of Banana Leaf" – consolidation prize.

5. Sanggar Sanjung Award (Research Product Category) for the year 2009.

6. Sanggar Sanjung Award (Journal Publication Category) for the year 2009.

KEJURUTERAAN PERMUKAAN LOGAM TITANIUM UNTUK TINDAKBALAS SEL

ABSTRAK

Penyelidikan ini fokus kepada pengubahsuaian permukaan titanium dengan morfologi topografi tiub-nano TiO_2. Tindak balas tiub-nano TiO_2 dan sel stromal tulang PA6 dikaji bagi memahami pengaruh struktur tiub-nano terhadap pertumbuhan sel. Bagi menjayakan objektif penyelidikan ini, kerajang titanium telah diubahsuai kepada tiub-nano TiO_2 yang mempunyai pelbagai dimensi melalui kaedah penganodan dan dicirikan. Tiub-nano TiO_2 bersaiz 25 - 110 nm berjaya dihasilkan di antara 10 V dan 40 V. Rintangan kakisan adalah tinggi bagi sampel yang dianodkan pada 10 V (25 nm-diameter). Panjang tiub-nano TiO_2 adalah 2.2 μm apabila dianodkan selama 3 jam. Fasa anatas, anatas-rutil dan rutil dihasilkan apabila tiub-nano TiO_2 disepuhlindap pada 300 °C, 600 °C dan 700 °C. Struktur tiub juga didapati musnah apabila disepuhlindap pada 700 °C. Fasa anatas mempunyai rintangan kakisan yang tinggi kerana lapisan oksida yang telah dihablurkan menghalang aktiviti kakisan (kadar kakisan = 0.31 nm/tahun). Morfologi sel, perlekatan, kebolehhidupan, immunokimia, aktiviti phosphatase alkali, pemendapan kalsium, *Western Blot* dan *immunophenotyping* dijalankan untuk menilai kesan biologi bagi sel PA6 apabila dikultur di atas tiub-nano TiO_2. Dari kajian ini, tiub-nano dengan 45 nm-diameter, 2.2 μm-panjang dan mengandungi campuran fasa anatas-rutil meningkatkan pertumbuhan sel PA6. Tiada bahan yang dibebaskan semasa tempoh pengeraman sel PA6 diperhatikan. Kepekatan protein di atas

permukaan tiub-nano lebih tinggi berbanding bahan kawalan kerana luas permukaan dan tapak perlekatan untuk sel memegang substrat adalah lebih besar. Ekspresi *immunostaining* untuk *cytokeratin, Bromodeoxyuridine,* CD34, IBMR3 dan propidium iodida adalah positif bagi kesemua sampel. Bagi analisa *immunophenotyping*, sel PA6 adalah positif untuk CD49e, CD51 and CD73. Ini mencadangkan sel PA6 di atas tiub-nano TiO_2 terlibat dalam perlekatan matrik sel luaran, interaksi sel stromal tulang, sistem imun dan pembahagian sel asas *mesenchymal*. Yang pentingnya, sinaran *fluorescence* menunjukkan sel PA6 yang dikultur di atas tiub-nano TiO_2 tidak mengalami perubahan yang mendadak berbanding bahan kawalan. Selepas 14 hari, hydroxyapatite didapati menyelaputi keseluruhan permukaan tiub-nano dan struktur ini meningkatkan pertumbuhan sel PA6. Penemuan ini menjelaskan bahawa tiub-nano merupakan faktor yang penting untuk mengoptimakan interaksi sel PA6.

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ABSTRACT

This research focused on the titanium surface modification with nanotopography morphology of TiO_2 nanotubes. Cell-metal interaction between TiO_2 nanotubes and PA6 bone marrow stromal cells were studied to understand the TiO_2 nanotubes parameters that affect the cell growth. To achieve objective of this research work, titanium foil was transformed into different dimensionalities of TiO_2 nanotubes via simple anodization method and characterized. TiO_2 nanotubes with inner diameter of 25 nm to 110 nm were successfully developed within 10 V to 40 V. Corrosion resistance was higher for sample anodizes at 10 V (25 nm-diameters). The length of the TiO_2 nanotubes arrays were 2.2 μm after 3 hours anodization. Anatase, anatase-rutile and rutile phase was observed when TiO_2 nanotubes subjected to anneal at 300 °C, 600 °C and 700 °C. Tubular structure destroy when anneal at 700 °C. Anatase phase give higher corrosion resistance because crystallized barrier oxide layer hinder the corrosion activity (corrosion rate = 0.31 nm/year). Cell morphology, adhesion, viability, immunocytochemistry, alkaline phosphatase activity, calcium deposition, Western Blot and immunophenotyping were done to evaluate PA6 cells interaction on TiO_2 nanotubes accordingly. From this study, 45 nm-diameter, 2.2 μm-length nanotube and anatase-rutile mixture phase enhanced the PA6 cells growth. No materials elution after 3 days incubation with PA6 cells observed. The protein concentrations on TiO_2 nanotubes were significantly higher than control due to large

surface area and binding sites for cells to anchorage the substrate. Immunostaining expression for *cytokeratin, Bromodeoxyuridine*, CD34, IBMR3 and PI was positive on entire samples. From immunophenotyping analysis, PA6 cells were positive on CD49e, CD51 and CD73, suggested that PA6 cells on TiO_2 nanotube arrays positively involved in extracellular matrix adhesion, bone marrow stromal cells interaction, immune system and mesenchymal stem cells differentiate. Importantly, fluorescence image shows PA6 cells cultured on TiO_2 nanotubes did not have much alteration as compared to control with regard of no significant different from the fluorescence intensity. After 14 days, hydroxyapatite fully covered TiO_2 nanotubes surface and enhance the PA6 cell growth and viability. These findings indicate that fine-tuning TiO_2 nanotubes will be essential parameter in optimizing PA6 cell interaction.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Over the past 20 years, titanium (Ti) and it alloys have been used as implant materials (Park *et al.*, 2010). Biocompatible nature (Vega *et al.*, 2008), excellent mechanical properties and chemical stability (Lee *et al.*, 2009) of Ti makes it a perfect candidate to be used in implant applications. However, implant materials for clinical applications tend to fail because of their poor surfaces characteristic that enable to support new bone growth and this will lead insufficient bonding to juxtaposed bone (Ma *et al.*, 2008), thus slow osteoconductivity (Thian *et al.*, 2006) and healing process. In this case, juxtaposed bone refers to natural bone bonding to implant material. Therefore, lately considerable attention has been focused on Ti surface modification (Chang *et al.*, 2009) such as plasma coating (Hauser *et al.*, 2009 and Wei *et al.*, 2008), etching (Das *et al.*, 2007) and anodization (Yu *et al.*, 2009) to improve surface characteristic for implant material.

Recently, Ti surface was modified to form self-ordered layer of vertically oriented titanium dioxide (TiO_2) nanotubes with diameters ranging from 25 and 100 nm by anodization process (Lan *et al.*, 2014). The results revealed that proliferation and cytocompatibility of cells on vertically aligned TiO_2 nanotube surfaces are nanotubes diameter dependent. A nanotube with diameter of 25 nm seems to have high biocompatibility of epithelial cells in comparison to 50 and 100 nm. Such results indicate that the surface nanostructure of an implant is an important factor for surface cell adhesion and growth. In line with this result, Zhao and co-workers

(2013) observed 30 nm-diameter TiO_2 nanotubes promotes the spread of mesencymal stem cells (MSC) into polygonal osteoblastic shape. The TiO_2 nanotubes samples promote osteogenesis in absence of an extra osteogenic agent. A 30 nm-diameter TiO_2 nanotubes also generates big nodular alkaline phosphatase (ALP) product and induce extracellular matrix (ECM) mineralization. However, two years ago, Zhao *et al.,* (Zhao *et al.,* 2012) reported 80 nm-diameters of TiO_2 nanotubes give best ability to simultaneously promote MSC proliferation and osteogenic differentiation simultaneously. In 2011, Choe has demonstrated that 50 nm-inner diameter of TiO_2 nanotubes provided good osseointegration such as cell proliferation, migration and differentiation (Choe *et al.,* 2011). Yang *et al.*, suggested that surface treatment with nanotubular TiO_2 surface enhanced the early osteoblast response, such as cell spreading and cytokine release, which is an important factor for subsequent cell functions and bone healing *in vivo* (Yang *et al.,* 2008a). Previous studies by Brammer *et al.*, demonstrate that nanotopography provided nanoscale cue that facilitate cellular probing, cell sensing if more actin cytoskeletal filaments formed lamellipodia and locomotive morphologies (Brammer *et al.,* 2011b). Park's group also showed that adhesion, spreading, growth and differentiation of MSC are critically dependent on the tube inner diameter (Park *et al.,* 2007). Spacing between 15 – 30 nm provided an effective length scale for accelerated integrin clustering/focal contact formation and strongly enhanced cellular activities compared to smooth TiO_2 surfaces. Cell adhesion and spreading were severely impaired on nanotube layers with tube diameter larger than 50 nm resulting reduced cellular activity and experienced programmed cell death. So, Park's group suggested TiO_2 nanotubes with 30 – 50 nm inner diameter represents critical borderline for cell to survive (Park *et al.,* 2007). The cell function altered if

the inner diameter of TiO_2 nanotubes were less than 30 nm and more than 50 nm. The above-mentioned findings are valid generally for the cell response to different topographical nanorough surface and have an important impact on the design and composition of implant surfaces (Gongadze *et al.*, 2011).

1.2 Problem statement

Recently, surface topography such as TiO_2 nanotubes have been shown to alter cell behaviors such as adhesion, orientation, differentiation and migration significantly (Koo *et al.*, 2013). It is due to nanotubes topography that can provide more abundant topographical cues similar to dimensional scale of bone collagen fibrils and elasticity resembling bones (Wang *et al.*, 2013). However, the dimensionality (diameter and length) of TiO_2 nanotubes on cell interaction is not well understood. In addition, there have been some inconsistencies in the literature regarding the optimal size of TiO_2 nanotubes for eliciting maximal adhesion, proliferation and cell functionality (Moon *et al.*, 2011; Rajyalakshmi *et al.*, 2011; Lan *et al.*, 2013 and 2014). Therefore, in this research work the effect of diameter and length of TiO_2 nanotubes on cell interaction were systematically studied.

Another factor attribute to the drawback of Ti as implant materials is TiO_2 phases. Among three different crystalline phases of TiO_2, anatase phase is more favorable for cell adhesion and proliferation due to lower surface contact angle (hydrophilic) and wettability (Koo *et al.*, 2013). In contrast, high surface contact angle (the water contact angle is larger than 90 °) lead to hydrophobic surface, which mimic biological surface such as lotus leaf (Rosario *et al.*, 2004). However, An and group reported that mixture anatase-rutile phase was more favorable for cell

interaction (An *et al.*, 2011). Such contradicting outcomes among research groups cause difficulty for researchers to select the best phase for implant materials. Thus, in order to understand effect of crystal structure on cell interaction, considerable efforts have been devoted to produce stable TiO_2 nanotubes phase that suits cells interaction requirement.

Besides, the selection of cells has also drawn an essential role in determining the cell-metal interaction. Many cells cannot adapt and poorly survive *in vitro* or implanted in the foreign body. This is because foreign material cannot interact properly with cells as they are lack of ECM (Llopis-Hernández *et al.*, 2011). Some efforts have been devoted in the literature to correlate the surface properties to protein adsorption and cell adhesion (Wang *et al.*, 2012). There is still lack of understanding of the cell-metal interaction from an integrated point of view that includes cell adhesion, cell viability and biocompatibility, adsorbed proteins on the nanomaterials surface such as TiO_2 nanotube arrays regarding their dimensionality. The different cells been used in the literature (Huo *et al.*, 2013; Neupane *et al.*, 2011; Roy *et al.*, 2007) also make the analysis on the cell-metal interaction became more complicated. Therefore, detail study on cell-metal interaction specifically on TiO_2 nanotube arrays need to be done by using single cell type (PA6 cells) will be primary concern of this study. PA6 bone marrow cells are well known for a good and main available source of MSC at the present time (Yang *et al.*, 2004 and Ayatollahi *et al.*, 2012a). It is also well ascribed that MSC are best candidates for tissue engineering and cellular therapy of orthopedic musculoskeletal tissues.

Ti has been introduced for biomaterials applications because it owns some of the good biocompatibility and high corrosion resistance. Yu *et al.* (2011) observed that 30 nm-diameter TiO_2 nanotubes had higher resistance of the barrier layer and

lower passive current density ($I_{pass)}$ compared to the smooth Ti. A previous study by Saji and co-workers indicated that the TiO_2 nanotubes surface exhibited passivation behavior and corrosion current density was considerably high. However, the relation within electrochemical corrosion behavior of TiO_2 nanotubes with cell-metal interaction was not reported. Indeed, comprehensive corrosion behavior study and cell-metal analysis would able to determine the best biomaterials implant.

Other laboratory concerns are materials elution from substrate to cell culture containing serum. This precipitation ions generated mineral nanoparticles with morphologically and chemically identical called nanobacteria (NB). NB is putative living entities are unusual for their small sizes (50-500 nm) have been implicated in numerous diseases involving extraskeletal calcification (Young *et al.,* 2009). Therefore, in the present study, materials elution from TiO_2 nanotubes were analyse after culturing with PA6 cells. This is to ensure that TiO_2 nanotubes are safe to use as implant materials.

The up-to-date biomarker to characterize cell behaviors has been study elsewhere (Oh *et al.,* 2013; Huo *et al.,* 2013; Peng *et al.,* 2009). However, no formal consensus has yet been reached on which markers may be best suited for PA6 bone marrow cells. To resolve the PA6 cells behavior during incubation with TiO_2 nanotubes, several biomarkers were tested and discussed.

The hydroxyapatite (HA) coatings formed upon immersion in Simulated Body Fluid (SBF) solution is believed to have similarities to bone apatite (Weng *et al.,* 1997). However, the relation of HA coating surface corrosion behavior and cell interaction study was not discussed by Weng *et al.* (1997). In the present study,

compositional and structural analyses are employed to reveal with the intention of gaining insight into the material response to cell.

In summary, many efforts have been made by researchers (Koo *et al.*, 2013; Llopis-Hernández *et al.*, 2011; Wang *et al.*, 2013; Weng *et al.*, 1997; Yu *et al.*, 2011) to improve biocompatibility of titanium as an implant material by developing TiO_2 nanotubes. However, the effect of nanotubes dimension, crystal structures and HA coating on biocompatibility of PA6 cells are less reported. Therefore, in the present research those parameters were studied.

1.3 Objectives

The objectives of this research are as follows:

i. To prepare TiO_2 nanotubes with different diameters by an anodization method by controlling potential, time and electrolyte pH.
ii. To prepare TiO_2 nanotubes with different crystal structures by annealing at different temperatures.
iii. To investigate TiO_2 nanotubes dimensionality and crystal structure to cell proliferation, viability, attachment, protein adsorption and mineralization.
iv. To prepare apatite coated TiO_2 nanotubes surface and investigate cell-metal interaction on developed TiO_2 nanotubes surface.

1.4 Research outcomes

The ultimate outcomes of this research project are detailed as follow:

a) Optimum anodization parameter to form TiO_2 nanotubes for cell-metal interaction is the main outcomes of this research work.

b) Data on annealing temperature to obtain specific crystal structure of TiO_2 nanotubes.

c) Optimum dimension and crystal structure for cell proliferation, viability, attachment, protein adsorption and mineralization.

d) Optimum duration of SBF immersion on apatite formation on TiO_2 nanotubes and its enhancement on cell-metal interaction.

e) Subsequently, by acquiring this knowledge on the formation of TiO_2 nanotube arrays and study on cell-metal interaction by altering the dimensionality and crystal structure, this research would reveal the viability of using TiO_2 nanotubes as a biocompatible implant material.

1.5 Structure of the Thesis

This dissertation is organized in five chapters consecutively. Chapter 1 is the introduction of the research project, problem statement, objectives and possible outcomes of this research. Chapter 2 introduces the important background of this research work, properties of titanium as implant materials, surface modification of titanium and cell-metal interaction between TiO_2 nanotubes with cells. This includes a comprehensive review on the improvement TiO_2 nanotubes dimensionality and

crystal structure by controlled anodization parameter. Reviews on cellular response to TiO_2 nanotube arrays are also presented. In chapter 3, detailed method of experimental work involved in the preparation and characterization of TiO_2 nanotubes are explained. Also, method to study cell interaction is presented in this chapter. This covers a brief explanation on the characterization equipment, operational principle and sample preparation.

Chapter 4 includes the discussion based on the results obtained from the experiment of TiO_2 nanotube arrays formation and cell-metal interaction study. The content consists of four main parts: (1) the detail investigation on the growth behavior of TiO_2 nanotubes by altering anodization parameter (e.g., applied potential, anodization period and electrolyte pH) and effect of structural characteristics on the surface properties; (2) the crystallization of TiO_2 nanotube arrays at different annealing temperature; (3) cell-metal interaction (cell proliferation, viability, attachment, protein adsorption, mineralization, biomarker (immunostaining and Western Blot) and immunophenotyping) with regard to the TiO_2 nanotubes dimensionality and crystal structure; and (4) HA coating on TiO_2 nanotubes by immersion in SBF and its interaction with PA6 cells. The potential of newly develop TiO_2 nanotube surfaces as implant materials are discussed in detail. Finally, Chapter 5 presents the conclusion of this research work and suggestions for improvement for future study.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

According to a recent report by World Health Organization (WHO), skeletal injuries that typically involves having patient lie in bed for up to 3 months, prevent most patient from working and thus places large burden on the patient's family (Matityahu *et al.*, 2014). Ideally, effective biomaterials implant is required to overcome aforementioned drawback to stimulate rapid wound healing (Brammer *et al.*, 2008).

Ti and its alloy have been well known implantable materials (Oh *et al.*, 2006). A number of reports have shown that the surface structure of titanium is critical for determining the success or failure of clinical titanium implantations for the purpose of bone, joint, or tooth replacements (Webster and Ejiofor, 2004; Raimondo *et al.*, 2010; Jacobi-Gresser *et al.*, 2013). In the past, numerous studies on implant surface modifications have been performed at the micrometer scale to optimize the surface geometry and profile to best fit cell interactions for adequate bone growth (Kawahara *et al.*, 2004; Li *et al.*, 2005; Pisarek *et al.*, 2011). Recently efforts have been made to improve cell stimulating, biomimetic activities by designing new surface geometries at nanoscale (Lim *et al.*, 2008 and Chiang *et al.*, 2009). Therefore, for the past decade, Kubota have suggested that TiO_2 nanotube arrays grow on Ti metal would be the best candidate as an implant material. The excellent biocompatibility appears to depend on the presence of a passive oxide layer (TiO_2 layer) formed on the surface (Sasaki *et al.*, 2006). In an effort to enhance the

cell implant material interaction and increase lifetime, bioactive ceramic based coatings have been applied to Ti implants (Crawford *et al*., 2007).

In this chapter, the properties of Ti as implant material is first introduced and reviewed. Then, technique for surface modification of Ti as implant device is discussed. A section on the interaction of TiO_2 nanotubes and cells is also presented.

2.2 Ti as biomaterials

European Society for Biomaterials Consensus Conference defined that biomaterial is a non-viable material used in a medical device intended to interact with biological systems (Carter and Norton, 2007). Recently, demand on the manufacture of synthetic biomaterials arises tremendously in the form of implant and medical devices (Simchi *et al.*, 2011). The aim of biomaterials design is to mimic the biomechanical properties of host tissue (Von Der Mark *et al.*, 2010) and suits it applications. Some of biomaterials and its application are listed in Table 2.1. Among this materials, Ti and its alloys such as Ti-6Al-4V and Ti-6Al-7Nb are widely used in bio-medical applications for instance (e.g. artificial hip, orthopedic or dental implants) because of their high strength-to-weight ratio, good mechanical properties (Table 2.2), biocompatibility (Crawford *et al*., 2007), high corrosion resistance (low metal ion release) (Macak *et al.,* 2005b and Tsuchiya, 2006), processibility and availability (Freese *et al.*, 2001). Even though Ti commonly use as implant material, they are also utilized as anti-bacteria (Baram *et al.*, 2009), anti-cancer (Kalbacova *et al.*, 2008), drug delivery (Peng *et al.*, 2009) and biosensor (Chen *et al.*, 2010). Figure 2.1 shows the schematic comparison of natural tooth and implant tooth. However, Ti cannot bond directly to living bone after implantation into a host body (Park *et al*.,

Table 2.1 List of biomaterials and its application.

Implant material	Morphology	Application	Author and Year
Polymer demixing of polystyrenes	Nanometrically high islands	Stents, conduits, and bone repair	De Graaf *et al.*, (1995) Dalby *et al.*, (2002) Berry *et al.*, (2006) Lim *et al.*, (2012)
Tantalum	Porous	Implant	Bobyn *et al.*, (1999); Zhang *et al.*, (1999); Koutsostathis *et al.*, (2009)
Carbon	Nanotubes	improved tracking of cells, sensing of microenvironments, delivering of transfection agents, and scaffolding for incorporating with the host's body	Harrison and Atala, (2007) Saito *et al.*, (2009)
Polyaryletherketones (PAEKs)	Porous	orthopedic, and spinal implants	Kurtz and Devine, (2007)
Fe_3O_4	Nanoparticles	pH-responsive drug release antibacterial biomaterials for biomedical devices and implants	Gan *et al.*, (2011) and Chen *et al.*, (2013) Das *et al.*, (2013)
HA	Powder compaction	Bone replacement	Saha *et al.*, (2012)
TiO_2	Foam	Scaffold	Haugen *et al.*,(2004)
	Thin film	Bone implant	Park *et al.*, (2011)
	Nanotubes	Nasal surgery (nasal septal perforation repairmen, nasal reconstruction or rhinoplasty and cerebral spinal fluid (CSF) rhinorrhea repairment)	Lan *et al.*, (2014)

Table 2.2 Mechanical properties of selected titanium biomaterials (Freese *et al.*, 2001).

Grade Designation and type	Tensile Strength (MPa)	0.2% Yield Strength (MPa)	Elongation %	Reduction in Area %	Typical Hardness (Rockwell)
Ti CP-1 (Alpha)	241	172	24	30	70 HRB
Ti CP-2 (Alpha)	345	276	20	30	80 HRB
Ti CP-3 (Alpha)	448	379	18	30	90 HRB
Ti CP-4 (Alpha)	552	483	15	25	100 HRB
Ti-6Al-4V (Alpha/Beta)	931	862	15	30	36 HRC
Ti-6Al-7Nb (Alpha/Beta)	862	793	10	25	32 HRC
Ti-15Mo	793	655	22	60	24 HRC
Ti-12Mo-6Zr-2Fe (Beta)	1000	965	15	40	33 HRC
Ti-35Nb-7Zr-5Ta	827	793	20	55	35 HRC

HRB = Hardness, Rockwell B Scale, *HRC* = Hardness, Rockwell C Scale.

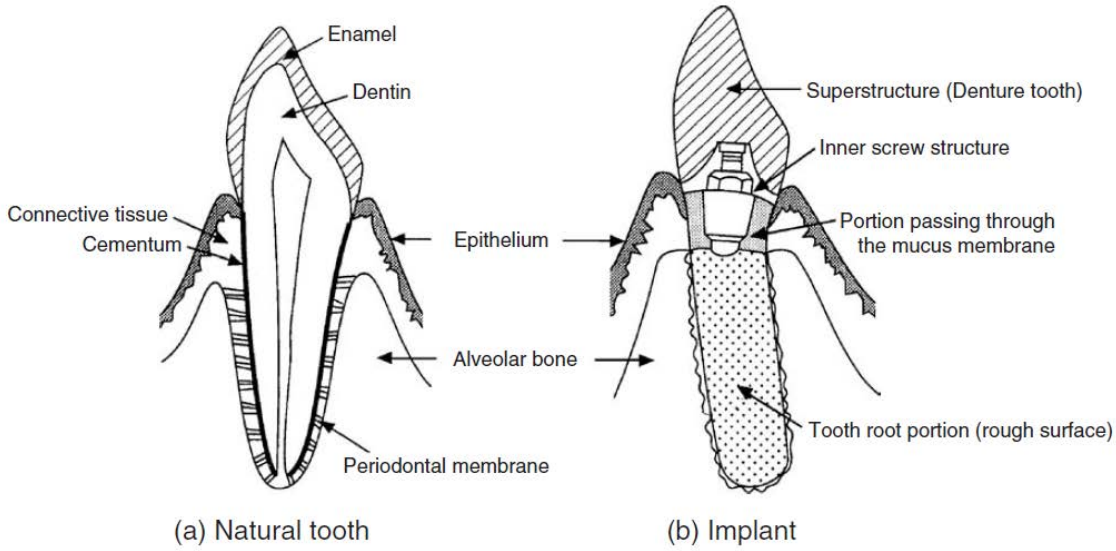

Figure 2.1 Schematic comparison between natural tooth and implant tooth (Oshida, 2006)

2010). Therefore, the following section provides a method for improving the bone-bonding ability of an implant by modifying Ti surface as investigated in the present study.

2.3 Physiological response to implanted materials

Recently, few studies have reported a correlation between nanoscale surface topography and cell interaction (Conforto *et al.*, 2008; Lamolle *et al.*, 2009; Chamberlain *et al.*, 2011). There are a few biological activities involves before the cell-metal interaction take place. Figure 2.2 shows biological response to material surfaces. Immediately after biomaterial implantation, interaction between water molecules and surface material occur. Then, protein adsorption takes place. Protein that bound to biomaterial surface act as detection sites via specific cell receptors for cell to adhere (integrins) (Jell *et al.*, 2009). Surface chemistry and topography affects the protein quantity, conformation, direction and distribution that bound to biomaterial surface. For instance, direction of adsorbed protein and conformation may hinder cell receptor detection. Cell anchorage to biomaterial surface is crucial for majority of cell type to survive. Focal adhesion sites are combination of proteins that bound to biomaterial surface, receptors at cell membrane and cytoplasmic proteins. Interaction between focal adhesion and cytoskeleton stimulate signal transduction, protein production, gene expression and ECM remodeling. These critical factors subsequently affect cell behavior. These reveal that adaptation of cell adhesion and behavior towards biomaterial surface depends on the type of cell, materials surface and environment.

2.4 Biocompatibility of nanomaterials

Ti is acceptable worldwide for its excellent in implant application due to the biocompatibility properties of Ti. Biocompatibility can be address by an ability of a material to perform with an appropriate host response in a specific application

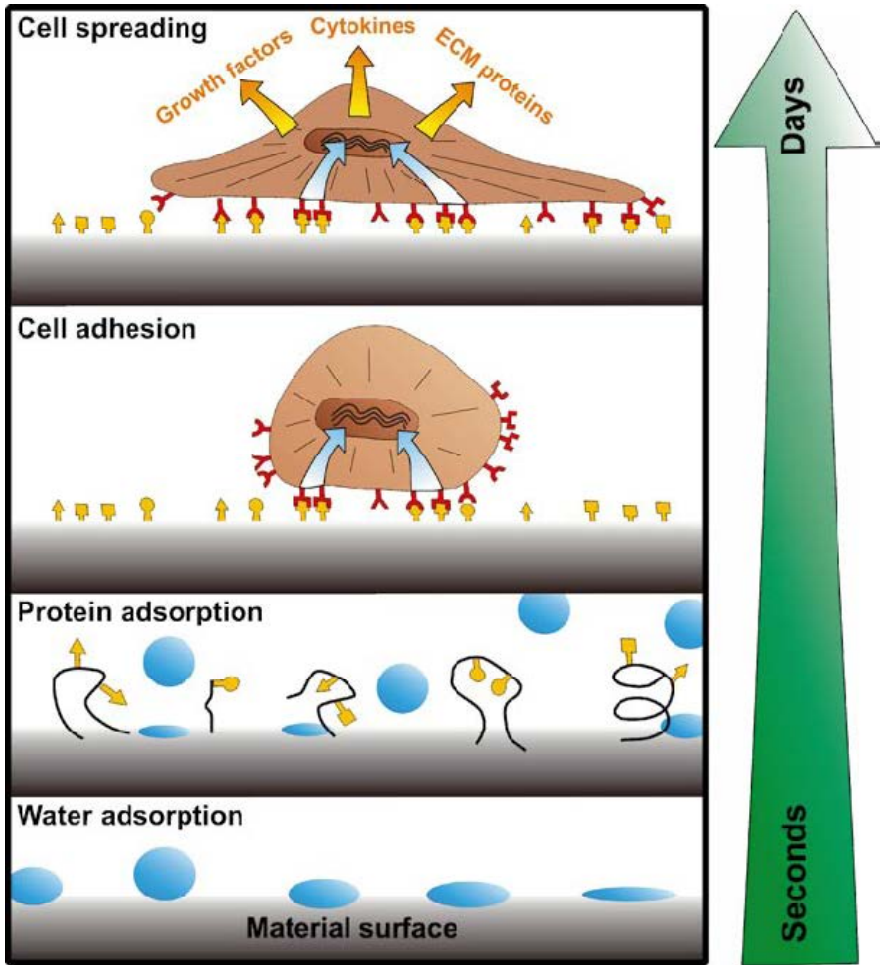

Figure 2.2 Time line of the biological response to material surfaces (Jell *et al.*, 2009).

(Ratner, 2001). Cytocompatibility and healing process can be improved by modifying Ti surface (Balasundaram *et al.*, 2008).

The high degree of Ti implant biocompatibility is usually ascribed to their ability to form stable and dense oxide layers consisting mainly of TiO_2. The native oxide layer on Ti is spontaneously grown in most environments whenever has mechanically damaged. These native oxide layers are usually 2-5 nm thick, depending on the redox potential of the surrounding environment. Based on previous

experience with Macak and co-workers (2005a), thicker oxide layers can be grown on the alloys by electrochemical anodization in various solutions.

Nanomaterials formulation exhibited a greater real surface area compared with conventional materials. It is because nanomaterials may significantly influence nanoimplants corrosion behavior (Yu *et al.*, 2009). TiO_2 nanotubes layer has larger surface area compare to conventional materials surface. This matter will affect titanium corrosion resistance (Saji and Choe, 2009). One important characteristic for implant materials is corrosion behavior. TiO_2 nanotubes possessed better corrosion resistance than bare alloys or pure Ti metal (Al-Mobarak and Al-Swayih, 2014). Hollow structure act as perfectly pits because can behave as effective channels for electrolyte to reach implant materials surface. Lower corrosion resistance of these TiO_2 nanotubes resulting from concave shape of tubes bottom and distinctly separated tube bottom with barrier oxide interface (Saji *et al.*, 2009). Thin oxide layer approximately 4 nm on Ti surface make Ti relatively inert and corrosion resistance metal (Ratner, 2001). Pure Ti metal has positive ions that tend to oxidize upon exposure to the environment. Therefore, a systematic research on TiO_2 nanotubes corrosion resistance is a must before clinical trial. Corrosion behavior will be defined either implant materials is biocompatible or not.

2.4.1 Titanium osseointegration

For the past 30 years, many researches have been developed for improving osseointegration (Ehrenfest *et al.*, 2009). In clinical terms, osseointegration is defined as the stability and stiffness of a joint due to abnormal adhesion and rigidity of the bones which may be the result of injury or disease (Arakeri *et al.*, 2011) of an implant in bone. Fibrous tissue isolates Ti from surrounding bone after implantation

process (Das *et al.*, 2007) by a process known as the foreign body reaction (van den Beucken *et al.*, 2005). Oxidative stress from surgical trauma during and after implantation will create overproduction of free radical and oxygenated derivatives. This phenomenon will thicken TiO_2 layer. Calcium and phosphorus ion from bone matrix are then incorporated within TiO_2 porous layer resulting interface between bone and implant to be highly dynamic (Khor *et al.*, 2006). Unfortunately, contamination and destruction of TiO_2 layer leads to peri-implantitis process. This process is destructive inflammatory process affecting the soft and hard tissues surrounding the implant materials (Ehrenfest *et al.*, 2009). To facilitate osseointegration, an anodization treatment of titanium and its alloys to achieve thicker and more stable TiO_2 based oxides, which are generally favorable for the surface bioactivity (Macak *et al.*, 2005) were studied in the present study.

2.5 Cell-metal interaction

Implantable biomaterials are subjected to several interacting forces whenever they come in contact with the physiological systems (blood, immune system, nervous, digestive, respiratory, reproductive and urinary) and organ in human body. The interaction include the effects of body temperature, body physiological fluids containing several ions and bio-molecules, proteins and cells with various functions (Jackson and Ahmed, 2007). The first interactions are between the cell and surface defines the quality of the cell-metal interaction (Anselme *et al.*, 2010). Integrin receptor acts as an interface between the intracellular and extracellular compartment in cell-metal interaction process. On the extracellular side, integrin interact with ECM and on intracellular side, integrin interact with cytoskeleton and signaling molecules at the adhesion site, called focal adhesion. Figure 2.3 ascribed the

filopodia, lamellipodia and focal adhesion of cell on surface substrate. However, limited information has been discussed in literature on cell–materials interactions. Llopis-Hernández *et al*., (2011) describe cell-material interaction as a complex bi-directional and dynamic process that mimics biological function to a certain extent the natural interactions of cells with the extracellular matrix. Figure 2.4 shows example ECM covering the substrate surface. Surrounding cells tends to adhere and rearrange adsorbed ECM proteins on the materials surface in a fibril-like pattern. In various literatures, the filopodia act as cell's tools to explore its surrounding (Le Guehennec *et al.*, 2008; Das *et al.*, 2009; Yu *et al.*, 2010).

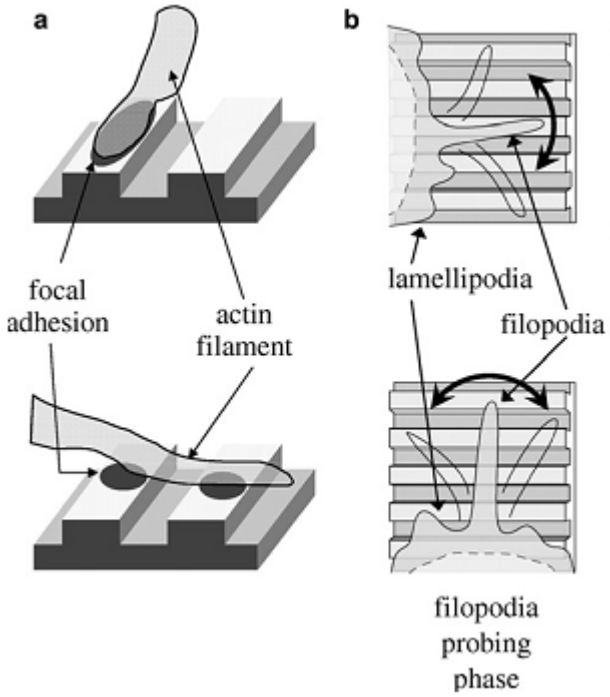

Figure 2.3 Model for cell alignment on surface substrate. (a) Focal adhesion and actin filament adhere to surface substrate. (b) Filopodia movements are isotropic on surface substrate. Adapted from Anselme *et al.*, (2010).

In general, cell-metal interaction study tends to focus on the initial cell adhesion phase. However, little attention is paid to cell-metal interaction after this stage. First, cells adhered within 24 h, called short-term adhesion. The long-term adhesion represents the strength of cell-metal interface formed within 3 weeks of culture period involving ECM proteins synthesized by the cells themselves and cell-cell interactions can be seen (Anselme *et al.*, 2010). However, cell-metal interaction mechanism which the topographical cue effects the functions of PA6 cells are still not well understood and this has hampered optimization of the biomaterials topography. Therefore, in this work, surface factors such as dimensionality, surface roughness, crystal structure and surface coating were investigated to understand the cell–materials interaction for the next generation orthopedic implants.

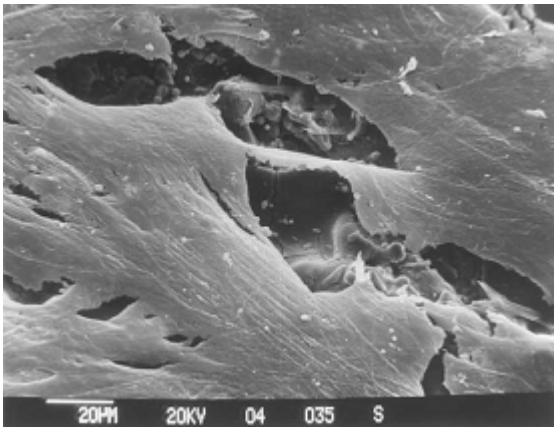

Figure 2.4 Scanning electron micrograph showing a multilayer of rat bone marrow cells and extracellular matrix covering the substrate surface (Knabe *et al.*, 2002). Bar = 20mm.

2.5.1 Cell

An adult human body consists of more than 50 trillion cells and most of these cells are specialized in structure and function (Wynsberghe *et al.*, 1995). Whatever their specific functions, most cells are capable of carrying on life-sustaining

activities such as breaking down food molecules for energy and generating energy-rich adenosine triphosphate (ATP), reproducing, synthesizing chains of polypeptides, engulfing foreign materials and creating new cell structures and getting rid of old ones. Each cells works together with other cells to provide an environment that is compatible with all the process of life (Wynsberghe *et al.*, 1995). Scientists divide cells into four basic parts:

1. The plasma membrane is the outer boundary of the cell. It selectively allows substances to pass into and out of the cell.

2. Cytoplasm is the portion of the cell outside the nucleus and within the plasma membrane. Metabolic reactions take place here with the aid of specialized structures called organelles. The fluid portion of the cytoplasm is called cytosol.

3. The nucleus is the control center of the cell. Within the nucleus are the chromosomes that contain the genes that direct reproduction, information flow and the heredity of cells. The nucleus is a clearly defined body that is separated from the surrounding cytoplasm by a double nuclear envelope.

4. Nucleoplasm is the material within the nucleus.

Table 2.3 shows the selection of cell types to investigate their response and interaction to biomaterials. From Table 2.3, it can be concluded that different cell types gave different cell responses. So, in present days, specific studies are focused to understand the influence of cell type, response and biological mechanisms to specific nanotopography pattern.

Table 2.3 List of cell type selection to study cell-metal interaction and its information.

Year	Author	Cell type	Information
2014	Lan *et al.,*	Human nasal epithelial cells (HNEpC)	Nasal application (nasal septal perforation repairmen, nasal reconstruction or rhinoplasty and CSF rhinorrhea repairmen).
2013	Lan *et al.,*	MRC-5 human fibroblasts	Stronger diameter dependence of cell activity
2012	Cao *et al.,*	Rat bone marrow stromal cells (BMSC)	Used as dental or orthopedic implants
2011	Brammer *et al.,*	MC3T3-E1 mouse osteoblast cells	Aiding in the design of orthopedic implants with improved osseointegrating interfaces.
2011	Chamberlain *et al.,*	Bone marrow cells differentiated into macrophage cells	Decreased inflammatory response in medical devices
2011	Choe	MC3T3-E1 mouse osteoblast cells	Used in dental and orthopedic implant materials
2011	Ma *et al.,*	Human gingival fibroblasts (HGF)	Use in dental implant abutment
2011	Narayanan *et al.,*	MG63 human osteosarcoma cells	Used as orthopedic implant materials
2011	Smith *et al.,*	Human dermal fibroblasts and human epidermal keratinocytes	Allow primary integration between the dermis and the transcutaneous implantable devices. Epidermal integration based on subsequent cell signaling and cell-cell attachment.
2011	Yang *et al.,*	Osteoblast from fetal rat calvarial cells	Osteoblasts are established cells that respond to the material substrate and have pivotal role at the surface of implant materials with the secretion of many cytokines involves in bone remodeling
2010	Yu *et al.,*	MC3T3-E1 mouse osteoblast cells	Used as implantation materials.
2008	Das *et al.,*	Osteoblastic precursor cell line (OPCI)	Preferential cell attachment on rough surface compare to smooth surface.

2.5.2 PA6 cells

PA6 or MC3T3-G2 cells are stromal cell line derived from newborn mouse calvaria (Turksen, 2002). Bone marrow stromal cells are a critical cellular element of the bone marrow microenvironment and support the production of blood cells from the bone cavity in adults (Milwid *et al.*, 2013). In certain situation, PA6 cells mono layer can be used as feeder cells that support sustained generation of various hematopoietic progenitor types. PA6 cells support the differentiation of cells resembling osteoclasts in co cultures with spleen cells (Krauser *et al*., 1994).

2.6 Nano-scale surface engineering on Ti

The term ‘surface engineering’ was used for the first time in England in the 70s. Different aspect of thermal spraying and welding are focusing at the beginning before progressively broadened its range of attention. Then, Wolfson Institute for Surface Engineering was formed at University of Birmingham. That institute mainly concern with problem stemming from surface diffusion treatment with vacuum technology at the beginning. Next, the activity broadening its scope to various technique of surface layers formation. Surface engineering is a science discipline including surface layers manufacturing processes (coating and exterior layer for the purposes of scientific and technology) related phenomenon and performance effects (Burakowski and Wierzchoń, 1999).

Surface modification can be derived by transformation of structure, morphology and material surface composition without leaving the bulk mechanical properties (Hanawa, 2009). The aim of Ti surface modification is to produce fine porous layer on biomaterials. Specifically, cavities and high surface area of

biospecies, precursor's adsorption and anchoring were exploited (Macak *et al.*, 2005b). To further improve Ti bioactivity, biocompatibility, the interface between bone and implant and implant anchorage to bone, different surface modification methods have been explored (Tsuchiya, 2006).

Typically, two different strategies have been developed. In the first approach by incorporating inorganic phases such as calcium phosphate on or into TiO_2 layer interface chemically was improved. This inorganic chemical modification, bone regeneration is stimulated and biochemical interlocking between bone matrix proteins and surface materials increase. Conversely, biochemical surface modification is differ from first strategy and refer to organic molecules incorporation such as protein, enzymes or peptides to persuade specific cell and tissues responses (Ehrenfest *et al.*, 2009).

For second approaches, the interface is improved physically by surface topography architecture. At micrometer stage, rough surface create higher developed area rather than smooth surface. This rough surface increase bone anchorage reinforced the biomechanical interlocking of the implant with bone up till certain level of roughness. At nanometer stage, the roughness increase surface energy to improve protein matrix adsorption, bone cell migration, proliferation and osseointegration (Ehrenfest *et al.*, 2009). To date, anodization of Ti has been investigated because can be easily create biological-inspired nanometer roughness (Balasundaram *et al.*, 2008). From Table 2.4, nano-scale topography of TiO_2 provides adhesion sites for protein help cell proliferation into 3D formation and facilitate in cell differentiation as compared to other surface morphology. Wu *et al.*, (2008) indicate the potential use of spin-coating materials for orthopedic and implant materials. Spin coating significantly improved adhesion strength, chemical stability

Table 2.4 List of surface engineering approaches to achieve geometrical structuring of TiO_2 surface.

Year	Author	Surface engineering	Morphology	Finding
2001	Casaletto *et al.*,	Metal organic chemical vapor deposition	Thin film	Higher amount of organic species found on the substrate surface
2005	Li *et al.*,	Polymeric sponge replication	Porous TiO_2 sponge	Nontoxic and favorable for cell attachment
2009	Chiang *et al.*,	Anodization	Nano-network layer of TiO_2	Greater amount of proteins on nano-scale TiO_2 network. Help human bone marrow stem cells (hMSC) proliferate to 3D formation in vivo Facilitate hMSC to differentiate toward osteogenic lineage
2010	Raimondo *et al.*,	Electron beam evaporation	Micron rough surface features and higher degree of nanometer surface features	Increase surface energy and promote surface osteoblast and endothelial cell adhesion

and able to form apatite layer in SBF compared to HA coat Ti-6Al-4V. Based on the literature review of this section, it is important to optimized implant materials regarding their surface characteristic (e.g. chemistry, topography, surface energy and morphology (Bauer *et al.*, 2008) before implantation.

2.6.1 TiO_2 nanotube arrays

An approach for synthesis of highly ordered and vertically oriented TiO_2 nanotubes on Ti and Ti-alloy substrates have been discovered in 1999. Basically, anodization of the substrates was performed in a fluoride containing electrolyte under specific electrochemical conditions that lead to self-organize TiO_2 nanotubes. Up to now, several generations of nanotubes have been brought forward (Kalbacova *et al.*, 2008) to further improve its dimensionality.

For pure Ti, the compact TiO_2 layer thickness increase gradually with the growth rate of 2.5 nm/V up to applied voltage where dielectric breakdown of the oxide occurs at 100 - 200 V. Ti anodization in electrolytes contain F^- ion at 10 - 20 V produced porous structures (π-TiO_2) consist of nanotubes with 100 nm range diameter (Macak *et al.*, 2005b) and a length of ~ 500 nm (Tsuchiya, 2006). Figure 2.5 shows TiO_2 nanotubes morphology and cross sectional view. Throughout anodization process, the Ti foil surface color generally transforms from purple to blue, yellow, red, and then lastly light red (Quan *et al.*, 2005). Through this technique, TiO_2 nanotubes with a diameter ~ 100 nm could be developed.

The construction of the nanotube structures on titanium during anodization is because of the competition between TiO_2 growth and TiO_2 dissolution. Throughout the Ti foil anodization nanotube structures are produced through two processes: field-enhanced Ti oxidation and field-enhanced oxide dissolution. There are two