BIOACTIVITY OF ROLLED TITANIUM

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Certificate

This is to certify that the thesis entitled "**BIOACTIVITY OF ROLLED TITANIUM**" by **Deepshikha Mahapatra (111BM0008),** in partial fulfillment of the requirements for the award of the degree of Bachelor of Technology in Biomedical Engineering during session 2011-2015 in the Department of Biotechnology and Medical Engineering, National Institute of Technology Rourkela, is an authentic work carried out by her under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/Institute for the award of any degree or diploma.

Place: NIT Rourkela **Dr. A. Thirugnanam**

Date: 11th May 2015 **Assistant Professor**

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Contents

List of Tables

List of Figures

Abstract

Metals have found wide applications in the field of implant fabrication for the replacement of human hard tissues. Titanium being biocompatible, possessing the high strength to weight ratio, having low density, low modulus and showing ease of fabrication is the new implant material. This study gives the microstructural characterization of conventional rolled titanium (Ti) deformed by cold rolling. The Ti samples were then characterized by optical microscopy, X-ray diffraction, Vickers hardness measurements, contact angle measurements and tensile test. In addition, in vitro bioactivity of the Ti samples was carried out in simulated body fluid (SBF) for 28 days. Protein adsorption was also studied using bovine serum albumin (BSA). Rolling has induced an increase in hardness, protein adsorption and tensile properties. X-ray powder diffraction (XRD) analysis shows the diffraction pattern of the samples corresponding to titanium along with those that correspond to apatite after bioactivity study in SBF. The above-mentioned properties are basic pre-requisites for bone-implant applications. Hence, from this study it can be concluded that the rolled Ti is a suitable biomaterial for biomedical applications.

Keywords: Titanium, rolling, SBF, bioactivity, protein adsorption, tensile properties.

CHAPTER 1

INTRODUCTION

1.1 Titanium

Titanium is an element represented by the symbol Ti and having the atomic number 22. It is a transition metal with low density, high strength and is lustrous by its appearance. It is highly resistant to corrosion due to sea water, chlorine and aqua regia. Pure titanium melts at 1670^{o}C. Titanium was discovered in Cornwall, Great Britain in 1971 by a person named William Gregor. It occurs with a number of mineral deposits mainly rutile and ilmenite. There are two allotropic forms and five naturally occurring isotopes of titanium, ^{46}Ti through ^{50}Ti, with ^{48}Ti being the most abundant (73.8%) [1]. The metal is principally obtained from its ore by Kroll's process.

1.2 Extraction of Titanium-Kroll's Process

Kroll's process is principally a pyro-metallurgical industrial process used to manufacture metallic titanium. Kroll's process is named after William J. Kroll as it was invented by him in Luxemburg. Titanium concentrates from mines (rutile) are put in a fluidized-bed reactor along with chlorine gas and carbon. The material is heated to 1000°C and the subsequent chemical reaction results in the creation of impure titanium tetrachloride ($TiCl_4$) and carbon monoxide. This step is known as extraction [2]. Next step is purification. The reacted metal is put into large distillation tanks and heated. Amid this step, the impurities are separated using fractional distillation and precipitation. The purified titanium tetrachloride is transferred as a liquid to a stainless steel reactor vessel. Magnesium is then added and the container is heated to about 800-850°C. The magnesium reacts with the chlorine producing liquid magnesium chloride. This leaves pure titanium in a solid state since the melting point of titanium is higher than that of the reaction [3]. The sponge is crushed and pressed before it is melted in a consumable electrode vacuum arc furnace. The melted substance is allowed to solidify in the presence of vacuum followed by further remelting to remove inclusions and bring uniformity.

1.3 Titanium Alloys and their Applications.

Recently, titanium alloys are getting much attention for biomaterials because they have excellent specific strength and corrosion resistance, no allergic problems and the best biocompatibility among metallic biomaterials. Commercially pure titanium and its alloys (mainly Ti–6Al–4V) are most widely used for the biomedical applications. They cover almost all of the biomaterial market of today. High corrosion resistance and excellent biocompatibility of titanium increase its suitability for biomedical applications. Titanium and titanium alloys possess low modulus

varying in the region 55 to 112 GPa [4]. Also its strength is approximately equal to the strength of 316L SS and its density being 55% lesser than 316L SS. The applications of Ti and Ti alloys include joint replacement, parts of hip, elbow, spine, knee, shoulder, dental implants, etc. even though Ti and its alloys are considered biocompatible but extended use can lead to release of Al and V ions which may cause health issues [5].

Beside commercially pure titanium (Cp-Ti) and Ti6-Al-4V, β-titanium alloys such as Ti-Ta alloys; Ti-Mo alloys; Ti-Nb and Ti-Ni shape memory alloys are very impressive as bioimplants [6, 7]. These are preferred due to high corrosion resistance and biocompatibility. Ti-Ta alloys have lower modulus and a good package of high strength and low modulus. They have the potential to become new entries for biomedical applications. The addition of Zr to Ti alloy lowers the Young's modulus and other mechanical properties thus making it more suitable for biomedical applications [6].

The α+β titanium alloys are also used in the biomedical applications. Among these alloys, Ti-6Al-4V was the first titanium alloy developed, and is the most widely used due to its biocompatibility and bioactivity. Another alloy of this category is Ti-6Al-2Sn-4Zr-6Mo [7]. But it has not found its use in the biomedical field due to the difficulty in its machining.

1.4 Biocompatibility and Bioactivity

The term biocompatible refers to the ability of a material to perform with an appropriate host response in a specific situation. The ambiguity of the term reflects the ongoing development of insights into how biomaterials interact with the human body and eventually how those interactions determine the clinical success of a medical device [8] i.e. not producing toxic compounds, injurious or immunologic response in biological tissues. Biocompatibility is the property of not reacting with the tissues whereas a material is considered bioactive if it has interaction with or effect on any cell tissue in the human body. Its pharmacological activity is usually taken to describe beneficial effects, i.e. the effects of drug candidates as well as a substance's toxicity. In common man's language, the effect of any substance on living tissues is known as its bioactivity. For any implant using metal that usually is used for orthopedic and dental purpose, the bone bonding ability is mainly evaluated by studying its ability to form apatite on its surface in a stimulated body fluid (SBF) in which the ion concentrations are kept

nearly equal to that of the human blood plasma. Apatite forming properties are used to analyze the in-vivo bioactivity of any substance. Any implant materials consisting of metal is believed to bond to living bone through a calcium phosphate layer and this apatite layer acts as the bone-like calcium phosphate layer. There also exist cases in which tissues bond to implants without the need for the apatite formation [9].

1.5 Rolling of Titanium

Titanium processing often involves steps of rolling, which plastically deforms its original structure. Rolling is a process of reduction of the cross-sectional area or shaping a metal piece through the deformation caused by a pair of rolls rotating in opposite direction. The gap between the rotating rolls is less than the thickness of the entering bar therefore a friction force is necessary to grab the bar and to pull it through the rolls. A metal bar passing through the rotating rolls is squeezed, and it elongates while its cross section area decreases [10]. A machine used for rolling metal is called rolling mill. A typical rolling mill consists of a pair of rolls driven by an electric motor transmitting a torque through a gear. The rolls are equipped with bearings and mounted on a stand with a screw-down mechanism. Rotating rolls mainly perform two functions i.e. drag the workpiece into the gap between the rolls and squeeze the workpiece for a reduction in cross sectional area. Cold rolling is when the process is carried out at room temperature. Cold rolling finds its application in this field is due to its property of material strengthening [11].

CHAPTER 2

LITERATURE REVIEW

This chapter depicts the importance of the role played by rolled Ti- medical implants used in clinical applications. The Ti implants must be biocompatible, osteoinductive and should possess proper young's modulus and mechanical strength. Such implants can be fabricated using various fabrication techniques. Titanium and titanium alloys are known have impressive mechanical properties and biocompatibility. On implantation, implants exhibit direct contact with bone. But smooth titanium implants fail to show strong bonding to bones even in unloaded conditions [12]. To combine mechanical properties with bone-bonding abilities, titanium metal has been coated with certain bioactive materials. One such preferred method for orthopedic implants is plasma spray hydroxyapatite (HA) coating [13]. Histological examinations have revealed that HA coated implants in direct contact with bone for 3-4 weeks after implantation produce areas of direct contact between implant and host-bone in addition to intervening fibrous tissue. Alkali and heat treated implants add to the osteoconductive nature of the HA coated implants as compared to those of untreated implants. There exist submicron level trabecular and irregular structures on the implant surface which can be put to use in orthopedic implants in terms of increasing roughness and oseointegration [14].

Other methods seen to have been used for modification of surface properties of titanium are calcium phosphate or oxide-coating, ion-implantation and alkali treatment. Plasma spraying and amino group (NH_2^+) ion implantation provide better bio-compatibility [15]. Cell behavior towards the implant surface has greatly been tested with proper examination of surface modified titanium by observing morphological behavior, cell proliferation and differentiation. Biocompatibility and bioactivity of a biomaterial is closely related to cell interaction and adhesion onto the surface. Plasma spray coating results in an increase in the surface roughness of amino-group (NH_2^+) ion implantation and leads to formation of thicker surface oxide layer even with a little amount of nitride sand-blasted. Cells spread, attach and proliferate on the surface of culture plates and display polygonal spindle shaped morphology on the surface of Ti. After few days, cells show colonized patches by spreading which improves after a week. Rolled Ti is non-toxic as it is untreated as well as a prerequisite for cell proliferation and osteogenesis [16-17].

In case of titanium, α and β phases coexist at room temperature consisting maximum α phase. With the rise in temperature up till 865^{o}C, phase transformation of α →β occurs. This

transformation determines however the high temperature state of the β phase and can therefore have a strong influence on the transformation at cooling. However, lately it was determined that a strong density increase of the inherited α phase when the starting a phase was cold deformed [18]. Cold rolling influenced the prior to a α→β→α transformation sequence, on the inherited texture in commercially pure titanium [19].

Hallab et al. demonstrated that surface free energy (SFE) was a more important surface characteristic than surface roughness for cellular adhesion strength and proliferation, and that the surface energy components of the various tested material were shown to be related to cellular adhesion strength [20]. Differences in surface roughness seem to influence the amount of adsorbed proteins. Deligianni et al.'s study showed higher amounts of fibronectin and lower amounts of albumin on rough Ti alloy compared to smoother samples [25]. In contrast, in another study increased surface roughness of Ti partly decreased the in vitro adsorption of fibronectin [26]. In Ruardy et al.'s study, showed that the spread area of human fibroblasts increased with wettability when going from the hydrophobic to the hydrophilic end of a surface composed of a wettability gradient [21]. Georgi et al. demonstrated increased cell proliferation with increasing material surface wettability (water sessile drop method). The relative importance of surface wettability on fibroblast spreading was studied by Webb et al. [23-24]. It was shown that cell attachment and spreading were significantly greater on hydrophilic surfaces than on hydrophobic surfaces, and that moderately hydrophilic surface promoted the highest level of cell attachment [27].

Vaudaux et. al.'s study showed that roughness has a significant influence on the wettability behavior of the metal surfaces i.e. initially the micro structured surfaces were hydrophobic but once wetted during the first emersion cycle shift to wettability [28]. There has been much effort for improving the bioactivity of titanium, such as coating with a Ca–P layer and modifying the natural oxide film [29-34]. The previous studies showed that the formation of apatite on titanium oxide films is in relation to the surface hydroxyl groups and surface energy, and not to the crystal phases of the surface oxide films [35].

The bone-bonding ability of a material is often evaluated by examining the ability of apatite formation on its surface when immersed in a simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma. Artificial materials implanted into bone defects are generally encapsulated by a fibrous tissue, leading to their isolation from the surrounding bone. However, in 1972, Hench et al. showed that some glasses in the $Na_2O–CaO–SiO_2–P_2O_5$ system, called Bioglass, spontaneously bond to living bone without the formation of surrounding fibrous tissue [36]. Since then, several types of ceramic, such as sintered hydroxyapatite, sintered β-tricalcium phosphate, apatite/β-tricalcium phosphate biphasic ceramics, and glass-ceramic A–W containing crystalline apatite and wollastonite have been also shown to bond to living bone, and they are used clinically as important bone substitutes [37-40].

On the other hand, the adsorption of proteins and the adhesion of cells on material surfaces also affect the bioactivity. It has been suggested that protein adsorption onto the surface oxide layer is important for the integration of a titanium implant-tissue, since reorganization of the tissue adjacent to titanium implants depends on adsorption of proteins from the liquid initially separating the implant and tissue [41]. Proteins adsorb onto a titanium surface following response of cells to the material [42-43]. In the presence of calcium and phosphate ions, the adsorption of bovine serum albumin (BSA) onto titanium powder is a function of protein concentration and pH level, which suggests possible conformational changes of the protein molecule [44-45].

Cell behavior on biomaterial surfaces depends upon implant–cell interactions, correlated with surface properties. Surface hydrophilicity, hydrophobicity, roughness, texture, chemical composition, charge and morphology strongly affect cellular responses in contact with the implants [18, 46]. Many studies have shown that implant success is dependent not only on the physio-chemical properties of the implant surface such as surface free energy or interfacial free energy, but also on its roughness [23-26, 47].

The aim of this present investigation is to obtain sub-micron grain by cold rolling of titanium. The effect of grain refinement on the sample was evaluated by metallurgical microscopy, micro-hardness testing, mechanical testing, wettability measurements, protein adsorption and in-vitro bioactivity.

CHAPTER 3

MATERIALS AND METHODS

3.1 Titanium Specimen Preparation

The commercially pure titanium i.e. (as received), (grade 2) [Composition: (in % weight) Carbon(C)-0.015, Oxygen(O)-0.115, Nitrogen(N)-0.0095, Hydrogen(H)-0.0013, Iron(Fe)-0.04, Titanium(balance)] procured from Midhani, Hyderabad was used as raw material. This wrought material was cold rolled by through the rolling mill with 10% of strain. Further these samples were cut into smaller pieces using the shear and hack saw.

3.2 Microstructure Analysis

The 2x2cm rolled titanium sample was taken and mounted using cold setting resin. The as received and processed samples were polished with 1/0, 2/0, 3/0, 4/0 emery sheet and cloth polisher for removal of passive oxide layer. Finally the samples underwent with diamond polishing. The samples were then etched with Kroll's reagent (92ml distilled water, 6ml nitric acid, 2ml hydrofluoric acid) for 30s. The sample is then removed from the solution and dried before observing its microstructure in a matallurgical microscope. The microstructural observation was carried out using the optical microscope (METISCOPE-I, Chennai Metco Pvt Ltd) at 100X magnifications. The grain size was measured using image analysis software (Innovision) as per ASTM-E112 standard.

3.3 Hardness Testing

The samples of same dimensions were taken and placed on the Vickers hardness testing machine. The micro-hardness measurements were made using the indentation load of 100gf for a dwell time of 15 seconds with the aid of a Vickers indenter tool. The indenter (made up of diamond) has a square-base pyramidal geometry with an included angle of 136◦. The indenter made an indent on the sample surface whose diagonal size was measured using a low magnification optical microscope. The hardness of the samples were calculated using the formula

$$HV = \frac{(2F\frac{\sin 136}{2})}{d^2}$$

where, F=applied force (in Newton)

d=average of the two diagonals (in mm)

The Vickers hardness number (H.V.) is the ratio of applied load to the surface area of the indent. Measurements were taken at nine locations across the rolling direction. Result is reported as the average value in units of kg/mm^2.

3.4 Mechanical Testing

The tensile property of the rolled titanium was evaluated using the Instron Universal Testing Machine (series-IX, automatic material testing system 1.26). The test machine was exercised or warmed up to normal operating temperatures (room temperature) to minimize errors that may result from transient conditions. The as received and rolled Ti samples were machined to the required tensile sample dimensions (the gauge length and diameter (width) were measured to be 25mm and 6mm respectively and thickness of the sample was 1mm). Then the Ti-samples were gripped in the test machine and the strain rate was fixed at $3.34 \times 10^4/s$. The tension test was carried out in triplicates at room temperature.

3.5 Contact Angle Measurement

The contact angle of the samples was measured using Drop Shape Analyzer (DSA) setup (KRUSS, Germany) to estimate the wettability of the processed sample. The as received Ti sample was first wiped with acetone and distilled water and then it was placed on the platform. The syringe head (needle), filled with distilled water was focused on the specimen. The distilled water droplet was poured on the Ti specimen and allowed to settle for 10 seconds. The obtained image was frozen and contact angle was analyzed using the baseline determination and profile extraction. The measurements were obtained at nine different locations on the polished sample under ambient conditions. The same procedure was repeated with the rolled Ti sample. Also the surface energy of as received and rolled was calculated using Young's equation:

$$E_s = E_{vl} \cos\theta$$

where, E_s=surface energy;

E_{vl}=surface energy between water and air under ambient conditions ($72.8 mJ/m^2$ at 20^oC of pure water)

θ = static contact angle

3.6 Protein Adsorption Study

Amount of protein adsorbed on a sample is a vital factor in influencing cellular interactions in vivo as well as in vitro. Here protein adsorption study was done by the optical density method. Ti-samples were treated with 1ml of bovine serum albumin (BSA) protein standard (1mg/ml protein in phosphate buffer solution (PBS) followed by placing in an incubator at 37^{o}C for 24h. The samples were then removed and washed using PBS for removing the non-adsorbed protein. Bradford assay was used to quantify the adsorbed protein amount. 50 µl of non-adsorbed protein was mixed with 500 µl of Bradford reagent. The protein concentration was determined by UV spectrophotometer (systronic double beam, UV spectrophotometer 2203) at 595 nm using a previously obtained standard curve. This helped in the accurate determination of concentration of adsorbed protein on different samples.

3.7 Assessment of In vitro Bioactivity (immersion in SBF)

Bioactivity of the rolled Ti-samples was evaluated by examining the apatite formation on the surfaces of the samples soaked in SBF (Table I). Reagents used for SBF preparation are NaCl, $NaHCO_3$, KCl, $K_2HPO_4.3H_2O$, $MgCl_2.6H_2O$, $CaCl_2$ and Na_2SO_4. SBF was prepared by dissolving these reagents into distilled water buffered at 7.30 with 1M-HCl and *Tris* buffer at 36.5^{o}C. The Ti-samples were immersed in SBF for 4 weeks. After soaking, the samples are dried in a desiccator without heating. The apatite formation was observed and confirmed using scanning electron microscopy (SEM) and x-ray diffraction (XRD) analysis.

Table 1. Ion Concentrations of Simulated Body Fluid (SBF) and Human Blood Plasma

	Concentration (mM)							
	Na^+	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**HCO^{3-}**	**HPO_4^{2-}**	**SO_4^{2-}**
SBF	142.0	5.0	1.5	2.5	148.8	4.2	1.0	0.5
Blood Plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Microstructure Analysis

The microstructures of rolled Ti-samples observed are shown in Fig. 1. The rolled Ti-samples were observed to possess average grains of size of 26.7 microns whereas the as received samples were observed to possess average grain size of 37.8 microns.

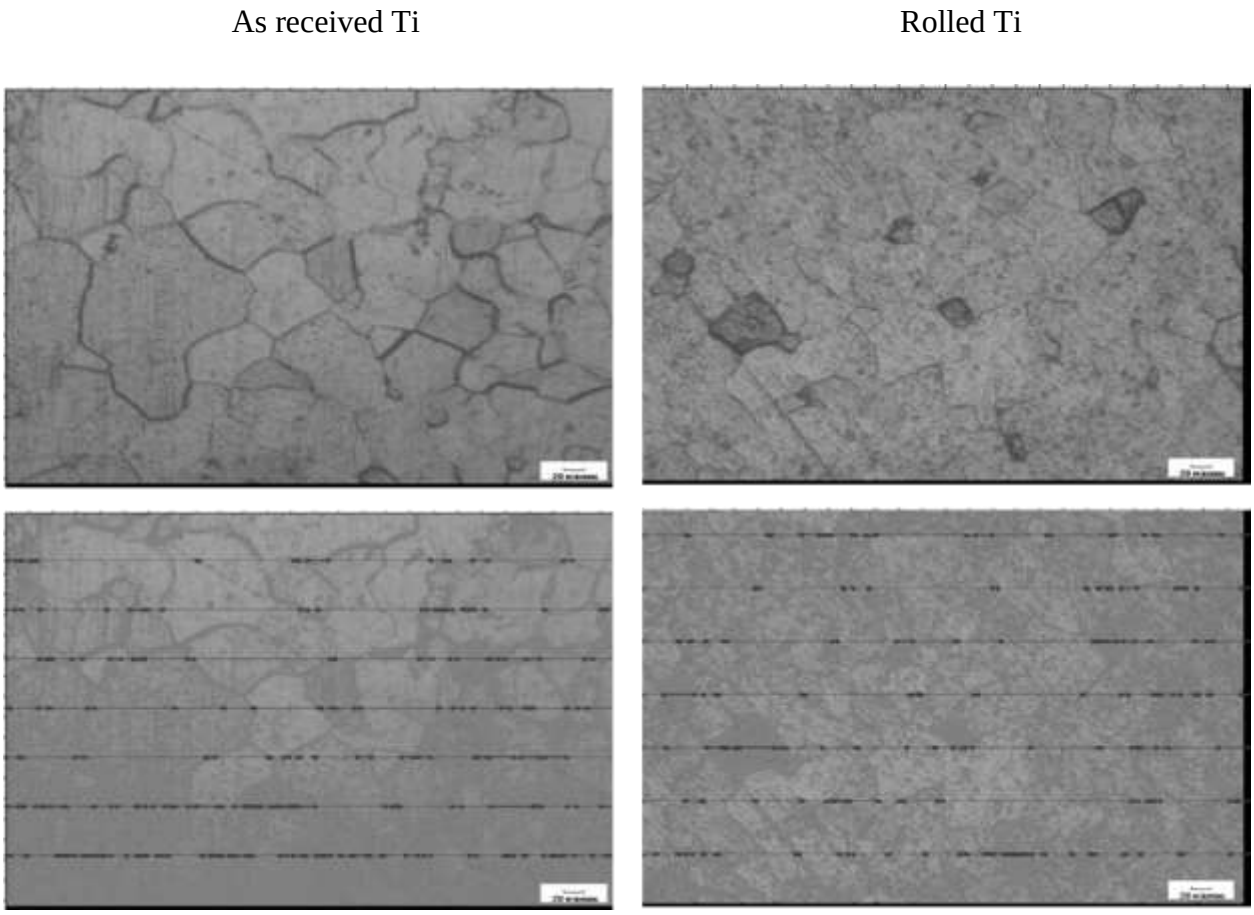

Figure 1: Optical micrographs of as-received and rolled Ti-samples showing grain size measurement using line intercept method.

The rolled titanium showed finer grain structure because of grain refinement which was observed during cold rolling of the material. More reduction by cold rolling made the grain shape elongated in the rolling direction and equiaxed in the transverse direction. The cold rolled sample took more time to etch the surface using Kroll's reagent.

4.2 Hardness Testing

Taking the average of the readings (i.e. Table 2) it is concluded that the hardness of the as received titanium sample was found to be 174.8 ± 7.18 kg/mm^2 and that of the rolled sample was

found to be 430.6 ± 23.88 kg/mm^2. The typical indentation made on the rolled Ti sample is shown in Figure 2.

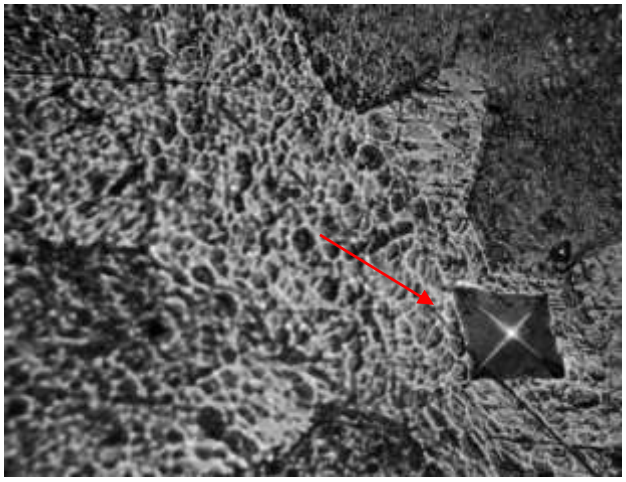

Figure 2: Typical indentation observed during Vickers hardness testing machine for rolled titanium samples.

Table 2. Vicker's hardness values of as received and rolled titanium at

Position	**Hardness values of as received Ti**	**Hardness values of Rolled Ti**
1	172.1	441.9
2	170.5	435.7
3	174.8	400.8
4	173.2	471.8
5	168.3	449.3
6	167	409.9
7	189.5	443.9
8	174.9	418.3
9	182.9	403.8
Average	174.8	430.6
S.D.	7.18	23.88

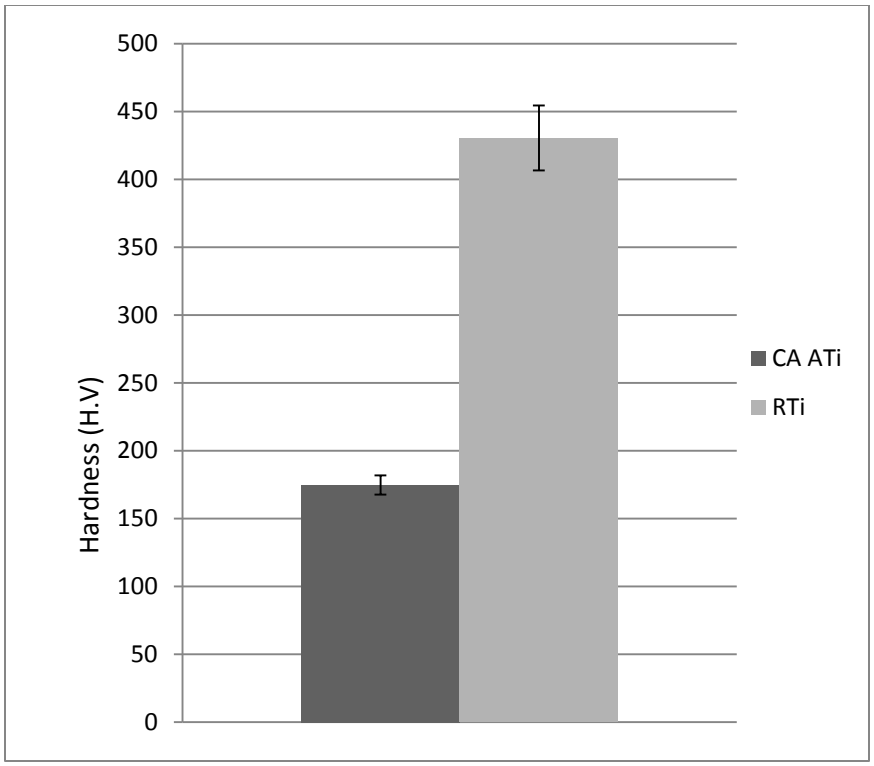

Figure 3: comparison between the hardness values of as received and rolled titanium at transverse directions.

The hardness of any material is an intrinsic as well as a characteristic property. Figure 2 shows the optical image after the hardness testing in the vicker's micro-hardness test setup and Figure 3 shows the comparison between the hardness values of as-received and rolled Ti sample. The plastic deformation resulted in the indentation of the cold rolling of titanium. The refining of the microstructure due to cold rolling made the as received titanium to significantly enhance the hardness (Table 2) as it was carried out at a temperature below its recrystallization temperature which increased the strength via strain hardening. Also it is to be noted that the amount of strain percentage during cold rolling process had a role to play in the hardness behavior of the as received titanium. The strengthening occurred because of the dislocation movements and dislocation generation within the crystal structure. This improvement in the hardness of the as received due to cold rolling can not only improve the mechanical property but also make it more analogues to the properties required for a bone implant.

4.3 Mechanical Testing

The tensile testing results are shown in Table 3. The images showing the samples before and after the tension testing are shown below (Fig. 4).

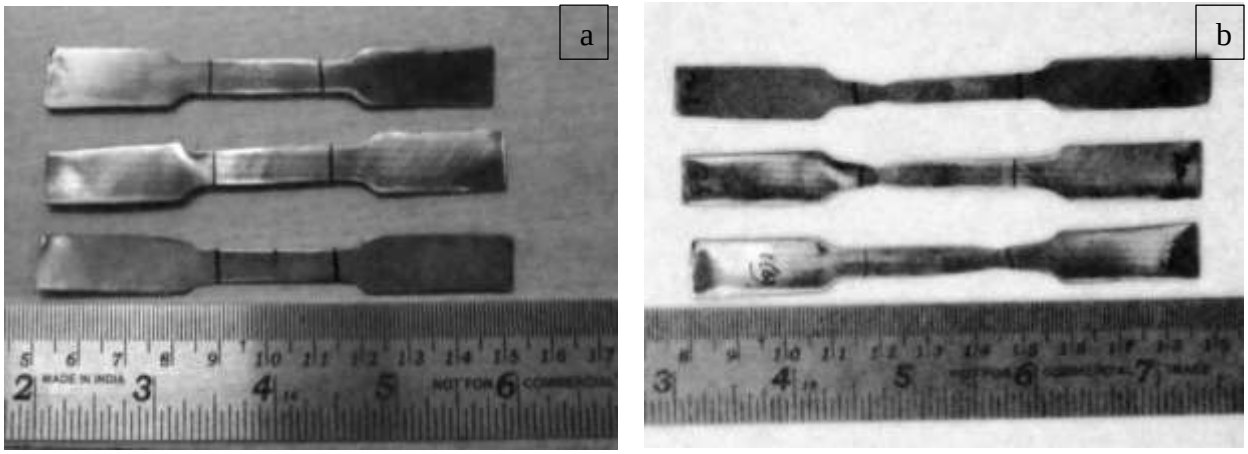

Figure 4: Image showing the rolled Ti samples (a) before and (b) after the tensile testing.

Table 3. Tensile testing of as-received and cold rolled titanium.

Sl. No.	**0.2 % Yield Stress [MPa]**		**Ultimate Tensile strength (UTS)[MPa]**		**% of Elongation**	
	AR Ti	RTi	AR Ti	RTi	AR Ti	Rti
1	328	410	460	523	30	24
2	345	402	485	510	28	21
3	341	415	488	533	27	20
Average	338	409	477.67	522	28.33	21.67
S.D.	8.88	6.55	15.3	11.53	1.52	2.08

Cold rolling can be considered as work hardening procedure where the strength of the material is influenced by the grain refinement. According to the Hall-Petch equation the relation between yield stress and the grain dimension is

$$\sigma_y = \sigma_o + \frac{k_y}{\sqrt{d}}$$

where, σ_y -yield stress

σ_o -resistance offered by lattice to dislocation movement

k_y – strengthening coefficient

d –average grain diameter

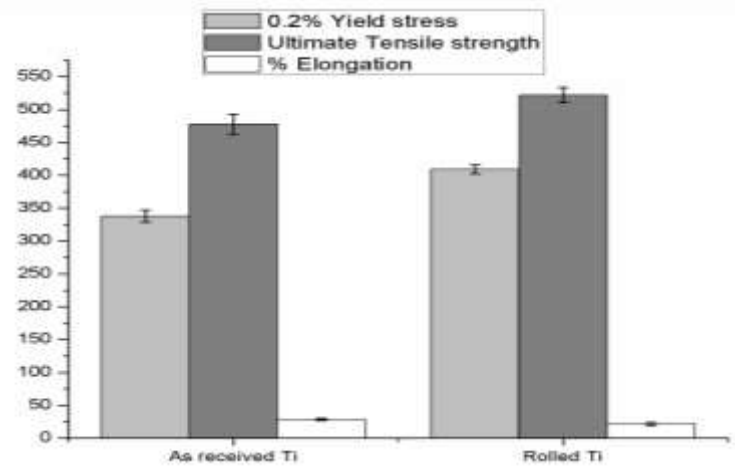

Figure. 5 Comparative study of tensile properties.

Thus, it can be inferred that the increase in the mechanical strength is a result of the granular refinement of titanium due to cold rolling. Figure 5 shows the comparative study of the tensile properties of both the samples.

4.4 Contact Angle Measurement

The contact angle measurements are shown in Table 4. Figure 6 shows a typical image of water droplet on as-received Ti surface. The contact angle was found to be $59.2^o \pm 6.5^o$ of the as received Ti sample and 45.9 ± 7^o of the rolled Ti. Using the Young's equation the surface energy was calculated to be $37.28 mJ/m^2$ for the as received Ti and $50.66 mJ/m^2$ for the rolled Ti samples.

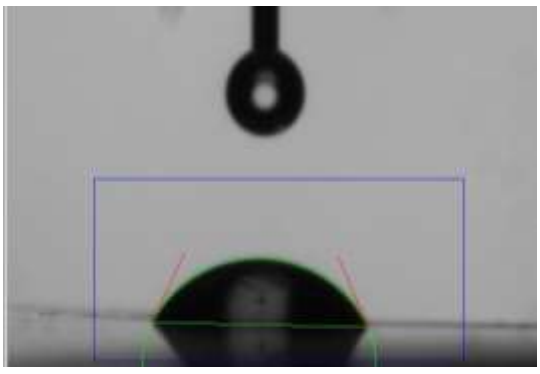

Figure 6: Frozen image of the liquid droplet and sample surface during contact angle measurement

Table 4. Contact angle measurements for as-received and cold rolled samples.

Sample Number	Static Contact Angle (deg)	
	received Ti	rolled Ti
1	67.7	20.2
2	64.2	42.1
3	63.9	38.6
4	61.7	33.6
5	63.0	64.3
6	58.5	53.7
7	53.5	59.6
8	50.9	53
9	49.4	48
Average	59.2	45.9
S.D.	6.5	7

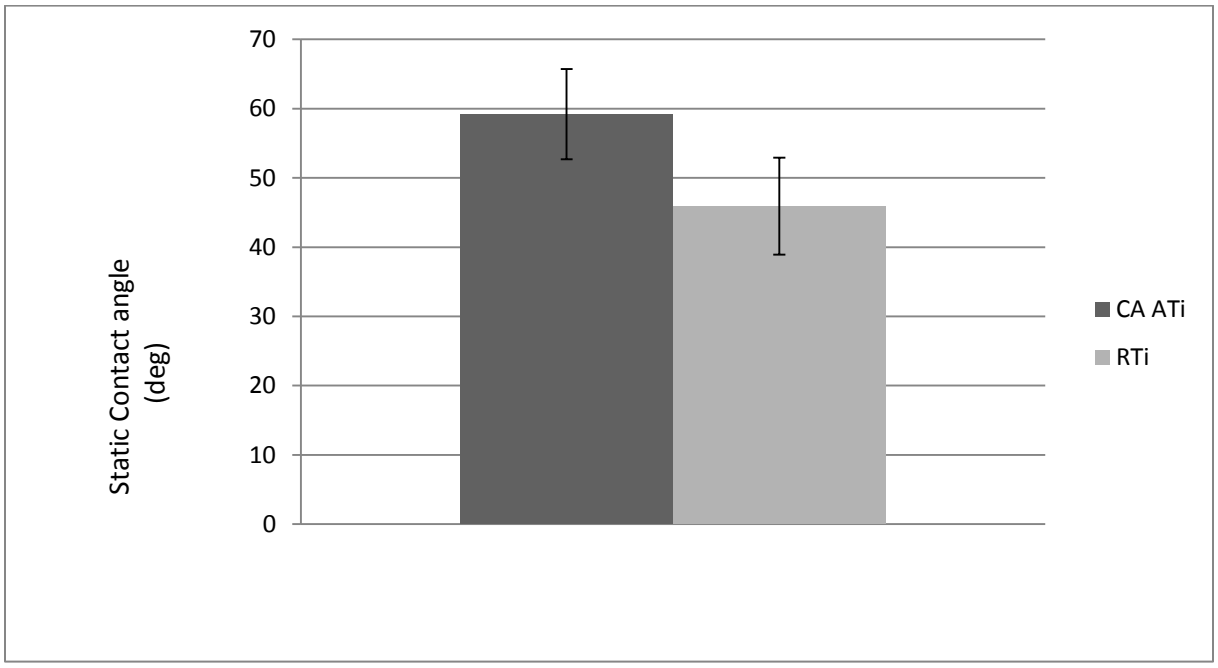

Figure 7: Comparison of static contact angle values of as received and rolled titanium.

The decrease in the contact angle measured depicts an increase in the hydrophilicity of the processed Ti sample. Also an increase in the surface energy was measured in case of the rolled sample when compared to that of the as received sample. This is mainly because of the small grain size achieved after rolling which has caused more surface bumps or micro-roughness is the prime reason behind increased surface energy. Materials with high surface energy are characterized by high wettability and these surfaces with high wettability help in adsorption of specific proteins and mineral phases which in turn promote a strong bonding between implant surface and tissue.

4.5 Protein Adsorption Study

Table 5 gives the data about protein adsorption values of as received and rolled Ti samples. The protein adsorption of the as received Ti sample was found to be 389.1 ± 2.3μg/ml whereas that of the rolled Ti sample was found to be 420.45 ± 3.0μg/ml, thereby marking an increase in the protein adsorption in case of rolled titanium.

Table 5. Protein adsorption of as received and rolled Ti-samples

Sl. No.	Adsorbed protein on as received Ti sample (μg/ml)	Adsorbed protein on rolled Ti sample (μg/ml)
1	390.69	429.65
2	387.5	411.25
3	389.7	423.32
Average	389.4	421.89
S.D.	1.6	9.3

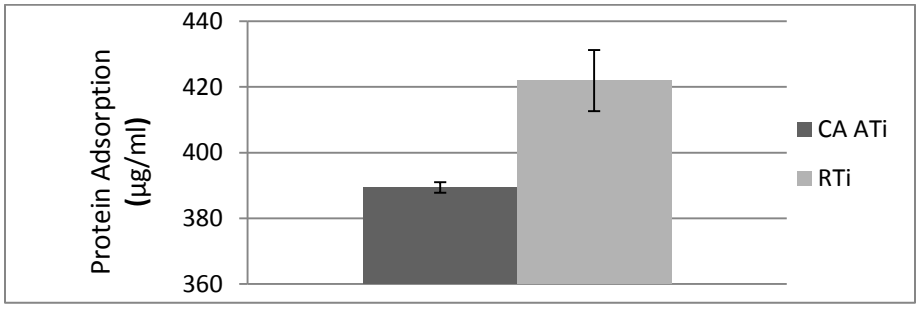

Figure 8: Comparison between the protein adsorption values of as received and rolled titanium.

BSA adsorption is favorable for cell attachment and proliferation which is prominent from the protein adsorption on rolled Ti-samples as compared to as received Ti-sample. Enhanced cell attachment in rolled Ti-samples is associated with greater binding affinity of cells for BSA. Also the presence of finer grains leads to the increase in wettability of the surface of rolled titanium which is another factor for the increase in protein adsorption. The increased protein adsorption of rolled Ti makes it a better suited for in vivo conditions as almost all biological activities are water based.

4.6 In vitro Bioactivity

The enhanced bioactivity is attributed to grain refinement that has caused during rolling when immersed in SBF; the apatite is formed by attracting the calcium ions from the SBF to form calcium titanate. As more calcium ions get attracted the overall net surface charge becomes positive and the phosphate ion gets attracted to form calcium phosphate (primary apatite). Once the primary apatite is formed, this acts as nucleating the sites for the secondary apatite. This secondary apatite has calcium to phosphate ratio close to that of bone mineral phase with globular in morphology.

The XRD pattern of as received and rolled sample in SBF for in-vitro bioactivity study after 2 weeks (Figure 9.) showed diffraction peak at 26° and 32° which correspond to hydroxyapatite phase (JCPDS#09_0432).

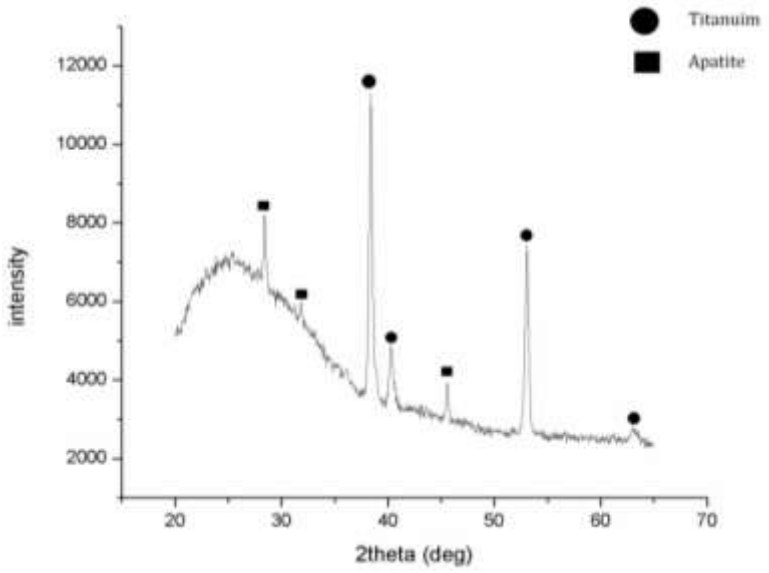

Figure 9: XRD patterns of rolled titanium sample immersed in SBF.

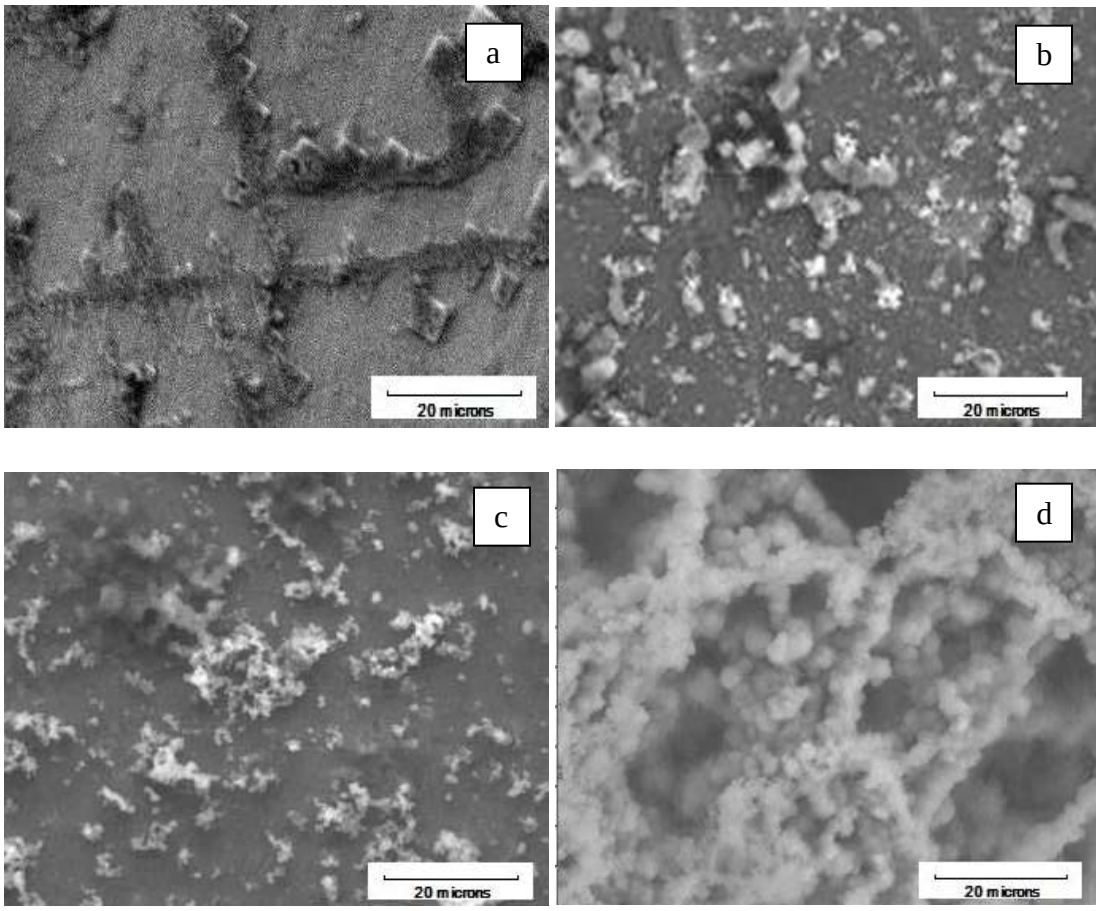

Figure 10. SEM micrographs of in-vitro bioactivity in SBF of (a) As-received Ti, 2weeks, (b) Rolled Ti, 2 weeks, (c) As-received, 4 weeks, (d) Rolled Ti, 4 weeks.

The in-vitro bioactivity of as received and rolled Ti immersed in SBF for about 2 weeks and 4 weeks were evaluated by SEM. Figure 10 (a and b) shows the SEM morphology of apatite formed on the as-received and rolled Ti surfaces after 2 weeks of SBF immersion. Deposits of spherical particles were observed on all sample surfaces whereas the untreated sample shows only few scattered apatite patches. From the SEM micrograph of Ti sample after immersion for 4 weeks (Figure 10 (c) and (d)) there is an observable increase in apatite deposition. The Ca/P ratio was found to be 1.66 by EDAX which is close to bone stoichiometric hydroxyapatite (1.67).

CONCLUSION

Rolled titanium showed grain refinement when compared to as-received titanium. It also resulted in superior hardness, mechanical strength, surface energy, protein adsorption and in-vitro bioactivity. The increase in in-vitro bioactivity is due to grain refinement. With grain refinement there was an increase in grain boundary area which resulted in more apatite nucleation. Hence, from this study it can be concluded that the rolled Ti is a suitable biomaterial load bearing sites. The enhanced bioactivity indicates that the healing time of the bone and implant in the patient can be reduced and thus, the Cp-Ti can be used in hard tissue replacement due to improved mechanical properties.

REFERENCES

1. Holleman, A.F., Wiberg, E., and Wiberg, N., *Lehrbuch der anorganischen Chemie*, Walter de Gruyter 1995.
2. Heinz, S., *Titanium Handbook of Extractive Metallurgy vol. II. Habashi, F*., Weinheim, Wiley-VCH, 1997.
3. Huiskes, R., Weinans, H., and Van Rietbergen, B., *The relationship between stress shielding and bone resorption around total hip stems and the effects of flexible materials.* Clinical Orthopaedics and Related Research, 1992. 274: p. 124-134.
4. Chen, Y., *Fabrication of porous titanium implants with biomechanical compatibility.* Materials Letters, 2009. 63(30): p. 2659-2661.
5. Ryan, G., Pandit, A., and Apatsidis, D. P., *Fabrication methods of porous metals for use in orthopaedic applications.* Biomaterials, 2006. 27(13): p. 2651-2670.
6. Geetha, M., *Ti based biomaterials, the ultimate choice for orthopaedic implants–a review*. Progress in Materials Science, 2009. 54(3): p. 397-425.
7. Mao, W., Hirsch, J., and Lücke, K., *Influence of the Cube Starting Texture on Rolling and Recrystallization Texture Development*, Proceedings of 8th International Conference of Textures of Materials.
8. Black, J., *Biological Performance of Materials*, 2006. p. 363-416.
9. Kokubo, T. and Takadama, H., *How useful is SBF in predicting in vivo bone bioactivity?* Biomaterials, 2006. 27(15): p. 2907-2915.
10. Larke, E. C., *The Rolling of Metals and Alloys*, Sheet Metal Industries, July 1946 *to* October 1953.
11. Umierwood, L. R., *The Rolling of Metals*, John Wiley and Sons, Inc., New York, Volume-1, *1950.*
12. Kangasniemi, I., *In vivo tensile testing of fluorapatite and hydroxylapatite plasma-sprayed coatings.* Journal of biomedical materials research, 1994. 28(5): p. 563-572.
13. Geesink, R., N. Hoefnagels, *Six-year results of hydroxyapatite-coated total hip replacement.* Journal of Bone & Joint Surgery, British Volume, 1995. 77(4): p. 534-547.
14. Nishiguchi, S., *Titanium metals form direct bonding to bone after alkali and heat treatments.* Biomaterials, 2001. 22(18): p. 2525-2533.

15. Yang, Y., *Preparation of graded porous titanium coatings on titanium implant materials by plasma spraying.* Journal of biomedical materials research, 2000. 52(2): p. 333-337.
16. Yang, Y., *Surface modification of titanium through amino group implantation.* Journal of biomedical materials research, 2001. 55(3): p. 442-444.
17. Yang, Y., *Morphological behavior of osteoblast-like cells on surface-modified titanium in vitro.* Biomaterials, 2002. 23(5): p. 1383-1389.
18. Proceedings of "ICOTOM, Eighth International Conference on Textures of Materials": p. 997–1004.
19. Gey, N., Humbert, M., *Characterization of the variant selection occurring during the α→β→α phase transformations of a cold rolled titanium sheet*.
20. Oshida, Y., Hashem, A., Nishihara, T., Yapchulay, *Fractal dimension analysis of mandibular bones: towards a morphological compatibility of implants*, Bone Material Engineering, 1994. p. 397– 407.
21. Murray, D. W., Rushton, N., *The influence of the surface energy and roughness of implants on bone resorption*, Bone Joint Surgery, 1989. p. 632–637.
22. Hallab, N., Bundy, K., *Evaluation of metallic and polymeric biomaterial surface energy and surface roughness characteristics for directed cell adhesion*, Tissue Engineering, 2001. p. 55–71.
23. Schakenraad, J. M., *Thermodynamic aspects of cell spreading on solid substrata*, Cell Biophysics, 1988. p. 75– 91.
24. Ruardy, T. G., Schakenraad, J. M., Van der Mei, H. C., Busscher, H. J., *Adhesion and spreading of human skin fibroblasts on physocochemically characterized gradient surfaces*, Journal of Biomedical Materials Research, 1995. p. 1415–1423.
25. Georgi, A., *Studies on the biocompatibility of materials: fibroblast reorganisation of substratum-bound fibronectin on surfaces varying in wettability*, Journal of Biomedical Material Research, 1996. p. 385– 391.
26. Webb, K., Hlady, V., Tresco, P. A., *Relative importance of surface wettability and charged functional groups on NIH 3T3 fibroblasts attachment, spreading, and cytosqueletal organisation*, Journal of Biomedical Material Research, 1997. p. 1156-1178.

27. Deligianni D. D., Katsala N., Ladas S., *Effect of surface roughness of the titanium alloy Ti–6Al–4V on human bone marrow cell response and on protein adsorption*. Biomaterials Chapter-22, 2001. p. 1241–1251.
28. Francaois P., Vaudaux P., Taborelli M., Tonetti M., *Influence of surface treatments developed for oral implants on the physical and biological properties of titanium. (II) Adsorption isotherms and biological activity of immobilized fibronectin*. Clinical Oral Implants Research, 1997. p. 217–225
29. .L. Ponsonnet, K., Reybier, N., Jaffrezic, V., Comte, C., *Relationship between surface properties (roughness, wettability) of titanium and titanium alloys and cell behavior*, Materials Science and Engineering, Elsevier, Chapter-23, 2003. p. 551–560.
30. Rupp, F., Scheideler, L., Rehbein, D., Axmann, D., Geis-Gerstorfer, J., *Roughness induced dynamic changes of wettability of acid etched titanium implant modifications*, Biomaterials, Chapter-25, 2004. p. 1429–1438.
31. Shirkhanzadeh M., Sims, S., *Immobilization of calcium phosphate nano-clusters into alkoxy-derived porous TiO2 coatings*. Journal of Material Science, 1997. p. 595– 601.
32. Li P., De Groot K., *Calcium phosphate formation within sol – gel derived titania in vitro and in vivo*. Journal of Biomedical Material Research 1993. p. 1495– 1500.
33. Chen J., Tong W., Cao Y., Feng J., Zhang X., *Thermal decomposition of hydroxyapatite structure induced by titanium and its dioxide.* Journal of Biomedical Materials Research, 1997. p. 15– 20.
34. Wen H. B., Wolke J. G. C., De Wijn J. R., Liu Q., Cui F. Z., De Groot K., *Fast percipitation of calcium phosphate layers on titanium induced by simple chemical treatment*. Biomaterials, 1997. p. 1471– 1477.
35. Feng B., Chen J. Y., Zhang X. D., *Calcium phosphate coating on titanium induced by phosphating*. Engineering Materials, 2001, p. 167–170, 192– 195.
36. Diana T., Wassell H., Embery G., *Adsorption of bovine serum albumin on to titanium powder*. Biomaterials, 1996. p. 859– 864.
37. Feng, B., Weng, J., Yang, B. C., Chen, J. Y., *Surface characterization of titanium and adsorption of bovine serum albumin*, Biomaterials, 2006. p. 2907–2915.

38. Hench, L. L., Splinter, R. J., Allen W. C., Greenlee T. K., *Bonding mechanisms at the interface of ceramics prosthetic materials*. Journal of Biomedical Materials Research, 1972. p. 117–141.
39. Jarcho, M., Kay, J.I., Gummaer, R. H., Drobeck, H. P., *Tissue, cellular and subcellular events at a bone–ceramic hydroxyapatite interface*. Journal of Bioengineering, 1977. p. 179–192.
40. Rejda B. J., Peelen J. G. J., de Groot K., *Tricalcium phosphate as a bone substitute*. Journal of Bioengineering, 1977; p. 93–97.
41. Feng B., Chen J.Y., Qi S. K., He L., Zhao J. Z., Zhang X. D., *Carbonate apatite coating on titanium induced rapidly by precalcification*. Biomaterials, 2002. p. 173–179.
42. Feng B., Chen J. Y., Qi S. K., He L., Zhao J. Z., Zhang X. D., *Characterization of surface oxide films on titanium and bioactivity*. J Mater Sci Mater Med 2002; 13:457– 64.
43. Healy K. E., Ducheyne P., *Hydration and preferential molecular adsorption on titanium in vitro*. Biomaterials, 1992. p. 553–561.
44. Dion I., Baquey C., Monties J. R., Havlik P., *Haemocompatibility of Ti6Al4V alloy. Biomaterials*, 1993. p. 122–126.
45. Zeng H., Chittur K. K., Lacefield W. R., *Analysis of bovine serum albumin adsorption on calcium phosphate and titanium surfaces*. Biomaterials, 1999. p. 377–384.
46. Legeros R. Z., Lin S., Rohanizadeh R., Mijares D., Legeros J. P., *Biphasic calcium phosphate bioceramics: preparation, properties and applications*. Journal of Material Science and Medical Materials, 2003. p. 201–209.
47. Kokubo T., Shigematsu M., Nagashima Y., Tashiro M., Nakamura T., Yamamuro T., et al. *Apatite- and wollastonite-containing glassceramicfor prosthetic application*. Bulletin of the Institute of Chemical Research, Kyoto University, 1982. p. 260–268.