臺北醫學大學口腔醫學院

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鈦基合金表面生成二氧化鈦薄膜之生物相容性研究

Research of biocompatibility on titanium-based alloy with titanium dioxide film

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論 文 摘 要

論文名稱：鈦基合金表面生成二氧化鈦薄膜之生物相容性研究

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內文

牙科植體漸漸成為治療缺牙區的選項之一時，許多學者便致力於研究植體與組織的交互作用，經由表面形態的改變，追求更有效率的骨整合。已有研究提出鈦金屬表面形成的氧化層跟其優良的生物相容性有關，因此瞭解氧化層的性質與改變氧化層的結構、形態、厚度等，讓人甚感興趣。

本實驗利用電漿處理的表面處理方式，得到不同表面氧化層的鈦金屬試片，以人類骨肉瘤細胞培養後觀察其所呈現的細胞形態以及與試片接觸的情形，同時比較血液相容性的差異。結果顯示經過處理的鈦金屬，其生物相容性與血液相容性都有改善的現象。

關鍵詞：**鈦金屬、表面處理、牙科植體**

Abstract

Title of Thesis：Research of biocompatibility on titanium-based alloy with titanium dioxide film

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The biocompatibility of titanium is closely related with the surface oxide film. The improvement of the biological activity of titanium with plasma immersion on implantation has been investigated. However, the effects of oxygen ion implantation on the titanium were still lack. In this study, the titanium plates were implanted with oxygen at different power conditions and subsequently analyzed for surface morphologies and phase composition. To observe the effect of oxygen ion implantation on cell behavior, MG-63 osteoblast-like cells were cultured on the treated titanium plates. The hemocompatibility was determined by measuring the adhesion of blood platelets and fibrinogen, P-selectine expression, and the blood clotting time on these modified titanium plates. The results revealed that MG-63 osteoblast-like cells expressed better response on the surface-treated titanium plates. Furthermore, the results of clotting time assay demonstrated titanium treated by oxygen ion power could promote blood coagulation. The titanium plates treated with 1 KW ion power revealed better expression of platelets and fibrinogen adhesion, which showed the higher average roughness. In conclusion, the biocompatibility and hemocompatibility of titanium-based alloy can potentially be improved by plasma immersion oxygen ion implantation.

Keywords: titanium, surface treatment, dental implant.

Chapter 1

Introduction

1.1 General background

Treatment options for patients with partial or complete edentulism are not limited to conventional removable prostheses in recent two or three decades. More and more people pay attention to the usage of dental implants for treatments of missing teeth. In the United States, more than 700,000 dental implants are inserted each year (Misch 2005). Between 1986 and 1990, a survey of U.S. dentists showed that dental implant use increased 73 percent (Stillman and Douglass 1993). Furthermore, the number increased more than tenfold from 1983 to 2002 (Misch 2005). Currently different implant brands and different designs have been identified in the markets. The goal of implantology research is to design devices that induce controlled, guided, and rapid integration into surrounding tissues (Puleo and Thomas 2006). Events leading to integration of an implant, and ultimately to success or failure of the device, take place mainly at the tissue–implant interface. Many factors may influence the tissue-implant interface, such as bone quality and quantity, implant materials, and implant surface characteristics. Among these factors, implant surface characteristics become the most important factor in implantology.

The concept of osseointegration was originally introduced by Brånemark et al. in 1977. Successful implant must have good osseointegration. Osseointegration means a direct – on the light microscopic level - contact between living bone and implant (Branemark, Hansson et al. 1977). Hereafter, it defined as a direct structural and functional connection between ordered,

living bone and the surface of a load-carrying implant (Branemark, Zarb et al. 1985). The establishment of osseointegration is according to Albrektsson et al. dependent on the following parameters: ❶ Implant material; ❷ Implant design; ❸ Implant finish; ❹ Status of the bone; ❺ Surgical technique; ❻ Implant loading conditions (Albrektsson, Branemark et al. 1981).

Titanium is the most widely used dental implant material for its excellent physical properties and biocompatibility. In order to achieve faster result of osseointegration, shorten the time of prosthetic treatment, researchers invested a lot of effort for the titanium implant to study the surface properties. The biocompatibility of titanium is related to the properties of the surface oxide film, in terms of its structure, morphology and composition. Many researchers have studied the effect of different surface properties of titanium implant to peri-implant bone formation (Schwartz, Lohmann et al. 1999; Ronold, Lyngstadaas et al. 2003). However, little knows about structural and chemical surface properties influence biological responses. The properties of the passive oxide have been pointed out as important factors. Other surface properties such as oxide composition and impurity layers, topography and roughness, oxide thickness and microstructure of titanium implants vary considerably, depending on the type of surface preparation used (Doundoulakis 1987; Keller, Stanford et al. 1994). Among all kinds of surface treatment, increasing surface roughness and titanium oxide formation were considered the most simple and effective methods. Therefore the current study will focus on these two options.

1.2 Motivation of this study

Since 1965, Dr. Brånemark had successfully done the implant surgery for the first patient, and until now it has been more than 40 years. Dr. Brånemark pronounced the concept of osseointegration at North America Dentist Meeting at 1982. He observed the contact between titanium implant and bone and found that pure titanium have excellent biocompatibility, it can long-term implant in the living bone tissue without any complications of inflammation or rejection (Branemark, Hansson et al. 1977). Titanium implant has been widely and successfully applied on dental implant. Its success rate up to 90% (Krennmair, Seemann et al. 2010). However for the patients with osteoporosis, especially at posterior area of maxilla, the properties of surface oxide layer play an important role at implant success rate. Therefore, improving the properties of implant surface oxide layer is absolutely necessary to achieve clinical faster and effective osseointegration.

1.3 Purpose of this study

Following implantation, implant directly contacts to host tissue; events take place both on the biological side and on the materials side. Thus the material characteristics and biocompatibility are the keys to establish suitable phase integration between implant surface and alveolar bone. The physical and chemical properties of implant surfaces are generally considered as decisive factors for the biocompatibility of biomaterials. Titanium implants have a thin oxide layer on its surface. The properties of this oxide layer may interpret the good biocompatibility of titanium implants. There are lots of methods altering surface properties to improve implant performance, such as sandblast, coating, acid etching and

anodizing. Much attention has been focused on changes in surface topography, including surface roughness and thickness of oxide layer. Implant with proper surface topography may make osseointegration more effective and rapidly. That is what the study is seeking for. The purpose of the study is to improve and maintain osseointegration of titanium implants by plasma implantation. The aim of the study is to increase surface roughness and thickness of oxide layer. Moreover by culturing cell on different surface treated titanium discs, we can observe the cell morphology to understand the cell activity at different surface to simulate the possible outcomes after implantation in human. This may be useful for clinical application and material manufacture.

Chapter 2

Literature Review

2.1 Properties of titanium

Whenever titanium comes in contact with body tissue fluid, a stable oxide layer will form on the surface. This oxide formation is the basis for the clinical excellent biocompatibility of titanium. Moreover when exposure to air, titanium can immediately (10^{-9} second) form the oxide layer on surface. This oxide layer reaches the thickness of 2-10 nm within one second and provides the ideal corrosion resistance. Meanwhile its elastic modulus is similar to that of bone. Thus titanium and its alloys have become the gold standard in implant materials. Generally commercially pure titanium can divide into four groups with the amount of impurity content (table 2-1). The oxygen content decreases from grade IV-0.4% to the lowest grade I-0.18%. This may contribute to various degree of impact on extension and strength of materials. The amount of impurity content influencing the mechanical and physical properties of titanium metal is list at table 2-2 (Powers, Sakaguchi et al. 2006). Titanium is a high strength but low density metal. It has excellent corrosion resistance, especially in the environment free of oxygen. It is as strong as steel, but 45% lighter than steel. It is 60% heavier than aluminum; however, it's twice as strong. The most noted chemical property of titanium is its excellent resistance to corrosion; it is almost as resistant as platinum, capable of withstanding attack by acids, moist chlorine in water but is soluble in concentrated acids.

From a materials view, titanium is a suitable choice. It is a relatively simple material, which means that it is possible to make a thorough physical and chemical characterization of the

material and its surface properties. Besides, the titanium surface can either be chemically or physically modified, or both, in order to improve biomaterial–tissue integration. The favorable biological performance is attributed to a thin native oxide film, which spontaneously forms on the titanium surface. Since the concept of osseointegration has been introduced in 1977, titanium and its alloys have gained more and more attraction as biomaterial because of their prominent mechanical, chemical, and biocompatible properties. It has been the most frequently used material for load-bearing dental implants in bone and also has been more frequently used as orthopedic implant material recently.

2.2 Characterization of TiO_2

The excellent biocompatibility of titanium comes from the dense oxide layer on surface. Regarding the surface oxide on commercially available turned c.p. titanium implant systems, previous spectroscopic studies have reported that oxide thicknesses are in the range of 1.8–17 nm, and that the chemical composition consisted mainly of TiO_2 (Binon, Weir et al. 1992; Machnee, Wagner et al. 1993; Olefjord and Hansson 1993; Lausmaa 1996). Walivaara et al. used Raman spectroscopy and found that the oxide structure was non-crystalline (Walivaara, Aronsson et al. 1994). Measuring by TopScan 3D measuring system, surface roughness varied from 0.53 to 0.67 μm in Ra values (Wennerberg, Albrektsson et al. 1993). This thin oxide film, naturally formed on a titanium substrate, is presumably responsible for the excellent biocompatibility of titanium implants due to a low level of electronic conductivity (Zitter and Plenk 1987), a high corrosion resistance and a thermodynamically stable state at physiological pH values (Solar, Pollack et al. 1979). In addition, titanium and its oxide(s) have a low ion-formation tendency in aqueous environments (Tengvall and Lundstrom 1992).

The responses of cell and tissues at implant interfaces have been shown to be affected by the chemical properties, topography, and roughness of the implant surfaces. It is of interest to research the influence of the oxide film structure, composition, and thickness on the corrosion behavior and on the biocompatibility. Consequently, knowledge of the chemical and physical properties of titanium oxide layers is important for the reaction of cells in contact with biomaterials which are made of titanium and titanium alloys.

2.3 Influence of the oxide film thickness

The roughness and porosity of titanium dental implant surface are considered to promote the chelation between bone and implant, and the surface oxide layer is also the important factor of osseointegration. Studies showed that bone healing around machined titanium implants takes place by a gradual mineralization process which is directed towards, but does not start at, the implant surface (Ma, Wei et al. 2008). Based on this, the (machined) titanium surface can be regarded as a permissive surface for gradual bone mineralization, but not as a bone-inducing surface. They also pointed out that treated implants present rougher surface to improve the growth of bone cells, accelerate osseointegration, and the composition of surface oxide layer was altered as well. Because machined titanium implant cannot achieve the ideal direct bone growth, researchers gradually pay attention to increase surface roughness and structure of oxide layer. Early in 1990s, researchers studied titanium surface properties and bone formation. They implanted machined and anodized implants in the tibia bone of New Zealand White rabbits, and found that the bone volume as well as bone-implant contact obviously lower at smooth (machined) surface. Bone formation was better at thicker and rougher oxide

layer (Larsson, Thomsen et al. 1996) (Larsson, Thomsen et al. 1994). After then, other researchers indicated stronger bone tissue reactions to implants having an oxide film thickness of 600–1000 nm compared to implants with an oxide thickness of 17–200 nm. They also demonstrated that the oxidized implants with oxide thicknesses of about 600, 800 and 1000 nm had significantly higher removal torque values than did implants with oxide thickness of 17 and 200 nm (Sul, Johansson et al. 2001; Sul, Johansson et al. 2002). The optimal oxidized implant should be composed of approximately 9% magnesium at relative atomic concentration in titanium oxide matrix and have an oxide thickness of approximately 1,000 to 5,000 nm, a porosity of about 24%, and a surface roughness of about 0.8 microm in Sa and 27% to 46% in Sdr, its oxide crystal structure should be a mixture of anatase- and rutile-phase crystals (Sul, Johansson et al. 2005).

2.4 Influence of the oxide film roughness

Already by the beginning of the 1980s, surface structure was identified as one of the six factors particularly important for implant incorporation into bone (Albrektsson, Branemark et al. 1981). Porous materials are examples of extreme surface roughness. Such materials have been used to allow growth of tissues into implants to enhance integration. Recently the methods of surface treatment develop into microrough surface. Several studies showed that titanium implants with a microrough surfaces achieve a faster bone integration, a higher percentage of bone implant contact, and a higher resistance to shear documented with higher removal torque values when compared with titanium implants with a polished or machined surface (Nasatzky, Gultchin et al. 2003). Early study with bioinert ceramics showed that pore sizes greater than 100 μm were needed for ingrowth of mineralized tissue (Hulbert, Morrison

et al. 1972). Pores in the range of 40 to 100 μm allowed formation of osteoid, and only fibrous tissue was present in 5 to 15 μm pores. The importance of pores exceeding 100 μm was also shown for metallic implants (Bobyn, Pilliar et al. 1980). More recent work with biomaterials indicates that bone may grow into smaller pores and that the size and volume density of interconnections is important because of the need for blood circulation and extracellular liquid exchange. A 20 microm interconnection size only allows cell penetration and chondroid tissue formation; however the size of the interconnections must be over 50 microm to favor new bone ingrowth inside the pores (Lu, Flautre et al. 1999). A recent electron microscopic examination of implants retrieved from humans revealed that mineralized bone had grown into the pores of the surface oxide layer, including pores with small diameters (< 2 microm) (Schupbach, Glauser et al. 2005). Another review article form Wennerberg mentioned a huge number of the experimental investigations have demonstrated that the bone response was influenced by the implant surface topography. Smooth (Sa< 0.5 μm) and minimally rough (Sa= 0.5-1 μm) surfaces showed less strong bone responses than rougher surfaces. Moderately rough (Sa> 1-2 μm) surfaces showed stronger bone responses than rough (Sa> 2 μm) in some studies (Wennerberg and Albrektsson 2009). Although the ideal surface roughness varies, it is convinced that surface roughness of materials play an important part in the interaction between implant materials and bone.

In order to understand the mechanism of effect of surface roughness to bone, researchers also pay attention to experiments of cellular responses. The results of these experiments suggest that the type of roughness produced on a c.p. Ti surface does affect initial biological responses such as cellular attachment and spreading. Significantly higher levels of cellular attachment were found using rough, sandblasted surfaces with irregular morphologies (Bowers, Keller et al. 1992). Decreased proliferation, increased ALPase and osteocalcin production and more

differentiated cell also can be identified on rough surface (Martin, Schwartz et al. 1995; Lincks, Boyan et al. 1998). Otherwise, surface roughness could modulate hormones.

On rough Ti surfaces, osteoblasts also generate an osteogenic microenvironment to regulate bone remodeling through autocrine and paracrine pathways (Boyan, Lossdorfer et al. 2003). MG-63 cells on rough surfaces secrete more prostaglandin E_2 (PGE_2) and transforming growth factor-β_1 (TGF-β_1) to promote osteoblastic activity (Kieswetter, Schwartz et al. 1996) and produce osteoprotegerin to decrease osteoclast formation and activity (Lossdorfer, Schwartz et al. 2004). The effects of 1α-estradiol and 1,25-dihydroxy vitamin D_3 [1α,25$(OH)_2D_3$] on differentiation and local factor levels are increased in a synergistic manner on microrough surfaces (Boyan, Batzer et al. 1998). Other study pointed out that total PGE_2 content in the media of cultures grown on the three roughest surfaces: EA（fine sand-blasted, etched with HCl and H_2SO_4）, CA （coarse sand-blasted, etched with HCl and H_2SO_4）, and TPS （Ti plasma-sprayed）was significantly increased 1.5-4.0 times over that found in media of cultures grown on plastic or smooth surfaces. When PGE_2 production was expressed per cell number, CA and TPS cultures exhibited six- to eightfold increase compared to cultures on plastic and smooth surfaces. There was a direct relationship between TGF-beta 1 production and surface roughness, both in terms of total TGF-beta 1 per culture and when normalized for cell number. TGF-beta 1 production on rough surfaces (CA and TPS) was three to five times higher than on plastic. These studies indicate that substrate surface roughness affects cytokine and growth factor production by MG-63 cells, suggesting that surface roughness may modulate the activity of cells interacting with an implant, and thereby affect tissue healing and implant success (Kieswetter, Schwartz et al. 1996). In order to assess the proliferation and differentiation of human bone marrow-derived cells cultured on titanium surfaces with different roughness characteristics, researchers compared the smooth surface and etching with

HF/HNO_3 for 15 and 30 minutes (rough surfaces). It showed that the expression of osteopontin and osteocalcin was greater at rough surface, indicating cells were differentiating into osteoblasts. Therefore it could infer that increased surface roughness accelerates the differentiation of undifferentiated mesenchymal cells into osteogenic lineage cells, but does not necessarily favor cell proliferation (Silva, Machado et al. 2009).

2.5 Ion implantation

Plasma immersion ion implantation (PIII) is a faster and more cost-effective modification that can obtain dense and pore-free films without adhesion problems (Conrad, Radtke et al. 1987; Mandl, Krause et al. 2001). Ion implantation comprises high-vacuum technology that can be applied under controlled temperature conditions. The ions disrupt the surface of the material due to their high kinetic energy, penetrating and becoming implanted within its atomic network – a phenomenon that implies modifications in the most superficial layers of the material. The implanted zone forms an integrated part of the material, thus avoiding the risk of delamination associated with coating techniques. Furthermore, there is no material loss with such processes – a fact that affords advantages over material removal techniques. Ion implantation is clean, versatile, highly controllable and reproducible, and induces intrinsic modifications within the more superficial layers, while preserving the structure and characteristics of the background material.

Several in vitro and in vivo studies have been performed using this method based on ion implantation. Previously published reports have shown better results when using this treatment, with greater bone–implant contact (BIC) when compared to simple machine-turned

titanium implants and diamond-like carbon (DLC)-coated implants (De Maeztu, Alava et al. 2003; De Maeztu, Braceras et al. 2008). Better results were also obtained over a short period of time in dogs with implants treated with ion implantation when compared to machine-turned titanium (De Maeztu, Braceras et al. 2007). Faster bone formation around implants treated using the ion implantation technique has been confirmed by other authors such as Bosetti et al. (Bosetti, Masse et al. 2001), who also demonstrated an improvement in surface-treated material in terms of resistance to corrosion, fatigue and metal ion release.

S. Mändl et al. investigated oxygen PIII treated titanium implants using a well-established rat model (Mandl, Krause et al. 2001). They concluded that using oxygen PIII, the formation of dense rutile layers with rather good adhesion on titanium is possible. Comparing the osseointegration of different titanium alloys in rat femurs with and without PIII treatment, it was observed that plasma immersion ion implantation can further improve the osseointegration of treated implants (Mandl, Sader et al. 2002).

Chapter 3

Materials and Methods

3.1 Materials and preparation

The grade II titanium substrates (Bio Tech One Inc., Taipei, Taiwan) used in these experiments were 1-mm-thick plates with a diameter of 14.5 mm. The titanium plates for the present study were polished using 600-grit SiC metallographic paper. After being polished, specimens were cleaned with methylethylketone solvent for 5 min, washed in distilled water for 20 min, acid pacified in 30% nitric acid for 30 min according to the American Standard Testing Materials (ASTM) procedure and rinsed again in ultrapure water for 20 min.

The plasma-oxidized samples were first cleaned in Ar plasma to remove the native surface layer (adsorbed contamination and impurities) and produce a repeatable starting condition for the subsequent oxidation procedure. The Ar plasma cleaning was immediately followed by different plasma treatments, using Ar and O_2 as the process gases. Samples were cleaned by argon-plasma treatment in the reactor. Plasma cleaning was performed at a working time of 10 min, and an argon flow rate of 100 sccm, after the base pressure was reduced to below 50 mTorr. The argon plasma treatment powers were the same as oxygen plasma treatment powers, which were at three different conditions (1kw, 2kw, and 3kw), denoted as Ti-1, Ti-2, and Ti-3, respectively. After plasma treatment, samples were annealed for 10 minutes in the reactor.

3.2 Samples analysis

To analyze the properties of treated titanium plates, the surface morphologies of the treated titanium plates were analyzed using an atomic force microscope (AFM, Nanosurf-Mobil S) with a Si probe. The AFM probe was scanned over an area of 5 μm x 5 μm, with 512 scans performed at a scanning rate of 1 Hz in the tapping mode. X-ray diffractometry (XRD, RIGAKU-2200) was used to identify the phases of the films in order to analyze the properties of titanium plates. Secondary ion mass spectroscopy (SIMS) was used to obtain the oxide thickness.

Surface wettability was evaluated by optical measurement of the static contact angle of water using a goniometer (KYOWR, CA-VP 150, Japan) at room temperature. For each measurement, a 5 μl droplet of distilled water was applied to the sample surface. The measurements were taken using a FTA-32 video contact angle system (First Ten Angstrom Inc., USA).

3.3 Cell culture

The cell culture experiments used MG-63 cells. Plasma treatment and control titanium plates were placed, modified side up, in 24 well culture plates. Then the titanium plates seeded at 5 $\times$ 10^4 cells/well in suspension on all titanium plates. After incubation, for all experiments, cells were cultured in different periods (12 hrs, 1 day, 3 days, 5 days, 7 days, and 9 days).

3.4 Glutaraldehyde- OsO_4 primary fixation

Cells were fixed with a 3% Glutaraldehyde/0.2M PBS solution for 15 min and washed with 0.1M PBS (5 min, 3 times). Then fixed with OsO_4 solution for 30 min and washed with 0.1M PBS solution (5 min, 3 times). Samples were dehydrated by gradient alcohol, and then sputter coated with gold, to be examined with scanning electron microscopy (SEM; Hitachi S4200).

3.5 Cell activity assay

Put the samples in 24 well culture plates, and seeded the MG-63 cell onto the surface of the specimens at a density of 5×10^4 cells/cm^2. After 4 hours cultured, washed with PBS (0.1M，pH7.2), then added 500μl medium and 50 μl MTT （3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide）. Cells were incubated at 37℃ in an atmosphere containing 5% CO_2. After 4 hours, the active cells formed the purple crystalline of formazan salt, then added the 10% SDS/ 0.01M HCl，500μl/ well. Take the culture overnight, and then the optical densities were measured using ELISA reader (ELISA reader; Csbiotech Anthos-2020) at 595 nm. Repeated the experiments for five times, and the mean results were compared with control group. Differences were considered significant for $p<0.05$.

Alkaline phosphatase activity of cell lysates was performed using extraction buffer, including 2mM ($MgCl_2$) and 1% TritonX-10, to dissolve cells. Place the samples in -20℃, 30 minutes then move to room temperature for 30 minutes, the procedures were preformed twice. The use of pipet tip helps the mechanical damage of cells, so that the cell membrane was perforated and the destruction was complete. Put 0.05 ml of samples in the 96 well plates, then added

0.05ml AB mixture (ALP buffer and phosphatase substrate solution) at 37℃ for 30 minutes reaction. The optical densities were measured using ELISA reader (ELISA reader; Csbiotech Anthos-2020) at 405 nm.

3.6 Hemocompatibility of the samples

The biocompatibility tests also use the blood to observe the clotting time, blood adhesion, and platelet adhesion. The optical densities were measured using ELISA reader to test the fibrinogen for plasma protein assay, CD 61 for platelet adhesion assay, and P-selectin for platelet activation assay. The details of experiments could be seen at Fig. 3-1-a, 3-1-b, 3-1-c, 3-1-d.

Chapter 4

Results

4.1 Atomic Force Microscope (AFM) surface analysis

Table 4.1 shows the surface roughness parameters of the control and the investigated samples. Under 5 μm resolution, the Rms and the Ra values for the control sample were 0.51 nm and 0.36 nm respectively, which were lower than other treated samples. Under 1 μm resolution, the Rms and the Ra values for the control sample were 6.05 nm and 4.73 nm respectively, which were larger than other treated samples. Besides, the Rms and the Ra values for the treated samples were observed to be between 2-3 nm under 5 μm and 1μm resolution. Average roughness of Ti\O_2 samples under 1, 2, and 3 KW ion power treated were showed in Fig4.1. The average roughness for Ti-1 was larger than other treated groups, especially the Ra values. There was no significant difference in Ra values between Ti-2 and Ti-3 groups.

4.2 Ti\O_2 Raman analysis

Raman analysis of Ti\O_2 samples under 1, 2, and 3 KW ion power treatments were shown in Fig 4.2. No differences in peaks among the investigated groups were observed.

4.3 $Ti\backslash O_2$ contact angle analysis

As shown in Fig 4.3, the larger of the mean contact angle was observed when the ion power was increased to treat the titanium disks. Ti-1 group showed the better hydrophilic properties when compared to the other groups.

4.4 $Ti\backslash O_2$ XRD analysis

Fig 4.4 shows typical XRD patterns. The XRD patterns show that all of the $Ti\backslash O_2$ groups were alpha Ti based crystal structure. No other crystal structure peaks were observed. And ion power treatment did not change the crystal structure of Ti disk.

4.5 The young's modulus and the hardness of different $Ti\backslash O_2$ samples

Fig 4.5.1 showed the young's modulus of the $Ti\backslash O_2$ samples. The results revealed no significant difference between all the $Ti\backslash O_2$ samples. Besides, Ti-1 group showed the lowest value of hardness. The hardness of the $Ti\backslash O_2$ samples increased with the increase of ion power (Fig 4.5.2).

4.6 Cell morphology of Ti\O_2 samples

Fig 4.6.1-3 OM images showed typical shapes of MG-63 cells cultured on the Ti\$O_2$ titanium disks after 3 days. The cells were observed to possess irregularly triangular or polygonal-shaped features. At 7 days of culture (Fig 4.6.4-6), the cells reached confluent at any Ti\$O_2$ titanium disks.

4.7 MTT values of MG-63 cells on different Ti\O_2 samples

The MTT values of the no specimens group show the typical growth curve of MG-63 cells (Fig 4.7). In addition, all of the different Ti\$O_2$ samples had significantly higher MTT values than the polished groups at 1, 3, 7 and 9 days of culture. At 9 days of culture, Ti-1 group revealed the greater MTT values than other Ti\$O_2$ groups, and the Ti-3 group showed the lowest MTT values than other Ti\$O_2$ groups.

4.8 ALP activity of MG-63 cells on different Ti\O_2 samples

As shown in Fig 4.8, ALP activity of MG-63 cells was lower for all the Ti\$O_2$ groups at 12 hours and 1 day of culture compared to the control group. However, there was no significant difference in ALP activity between control and all the Ti\$O_2$ groups at 3 and 7 days of culture.

4.9 Clotting time assay on different Ti\O_2 samples

The results of clotting time assay demonstrated titanium treated by O_2 ion power could promote blood coagulation (Fig 4.9). After 20 minutes, the mean optical density of solution from the treated disks was lower for all the Ti\O_2 groups compared to the control groups. However, there was no significant difference after 30 and 40 minutes.

4.10 Fibrinogen adhesion assay on different Ti\O_2 samples

The amount of fibrinogen adhesion was shown in Fig 4.10. The Ti\$O_2$ samples treated with 1 KW ion power revealed higher values of optical density than other Ti\$O_2$ and control groups. However, the differences between all the Ti\$O_2$ groups and the control group did not significant.

4.11 Platelet activation assay on different Ti\O_2 samples

The results of sP-selectin were shown in Fig 4.11. There were no significant different between Ti\O_2 samples treated with 1, 2, 3 KW ion power and the control group.

4.12 Platelet adhesion assay on different Ti\O_2 samples

Fig 4.12 shows the platelet adhesion assay of the control and experiment samples. The results of Ti\$O_2$ samples treated with 1 and 3 KW ion power were observed higher than the control group. However, The Ti\$O_2$ groups treated with 2 KW ion power showed the least optical density. The differences between all the Ti\$O_2$ groups and control group did not significant.

Chapter 5

Discussion

Titanium-based alloy is the most common material of implants. It is investigated that the air exposure of the titanium surface results in extremely rapid oxide formation, which contributes to its excellent biocompatibility (Kasemo 1983). Numerous surface modifications have been carried out to improve the biological activity and promote osseointegration by thickening the oxide layer (Larsson, Thomsen et al. 1994), increasing surface roughness (Martin, Schwartz et al. 1995) or increasing surface energy (Rupp, Scheideler et al. 2006). The major part of the study was performed using oxygen ion implantation method. The physical analysis results of Raman and XRD showed the main composition was titanium oxide. The hardness and young's modulus were almost the same, except that Ti-1 group showed lower value of hardness than other groups. It seems that oxygen ion implantation power did not significantly alter mechanical properties of titanium. It was contrast to the results of Yang et al. (Yang, Wang et al. 2011), who found that OPIII-treated Ti surfaces possessed higher surface hardness and young's modulus, lower I_{corr} and I_{pass} in SBP solution and better cell adhesion and spreading morphologies. On the other hand, it is worth noting that the TiO_2 coatings of 40 nm grains possess significantly lower stiffness than the other groups of 20 and 80 nm grains (Yang, Oh et al. 2006). Therefore, it may indicate that the surface roughness between 3-8 nm, its mechanical properties, such as hardness and young's modulus are not significantly affected.

Most studies investigated surface roughness in micro- and submicro-scale. Surface roughness in nano-scale could also play an important role in osteoblast differentiation and tissue regeneration because it directly corresponds to the sizes of proteins and cell membrane receptors (Gittens, McLachlan et al. 2011). Thus, nanostructure may have a potential opportunity for faster healing times and improved implant osseointegration in vivo. In this study, the Ra values of Ti\O_2 surface roughness were observed in nano-scale and were between 2-3 nm. However, as the ion power increased, the average surface roughness did not increase gradually. Yang et al. found that after O-PIII treatment, the surface topography and roughness of Ti specimens was not distinctly changed (Yang, Wang et al. 2011). This may implied that O-PIII treatment had no influence on surface topography and roughness of Ti specimens. In the present study, average roughness of Ti-1 group revealed the highest value than other groups. The difference between Ti-2 and Ti-3 groups did not significant. It can be suggest that ion power above 2 KW did not affect surface roughness.

To quantify the number of live cells on each surface treatment, we carried out an evaluation of the cellular viability by using an MTT test. This test makes it possible to quantify the mitochondrial activity and, as a consequence, to measure survival or the cellular proliferation. In addition, alkaline phosphatase activity is an early marker of osteoblast differentiation and relates to the matrix mineralized production. It was reported that increases in surface roughness lead to enhanced osteoblast differentiation and local factor production in vitro (Kieswetter, Schwartz et al. 1996; Raines, Olivares-Navarrete et al. 2010). In this study, it was found that the average roughness for Ti-1 group were larger than other treated groups. At 9 days of culture, higher ALP activity was observed for all the Ti\O_2 groups compared to the control groups. This results is consistent with the findings of Zhao et al. (Zhao, Schwartz et al.

2005) and Lincks and Batzer et al. (Batzer, Liu et al. 1998; Lincks, Boyan et al. 1998). Both the cellular alkaline phosphatase and cell layer alkaline phosphatase increased as the surface roughness increased. However, the results of other experiments also showed that on the rougher surfaces, cell number was decreased. In this study, Ti-1 group revealed the greater MTT values than other Ti\O_2 groups. On the other hand, Kim et al. investigated the effects of the process parameters of Ti alloy substrate on MG-63 osteoblast-like cell proliferation. It was found that as the surface roughness increases, the proliferation of osteoblast-like cell also increases (Kim, Jang et al. 2004). In the study of Webster et al., osteoblast proliferation was significantly greater on nano-phase (materials with grain sizes less than 100 nm) titanium than on conventional ones after 3 and 5 days. Moreover, the synthesis of ALP was greater on nano-phase titanium after 21 and 28 days (Webster, Ergun et al. 2000). It seems that rougher surface in this study not only affected cell differentiation but also proliferation. Furthermore, other studies found that as the increase of surface roughness, the bone-to-implant contact increased in vivo (Buser, Schenk et al. 1991; Park, Heo et al. 2007). It was suggested that rougher titanium surface may improve cell activity and implant osseointegration.

As a surface begins to contact with biological tissues, water molecules first reach the surface. Hence, surface wettability may play a major role in adsorption of proteins onto the surface, as well as cell adhesion. It was reported that hydrophilic titanium surfaces have a significant influence on cell differentiation and growth factor production. In addition, animal experiments have pointed out that hydrophilic surfaces improve early stages of soft tissue and hard tissue integration of titanium implants (Schwarz, Wieland et al. 2009). In this study, it was found that with the decreased of ion power, the measurements of contact angle decreased. The values of the control and treated surface groups were in the range of 70 to 100 degree, as

similar as the results of Zhu et al. (Zhu, Chen et al. 2004) , namely, all the surfaces were hydrophilic surfaces. When the MG-63 osteoblast-like cells were cultured on the Ti-1 plates for 9 days, the cell viability increased significantly (Fig 4.7). The results indicated that the hydrophilic surfaces have positive effects on cell viability. Hemocompatibility can be determined by measuring the adhesion of blood platelets, the sP-selectine expression, and of the blood clotting time on the samples. In the present study, the blood clotting time reduced after 20 minutes for all the Ti\$O_2$ groups. However, the measurements of platelets adhesion, activation and fibrinogen expressions revealed that all the Ti\$O_2$ groups and the control groups had no significant differences. In the experiment of Maitz et al., surface roughness below 50 nm has minor effects on the blood compatibility (Maitz, Pham et al. 2003). It may be the explanations of the results in this study.

Once the MG-63 cells attached on the surface, the spherical cells started to spread. During cell spreading, the shape of cells is changed and the cellular skeleton is reorganized. In the study of Zhu et al. (Zhu, Chen et al. 2004), the fully spread cells present flatten and extension of plasma membrane to all sides. The morphologies of cells were polygonal shape. It is reported that surface texture of the Ti substrate can affect the expression of fibronectin and vitronectin integrin receptors, modify their clustering or aggregation, and therefore determine variations in shape and spreading of cells (Degasne, Basle et al. 1999). On the rougher surface, cells were more likely to adhere to the surface and were found to project more small elongated processes, presenting a more elongated morphology. However, with the increase of culture time, cell number increased and became confluent on both rough and smooth surfaces (Zhu, Chen et al. 2004; Kim, Kim et al. 2005; Simon, Lagneau et al. 2005; Advincula, Rahemtulla et al. 2006). In the present study, cells grew well on the titanium surfaces. It was obviously to

observe osteoblasts adhered to all surfaces by means of thin cytoplasmic digitations or filopodia. The morphology of cells started to extend after 1 day. After 9 days of culture, cells became confluent to cover the surfaces. Different condition of treated power did not affect cell spreading significantly.

Chapter 6

Conclusions

The biocompatibility of titanium is closely related with the surface oxide film. The improvement of the biological activity of titanium with plasma immersion ion implantation has been investigated. However, the effects of oxygen ion implantation on the titanium were still lack. In this study, the titanium plates were implanted with oxygen at different power conditions and subsequently analyzed for surface morphologies and phase composition. Also, the study investigated the biocompatibility of surface modification on titanium-based alloys. The results revealed that different ion power treatments contributed to different surface roughness. In the oxygen ion implantation groups, titanium alloy treated with 1 KW ion power showed larger surface roughness than other groups. It also showed greater MTT values and ALP activity. It was suggested that with oxygen ion implantation treatment, titanium-based alloys could maintain or improved the physical properties and increase both cell differentiation and proliferation. In addition, the hemocompatibility of modified titanium alloys could maintain as untreated titanium alloys. Therefore, titanium modified by oxygen ion implantation may not only maintain its properties but also improve cell activity.

Table 2.1 Chemical compositions of titanium and its alloys (ASTM)

	Grade I	Grade II	Grade III	Grade IV
O (%)	0.18	0.25	0.35	0.40
N (%)	0.03	0.03	0.05	0.05
C (%)	0.10	0.10	0.10	0.10
H (%)	0.015	0.015	0.015	0.015
Fe (%)	0.20	0.30	0.30	0.40
Ti : 99.0-99.5%				

Table 2.2 Mechanical and physical property of titanium metal (ASTM)

Material	Ultimate tensile strength (MPa)	Proportional limit (MPa)	Elongation (%)	Density (g/cm3)	Elastic modulus (GPa)
Grade I	240	170	24	4.5	100
Grade II	345	235	20	4.5	100
Grade III	450	380	18	4.5	100
Grade IV	550	483	15	4.5	100
Cortical bone	**140**	**130**	**1**	**0.7**	**18**

Table 4.1 Surface roughness parameters of the control and the investigated Ti\O_2 samples.

Sample	5 μm resolution Rms (nm)	5 μm resolution Ra (nm)	1 μm resolution Rms (nm)	1 μm resolution Ra (nm)
Control	0.51	0.36	6.05	4.73
Ti-1	3.5	2.67	3.88	3.02
Ti-2	3.13	2.47	3.07	2.41
Ti-3	3.08	2.35	3.3	2.52

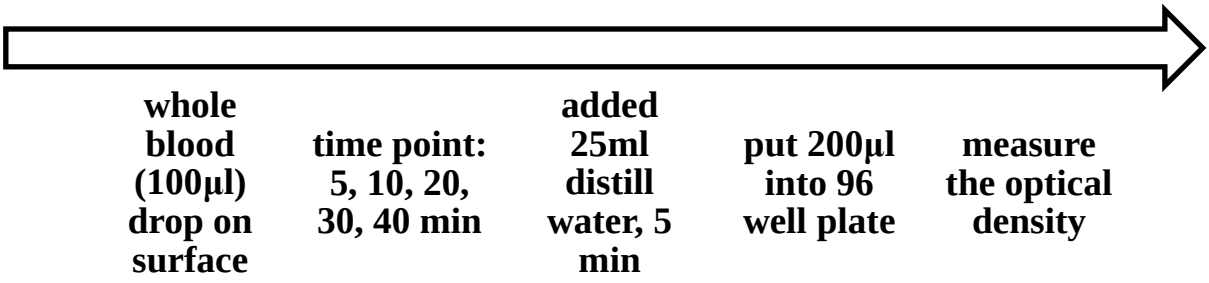


Figure 3.1-a Clotting time analysis experimental procedures

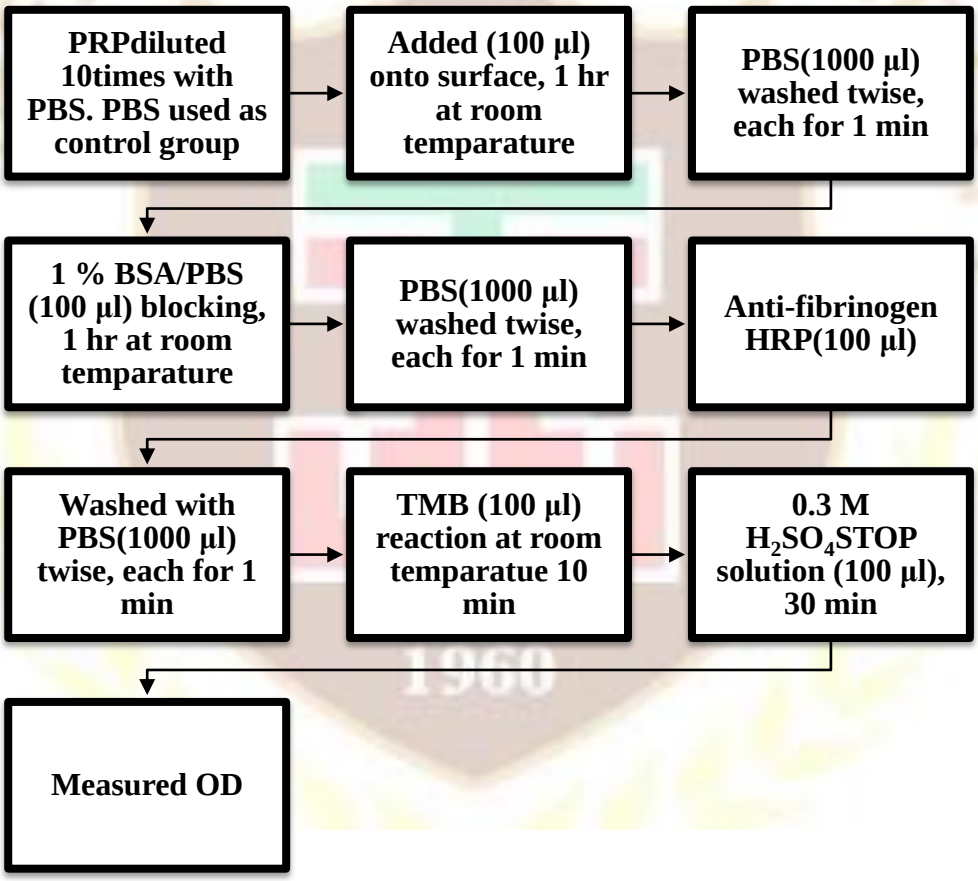


Figure 3-1-b Fibrinogen assay experimental procedures

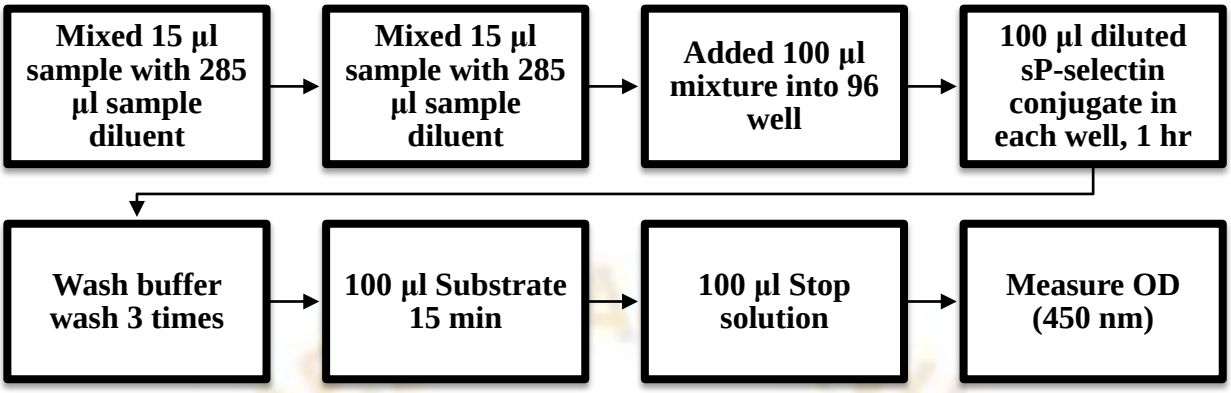


Figure 3-1-c sP-selectin assay experimental procedures

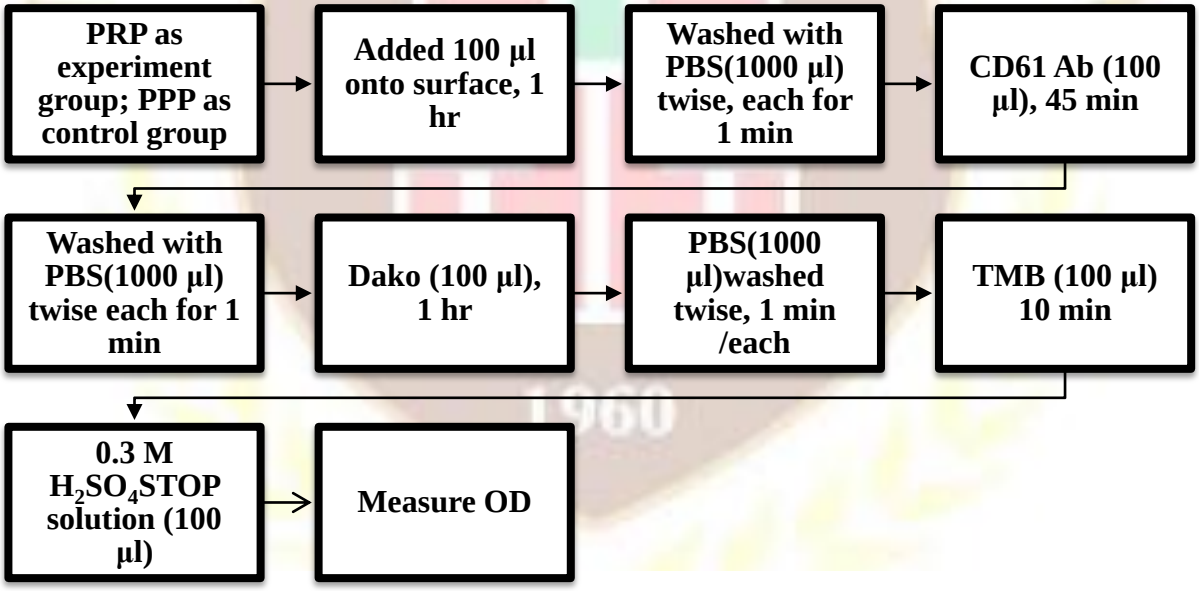


Figure 3-1-d CD 61 assay experimental procedures

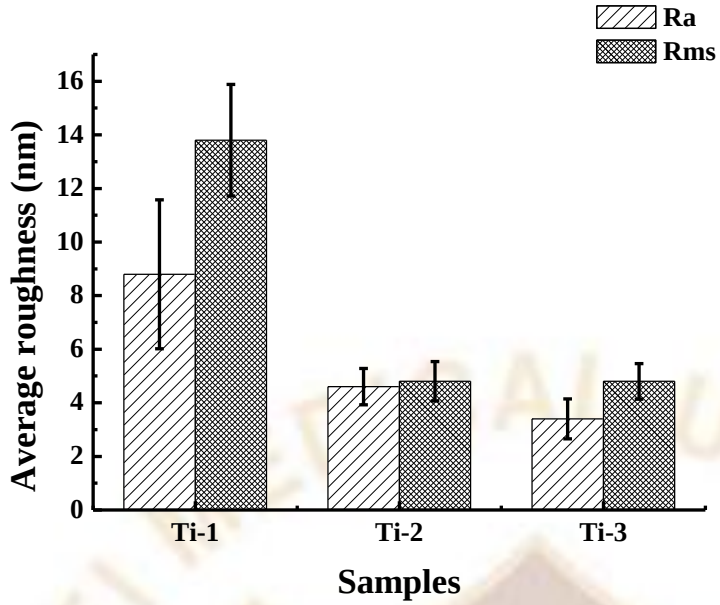


Fig 4.1 Average surface roughness of Ti\O_2 samples under 1, 2, and 3 KW ion power treatments.

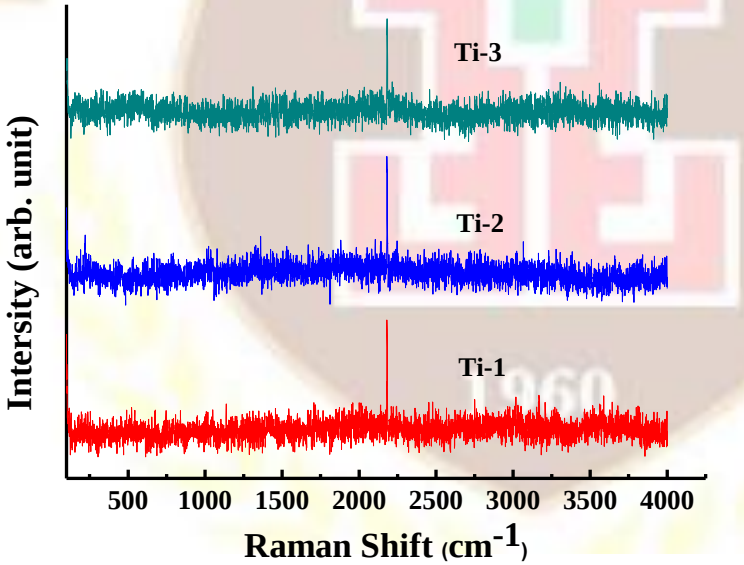


Fig 4.2 Raman analysis of Ti\O_2 samples under 1, 2, and 3 KW ion power treated

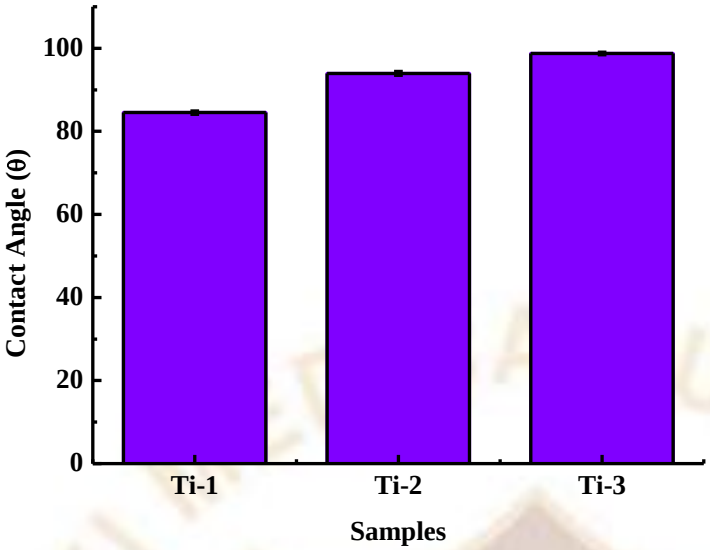


Fig 4.3 Average contact angle of different Ti\O_2 samples

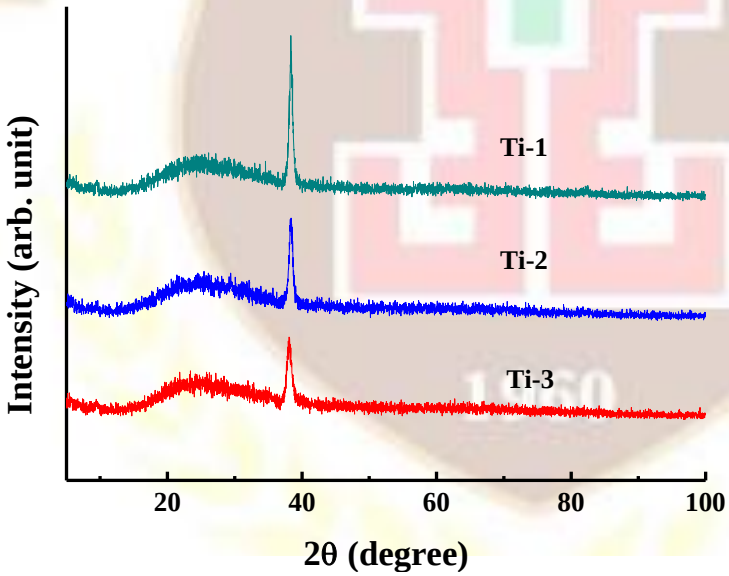


Fig 4.4 XRD patterns.

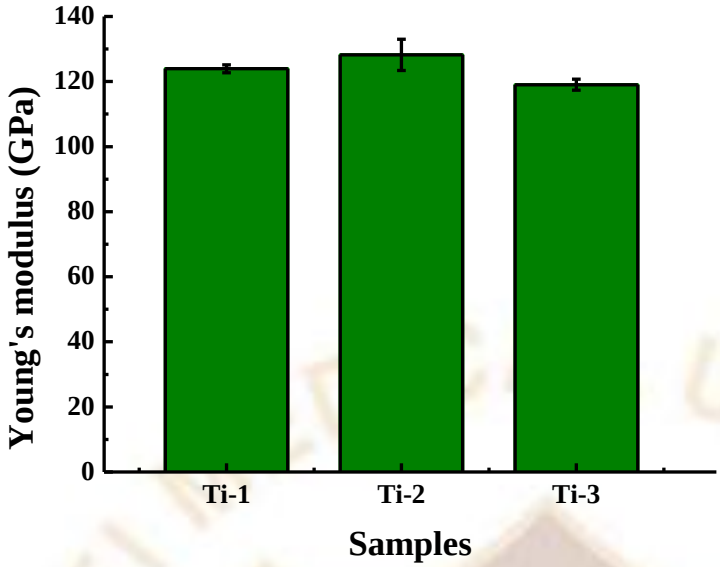


Fig 4.5.1 The young's modulus of different Ti\O$_2$ samples

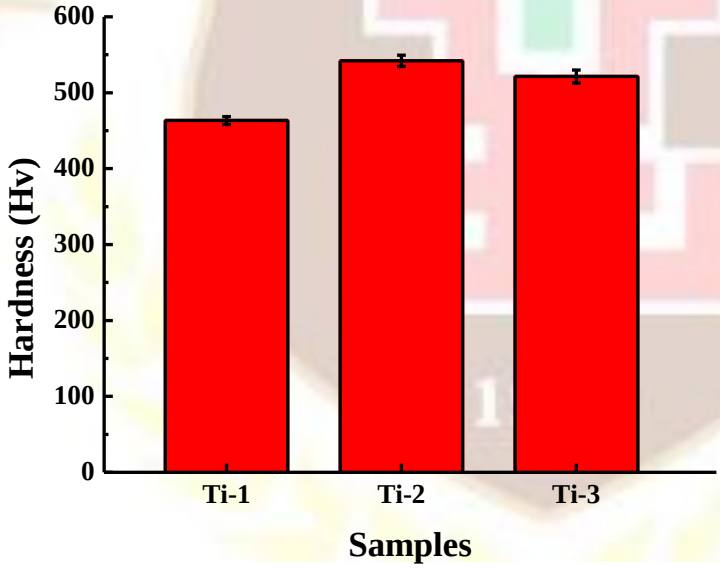
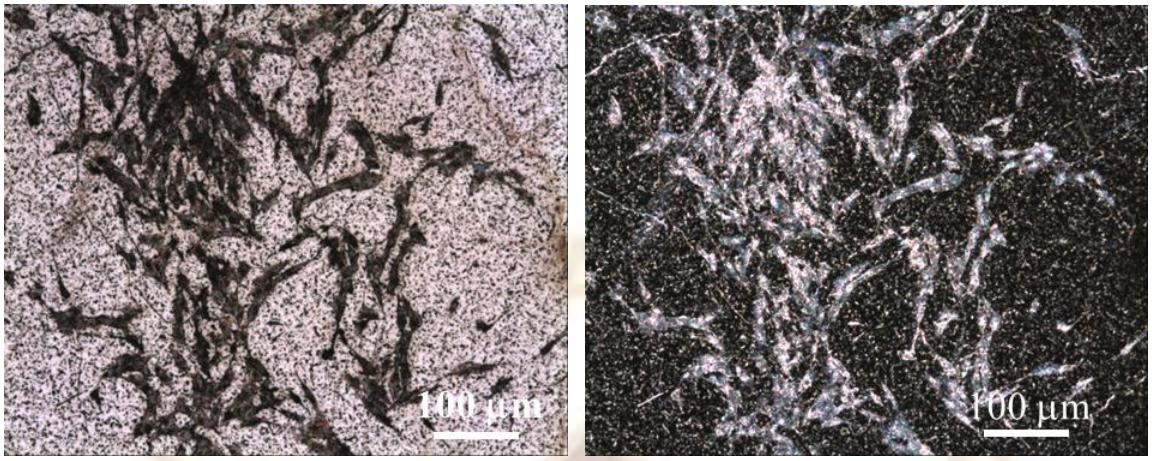
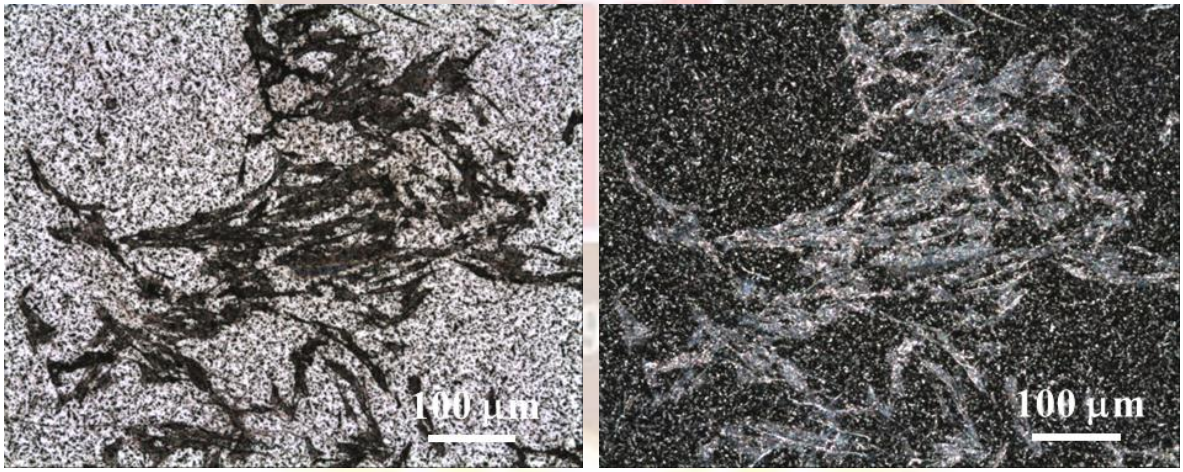


Fig 4.5.2 The hardness of different Ti\O$_2$ samples



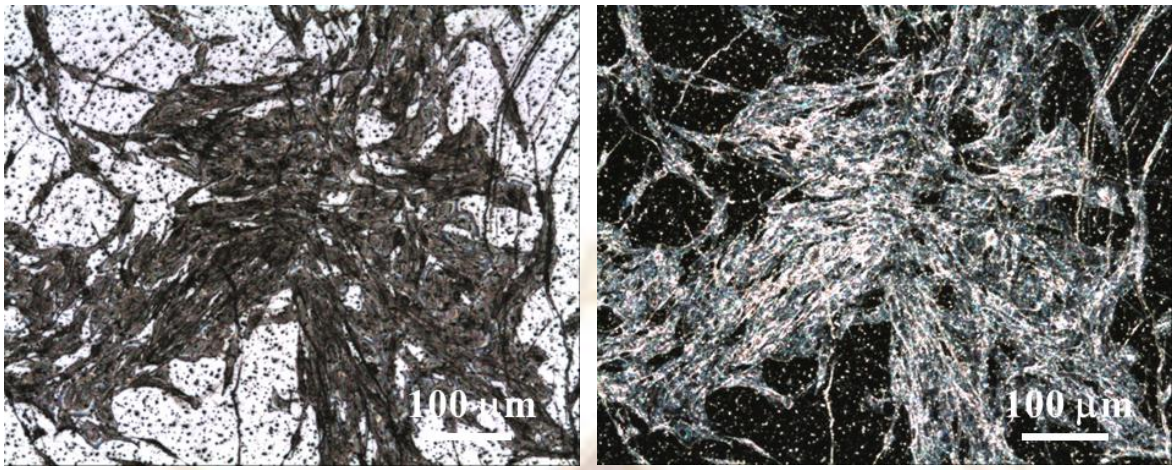
(a) Bright field (BF) (b) Dark field (DF)

Fig 4.6.1 OM images of MG-63 cells cultured for 3 days on Ti-1 titanium disk



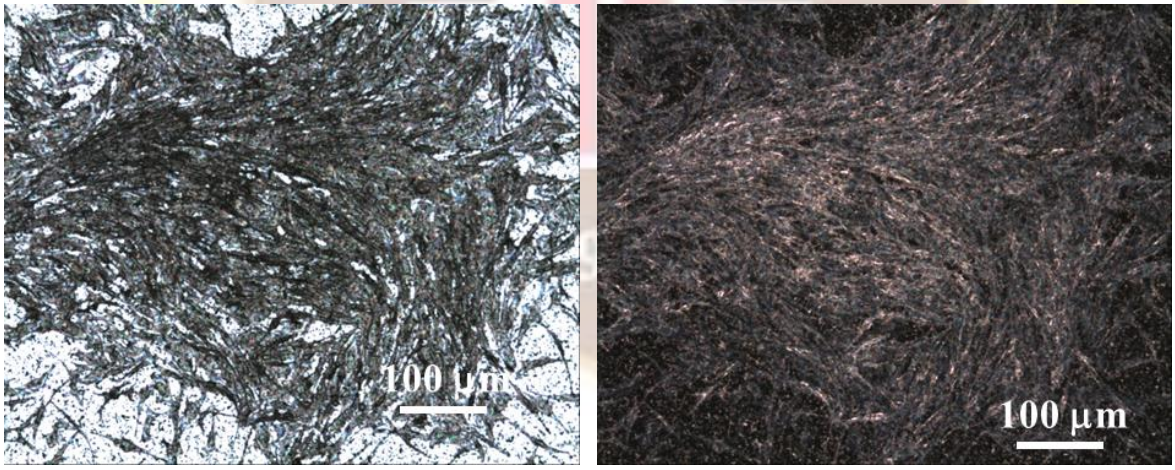
(a) Bright field (BF) (b) Dark field (DF)

Fig 4.6.2 OM images of MG-63 cells cultured for 3 days on Ti-2 titanium disk



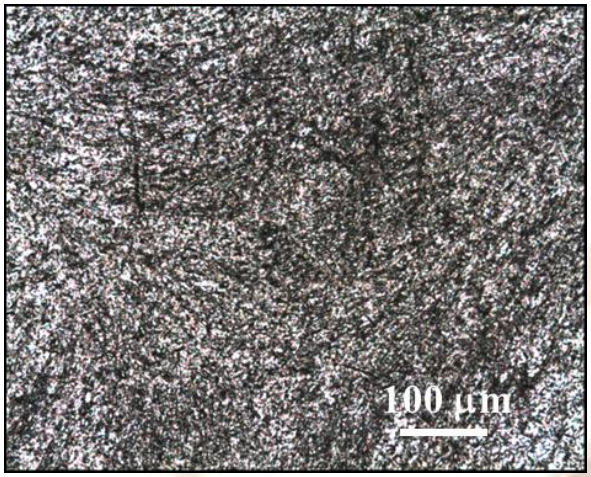
(a) Bright field (BF) (b) Dark field (DF)

Fig 4.6.3 OM images of MG-63 cells cultured for 3 days on Ti-3 titanium disk

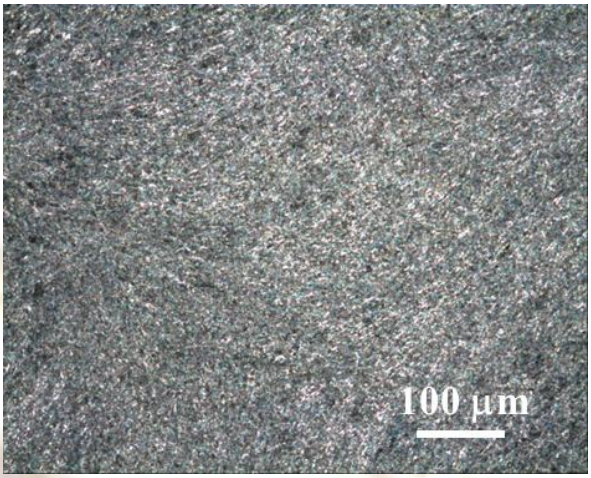


(a) Bright field (BF) (b) Dark field (DF)

Fig 4.6.4 OM images of MG-63 cells cultured for 7 days on Ti-1 titanium disk

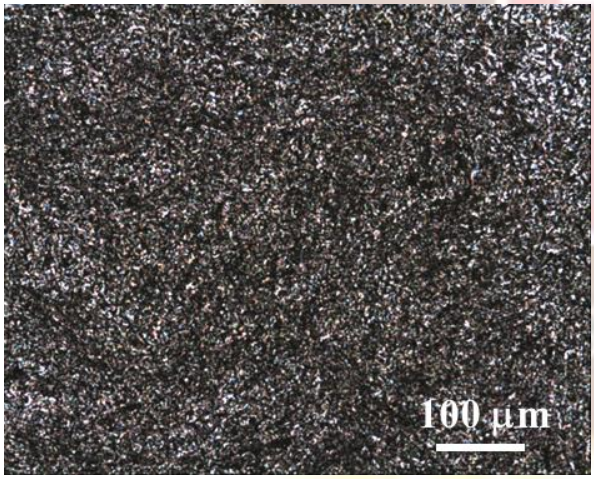


(a) Bright field (BF)



(b) Dark field (DF).

Fig 4.6.5 OM images of MG-63 cells cultured for 7 days on Ti-2 titanium disk



(a) Bright field (BF)

(b) Dark field (DF).

Fig 4.6.6 OM images of MG-63 cells cultured for 7 days on Ti-3 titanium disk

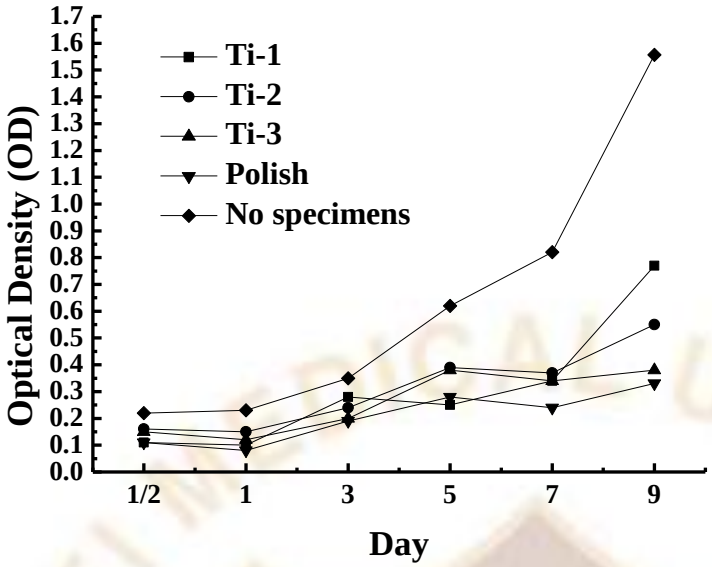


Fig 4.7 MTT values of MG-63 cells on different Ti\O$_2$ samples

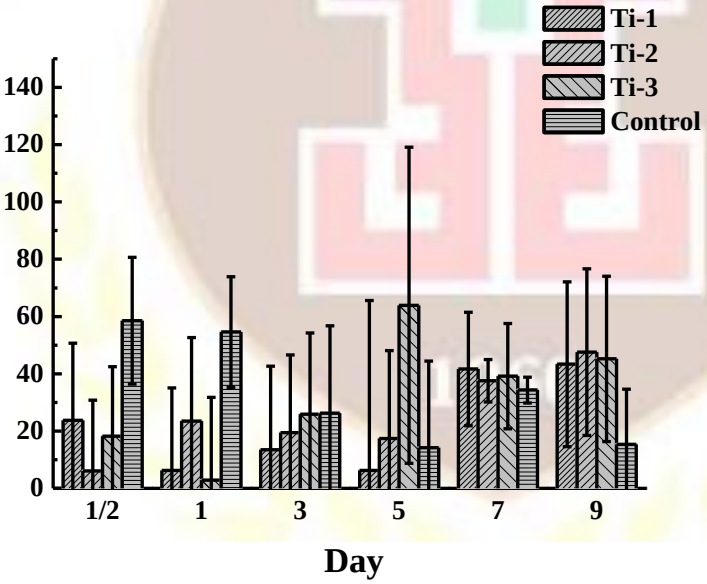


Fig 4.8 ALP activity of MG-63 cells on different Ti\O$_2$ samples

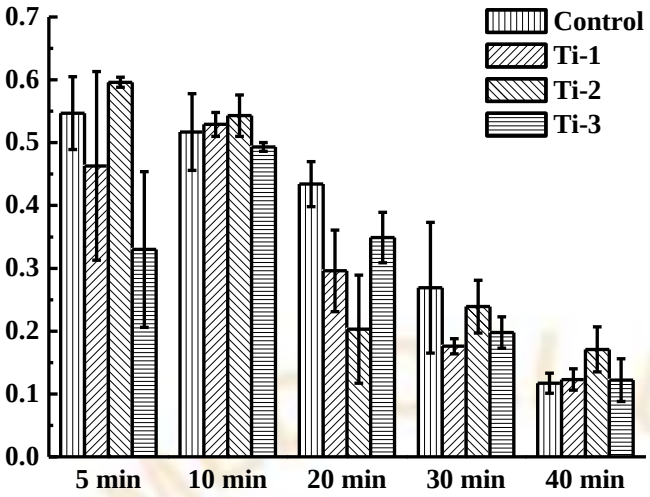


Fig 4.9 Clotting time assay on different Ti\O$_2$ samples

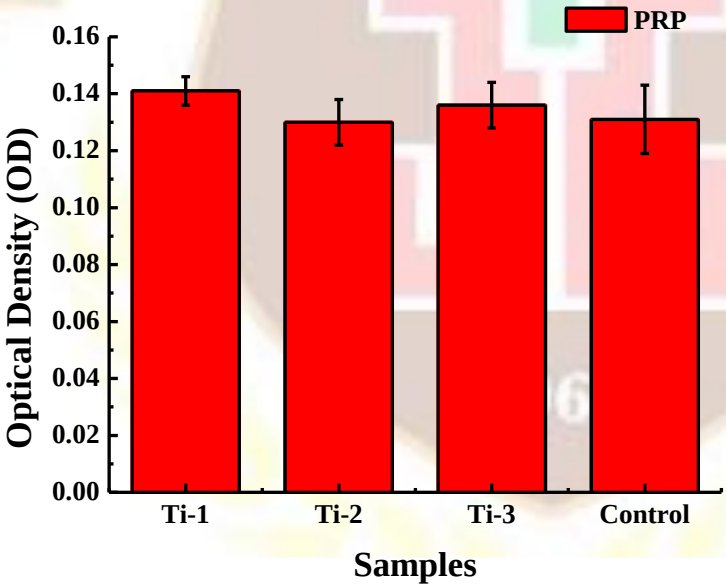


Fig 4.10 Fibrinogen adhesion assay on different Ti\O$_2$ samples

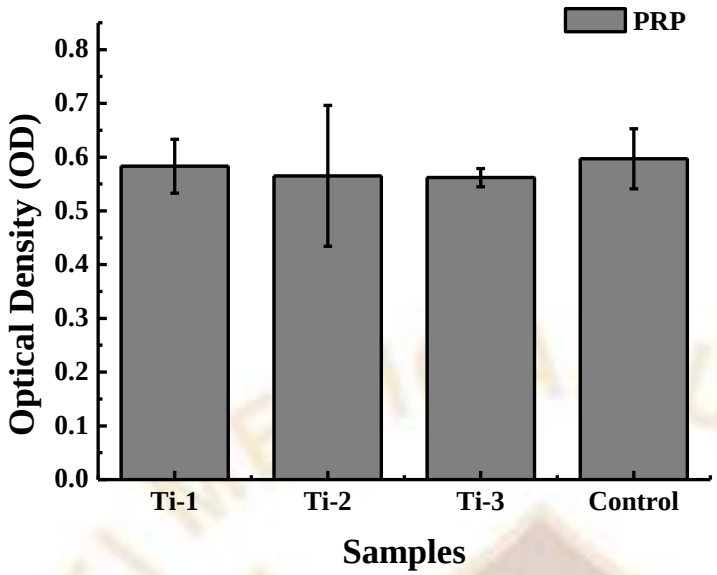


Fig 4.11 sP-selectin expression assay on different Ti\O$_2$ samples

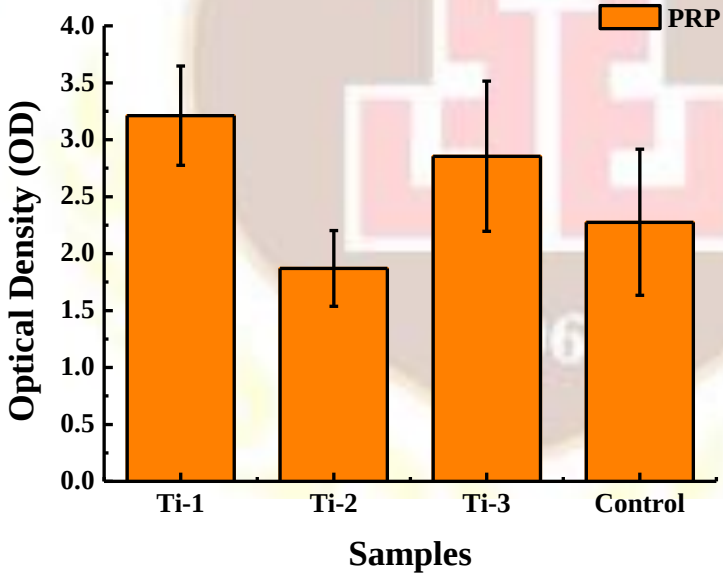


Fig 4.12 Platelet adhesion assay (CD-61) on different Ti\O$_2$ samples

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