1. Introduction

The overall reaction of the living system body to a foreign material implant is governed by a number of factors that determine whether the implant is accepted or rejected. Biocompatibility and biosafety are considered to imply that the clinical application of a biomaterial should neither cause any adverse reaction nor endanger the life of a patient. Generally, parameters determining the biocompatibility are: i- tissue as a host and ii- implant as a guest. Animal testing is an inherent component of biocompatibility testing. The use of in vitro methods can reduce the extend of animal testing and significantly reduce time and cost of testing. Knowledge of basic mechanisms of cell–material interaction and better understanding of ongoing processes at the cellular level during interaction of anchorage dependent cells can aid in the development of new biomaterials.

Factors affecting the tissue–implant interface from biocompatibility point of view include: general health, immunity factors, roughness, surface porosity, chemical reactions, corrosion and cytotoxicity. Thus, the surface characterization of biomaterials is particularly important if the biocompatibility of implants is to be understood. Possible mechanisms through which a biomaterial can interact with a metallic implant is illustrated in Fig. 1.

A variety of surface properties are believed to be responsible for the favorable performance of titanium implants, in particular the presence of a chemically very stable oxide film protecting the underlying metal from corrosion, the moderate charge of the surface under biological conditions, the very low concentration of charged species within the dissolution products and a dielectric constant $\varepsilon$ for titanium oxide close to that of water ($\varepsilon =78$). The result is that the titanium surface does not lead to excessively strong interaction (and denaturation) with proteins in the extra cellular matrix; rather the surface is in some way water like, interacting gently with the hydrophilic outer surface of the protein molecules. Nature of interaction between osteoblast cells and their substrate can influence the ability of these cells to produce an osteoid matrix around an implant which in turn will determine the fate of the implant. Attachment of anchorage dependent cells is the first step in the process of cell surface interactions which in turn can affect subsequent cellular and tissue responses.
Cells attach to substrates through contact sites which are classified as focal contacts. It is important to understand the nature of contact of cells interacting with biomaterials. Changes in cell morphology can be studied using different microscopic techniques like phase contrast and electron microscopy. In case of implants intended for orthopaedic application where close apposition with bone cells is required for better osteointegration, cell attachment and adhesivity play an important role.

2.1 Kinetics of cell-bioimplants interaction

**Protein adsorption**

Titanium implants were first introduced by Branemark in the early 1970s [Branemark et al., 1977] and have been widely used in the medical and dental fields with excellent clinical results. In addition, bone appears to bond with the titanium oxide layer following implantation, a phenomenon termed osseointegration. However, little is known about the specificity of this process and still less is known about the proteins that adsorb on the titania surfaces. In terms of the host tissue-biomaterial interaction the role of adsorbed proteins is likely to be an important one because subsequent events may depend on the composition and conformation of this protein layer and its tendency to change over time. The final protein film may trigger the activation of complementary coagulation cascades and other inflammatory response [Kazatchkine & Carreno, 1988; Ziats et al., 1988]. Macromolecules adsorption is a complicated process but for simplicity it can be summarized as follow:

1. The encounter of soluble molecules with surface which is determined by diffusion constants of concentration of different species.
2. A reversible binding of molecules to surface which means that bound molecules may be detached in a very short time.
3. Constant modification of the composition of adsorbed layers ie. some species may be replaced by more adhesive ones. This is the basis of Vroman effect.
4. Progressive conformation changes of adsorbed proteins hence strengthening adhesion.
5. Possibility of continues adsorption with formation of multiple protein layers.

As it is seen in Fig. 1 there are various forces such as Vander walls, bipolar, hydrogen, ionic and covalent that can play an important role in adsorption of protein molecules. The kinetics of adsorption of proteins to solid surfaces generally consist of a very rapid initial phase that is diffusion limited, followed by a slower phase upon approach to the steady-state value. In the initial phase, the proteins typically adsorb a quickly as they arrive at the relatively empty surface. In the later, slower phase, it is presumably more difficult for the arriving proteins to find and fit into an empty spot on the surface. In as short a time as can be measured after implantation in a living system (<1 sec), proteins are already observed on biomaterial surfaces. In seconds to minutes, a monolayer of protein adsorbs to most surfaces. The protein adsorption event occurs well before cells arrive at the surface. Therefore, cells see primarily a protein layer, rather than the actual surface of the biomaterial.

It is thought that the particular properties of surfaces, as well as the specific properties of individual proteins, together determine the organization of the adsorbed protein layer, and that the nature of this layer in turn determines the cellular response to the adsorbed surfaces. The soluble proteins differ from the insoluble proteins is many ways, including the

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fact that they are less regular in their amino acid composition and three dimensional structure. The soluble proteins are therefore difficult to describe, this diversity originates in the linear sequence of amino acid that uniquely characterizes each protein.

Fig. 1. Routes of protein adsorption to implant surface

It is important to note the particular structure assumed by a protein which in fact is a unique arrangement of the amino acid sequence in three-dimensional space exists for each protein. Furthermore, the spatial arrangement results in the hydrophobic residues preferentially located “inside” the protein where they are shielded from water, while the ionized and polar residues are usually on the outside of the proteins and in contact with the aqueous phase.

This spatial arrangement of the amino acids in proteins has a direct bearing on the interaction of proteins with surfaces because it means that the many residues “buried” inside the proteins may not be able to participate in bond formation with the surface. The folded proteins structures have densities of about 1.4 g/cm³ in comparison with water’s density of 1.0, or the density of most synthetic polymers of about 1.1, this basic fact about proteins reflects their tightly folded structure. Therefore, depending on the two major driving forces for adsorption, namely, the relative bulk concentration of each protein and its intrinsic surface activity, the outcome of the competitive process of adsorption is an adsorbed layer that is richer in some proteins than others; the surface composition differ from the bulk composition.

The thermodynamics of protein adsorption are not easily characterized because the process appears to be essentially irreversible. Because adsorption is irreversible, the calculation of an
equilibrium binding constant from a plot of adsorption versus bulk concentration, and its conversion to a free energy value in the usual way, is not a valid method to obtain thermodynamic information. The enthalpy of the adsorption process has been observed to vary a great deal. The observation of positive enthalpies upon spontaneous adsorption to certain surfaces must mean that the process is entropically driven in these cases. The net negative free energy characteristic of a spontaneous process means that $T\Delta S$ is greater than the positive $\Delta H$ term in the formula $\Delta G=\Delta H-T\Delta S$. More generally, all protein adsorption processes are thought to be strongly driven by entropic changes. The important of entropic factors in this process can easily be envisaged to arise from changes in water binding to the surface and the protein as well as limited unfolding of the protein on the surface.

The orientation of proteins in the adsorbed phase must also be considered because the proteins are not uniform in properties or structure across their surface. As far as is known, proteins are not very free to rotate once adsorbed, owing to multiple bonding is exposed to the bulk phase. The reactions of proteins in the adsorbed phase may be broadly classed into noncovalent reactions represented by structural transitions, and covalent reactions, such as those that occur with proteins complements.

**Cell – adhesion**

There is no need to emphasize the potential interest of controlling or even predicting the outcome of encounters between cells and artificial surfaces. Indeed, such knowledge would greatly facilitate the production and use of biomaterial. Three sequential steps of cell surface interaction are considered as: 1-protein adsorption, 2-formation of adhesive ligand–receptor bonds, 3-triggering specific cell function such as apoptosis, proliferation, migration (locomotion), differentiation or activation. Cell adhesion is a well-studied mechanism of cell communication. Adhesion is a form of mechanical linkage, of cells to cells and cells to the ECM, and is critically involved in cellular signaling events that control proliferation, survival, apoptosis, shape, polarity, motility, and differentiation. Adhesion is mediated by transmembrane proteins, which connect the interior of the cell to its extra cellular environment (Fig. 2). One major feature biological adhesion that is different from non-biological adhesion such as with household and industrial adhesives is that the former is mediated by chemical signals that often positively or negatively feedback to adhesion and subsequent cell behaviour. In other words, cell adhesion is more than a simple glue to hold cells and tissues together; it is also a critical signaling platform. Integrins and cadherins are two principal classes of molecules mediating primarily cell-ECM and cell-cell adhesion respectively.

Integrins are the best-understood class of adhesion receptors [Hynes, 2002]. They mediate both cell-ECM and cell-cell adhesion, differing based on the cell type and the type of receptor used in the interaction. Integrins, composed of $\alpha$-$\beta$ subunit heterodimer, assemble into 24 distinct integrins and bind to proteins in the ECM such as fibronectin, collagen and laminin [Hynes, 2002]. The binding of integrins initiates clustering of integrins to form structures and the recruitment of a host of signaling and adaptor proteins as well as the actin cytoskeleton [Geiger & Bershadsky, 2001]. These focal adhesions are involved in the signaling events that lead to proliferation, motility, cytoskeletal organization, and cell survival [Geiger & Bershadsky, 2001].

Cadherins mediate adhesive contact between cells in structures called adherens junctions, and play a vital role in morphogenetic events during development. The best studied of these
are the classical cadherins, which mediate adhesion between adjacent cells by forming homotypic junctions at sites called adherens junctions, and are linked intracellularly to the actin cytoskeleton through beta-catenin and alpha-actinin. Similar to integrins, cadherins too have a mechanical and signaling role. Along with the mechanical that adhesion plays in tissue cohesion, cadherins, like integrins, transmit specific signals to the cell interior through proteins at the adhesion site [Shay-Salit et al., 2002; Yap & Kovacs, 2003]. Both integrin and cadherin adhesions are biochemically regulated and are both dynamic and reversible. Normal cell processes such as the rounding of spread cells during mitosis, cell sorting and migration during embryogenesis, or disease processes such as cancer cell metastasis involve active changes in adhesion strength between cell-ECM and cell-cell contacts. Also, adhesive signals regulate many of the same cell function that soluble growth factors do. Adhesion and growth factor pathways are cooperative: for example, anchorage dependent cells do not grow in the absence of adhesion when they are placed in suspension even in the presence of saturating amounts of growth factors, neither do they grow in the absence of growth factors even when adherent. It is only when both adhesive and growth factor signals are present that growth pathways are optimally activated in cells [Zhu & Assoian, 1995].

Fig. 2. Representation of the cell–biomaterial surface interaction

However, the effects observed for a given protein have been found to vary substantially depending on the nature of the underlying substrate and the method of immobilization [Neff et al., 1999; Juliano, et al., 1993]. Because cells interact with ECM proteins through receptors that bind to localized regions within their proteins ligands, the biological activity of proteins on the surface will depend upon whether specific active peptide sequence in specific proteins are accessible to the arriving cells [Yamada, 1991]. Many of the ECM proteins, like fibronectin, laminin vitronectin, von Willebrand factor, carry a sequence of amino acid, arginine-glycin-aspartic acid (RGD) to which cells can bind using specific surface receptors called integrins [Pierschbacher & Ruoslahti, 1984; Ruoslahti &
Integrin-mediated binding of cells is the foundation for cell growth and differentiation and is the dominant mechanism by which cells communicated with noncellular surroundings [Ruoslahi, 1991].

In brief, cell adhesion is involved in various natural phenomena such as embryogenesis, maintenance of tissue structure, wound healing, immune response, metastasis as well as tissue integration of biomaterial. Surface characteristics of materials, whether their topography, chemistry of surface energy, play an essential part in osteoblast adhesion on biomaterials. Thus attachment, adhesion and spreading belong to the first phase of cell/material interactions and the quality of this first phase will influence the cells’ capacity to proliferate and to differentiate itself on contact with the implant.

Cell spreading is a combined process of continuing adhesion and cytoplasm contractile meshwork activity. Cell adhesion and spreading are influenced by the physicochemical characteristics of the underlaying solid surface. Other important parameters involved in cell-adhesion are: 1- surface charge, 2- topography, 3- porosity, 4- grooves, 5- mechanical forces, 6- texture and 7- cellular locomotion. The high ionic strength of the physiological environment and the rapid establishment of ionic equilibrium indicate that surface electrical properties do not significantly influence the formation of initially adsorbing protein layers or adhering cells. The surface topography of a biomaterial can be classified according to roughness, texture, and porosity. Porosity is used on a large scale to promote anchorage of biomaterials to surrounding tissue. Grooved substrata were found to induce a certain amount of cellular orientation and locomotion in the direction of the grooves. Applying grooved substrata will therefore induce cell contact guidance. Mechanical forces around an implant, especially in combination with a rough surface, induce abundant formation of fibrous tissue, owing to the constant irritation of the cells. The texture of an implant surface and its morphology can be adapted to the clinical purpose of the biomaterial by such approaches as changing the fabrication process (e.g., woven, knitted, fibrous, grooved, veloured, smooth). Cellular locomotion can be directed by various gradients in the cell environment. For example, after implantation of a biomaterial, granulocytes are attracted by a negative oxygen gradient. Fibroblasts are attracted by agents produced by macrophages.

**Orthopaedic implants**

Titanium and titanium alloys are largely used as implant materials because of their high in vitro and in vivo biocompatibility and load bearing. It is well established that osseointegration is an important property of titanium implant surfaces and that the current success rate is satisfactory [Feng et al., 2003; Puleo et al., 1989; Albrektsson et al., 1981]. Osseointegration is a term introduced by Beraceras et al., 1977 to describe a loaded, stable implant in direct contact with bone. Osseointegrated implants differ from ingrown ones that are dependent upon bone growth into surface macroscopic features or irregularities. By contrast, osseointegration is dependent on tissue ingrowth into minute surface features, such as the fundamental asperities of a smooth surface or as postulated for surfaces of various crystalline calcium phosphates (such as calcium hydroxyapatite) or amorphous, bioactive glasses, on direct chemical bonding between tissue and implant. Nevertheless, some concern remains as to the effects of vanadium and aluminum which are known to be cytotoxic [Khosroshahi et al., 2007; Deppe et al., 2005]. The chronology of the metal alloys is shown in table 1.
Table 1. Chronology of metal alloys in orthopaedic applications

| Alloy                | Year | Application       | Performance issues                               |
|----------------------|------|-------------------|---------------------------------------------------|
| Vanadium steel       | 1912 | Bone plates       | Corrosion problems                               |
| Cast Co-Cr-Mo        | 1937 | Dental devices    | Well accepted                                    |
| Cast Co-Cr-Mo        | 1938 | Orthopaedic implants | Well tolerated, adequate strength               |
| 302 stainless steel  | 1938 | Bone plates /screws | Corrosion resistance                             |
| 316 stainless steel  | 1946 | Trauma implants   | Better corrosions resistance and strength         |
| Titanium             | 1965 | Hip implants (England) | Corrosion resistance, tissue acceptance         |
| 316LVM stainless steel | 1968 | Trauma implants   | Further improvements in corrosion                |
| MP35N                | 1972 | European hip prostheses | High strength                                   |
| Ti-6Al-4V            | 1974 | Trauma implants   | High strength, biocompatibility                  |
| Ti-6Al-4V            | 1976 | Hip prostheses    | High strength, low modulus                      |
| Forged Co-Cr-Mo      | 1978 | Hip prostheses    | Highest fatigue strength                        |
| 22-13-5 stainless steel | 1981 | Hip implants, trauma | High strength, forgeability                    |
| Ti-6Al-7Nb           | 1982 | Hip implants      | High strength, biocompatibility                  |
| Cold-forged 316LVM   | 1983 | Compression hip screw | High strength                              |
| Zirconium 2.5 Nb (zirconia cated) | 1994 | Joint prostheses  | Improved wear resistance, Biocompatibility      |

Bone - cell adhesion

The attachment phase occurs rapidly and involves short-term events like physicochemical linkages between cells and materials involving ionic forces Van der Waal forces, etc and the adhesion phase occurs in the longer term and involving various biological molecules: extra cellular matrix proteins, cell membrane proteins, and cytoskeleton proteins which interact together to induce signal transduction, promoting the action of transcription factors and consequently regulating gene expression. Proteins involved in osteoblast cells adhesion are: 1-Extra cellular matrix proteins, 2- Cytoskeleton proteins, 3- Adhesion molecules.

1.2 Surface modification of Ti-6Al-4V alloy by long Nd:YAG laser for orthopaedic applications

It is possible to change localized areas of metals in order to obtain both compositions and microstructures with improved properties. Titanium and titanium alloys are the most frequently used material for load-bearing orthopaedic implants, due to their specific properties such as high corrosion resistance, surface oxidation layer, high strength and high-temperature resistance [Feng et al., 2003; Ducheyne & El-Ghannam, 1994; Kelly et al., 2003; Wang et al., 2003; Hsu et al., 2004; Tian et al., 2005]. Titanium and its alloys’ application like
any other biomaterials involve the creation of at least one interface between the material and biological tissues. Biocompatibility and bioactivity of biomaterials rely on the interactions that take place between the interface of the biomaterials and the biological system [Wang & Zheng, 2009]. It is generally believed that proteins adsorbed on implant surface can play an important role in cell-surface response. Different proteins such as collagen, fibronectin and vitronectin which are acting as ligands are particularly important in osteoblast interaction with surface. Ligands are the junctions which facilitate adhesion of bone cells to implant surface. In another word, more ligand formation implies a better cell-surface interaction [Tirrell et al., 2002; Anselme et al., 2000]. In vitro studies can be used to study the influence of surface properties on processes such as cell attachment, cell proliferation and cell differentiation. However, in vivo studies must be performed to achieve a complete understanding of the healing process around implants. Previous studies have shown that surface characteristics named above have a significant influence on adhesion, morphology and maturation of cultured osteoblasts [Davies, 1996; Thomas et al., 1997; Cooper et al., 1998; Masuda et al., 1998]. Also, it has been demonstrated that for primary bovine osteoblasts, the wettability is one of the key factors [Meyer, 1993]. In our studies [Khosroshahi(a) et al., 2007; Khosroshahi(b), 2007; Khosroshahi(a) et al., 2008; Khosroshahi(b) et al., 2008; Khosroshahi et al., 2009] it is shown that the wettability of the surface can provide a better spreading condition for osteoblast cells due to reduced contact angle. Bearing in mind that the adhesion of bone cells to implant surface consists of two stages. In primary stage the cells must get close enough to surface in an appropriate distance known as focal distance over which the cells can easily be spread over it. In this respect, the wettability can be effective in providing a preferred accessibility to surface and thus reaching the focal distance. The secondary stage includes cell-cell attachment which obeys the regular biological facts.

Interface reactions between metallic implants and the surrounding tissues play a crucial role in the success of osseointegration. The titanium and its alloys like some other medical grade metals are the materials of choice for long-term implants. The effect of implant surface characteristics on bone reactions has thus attracted much attention and is still considered to be an important issue [Albrektsson & Johansson, 2001; Buchter et al., 2005; Buchter et al., 2006]. So far as the surface characteristics of the implants are concerned, two main features that can influence the establishment of the osseointegration are the physico-chemical properties and the surface morphology. Cell adhesion is involved in various phenomena such as embryogenesis, wound healing, immune response and metastasis as well as tissue integration of biomaterial. Thus, attachment, adhesion and spreading will depend on the cell-material interaction and the cell’s capacity to proliferate and to differentiate itself on contact with the implant [Chung et al., 2003; Biggerelle & Anselme, 2005].

Cell behaviour, such as adhesion, morphologic change and functional alteration are greatly influenced by surface properties including texture, roughness, hydrophilicity and morphology. In extensive investigations of tissue response to implant surfaces, it has been shown that surface treatment of implant materials significantly influences the attachment of cells [Cheroudi et al., 1995; Curtis & Wilkinson, 1998; Brunette & Cheroudi, 1999; Lavose-Valereto et al., 2001; Curtis & Clark, 2001; Sowden & Schmitz, 2002; Heinrich et al., 2008]. Additionally, these modified surfaces must resist both the mechanical wear and the corrosion [Sighvi & Wang, 1998]. It is therefore important to evaluate systematically the role of different surface properties and to assess the biological performance of different implant materials.
Titanium as a biomaterial implant has an excellent biocompatibility due to the fact that it is highly inert and is not soluble in body fluids and forms a protective oxide layer on the surface [Burser & Schenk, 1991; Breme & Helsen, 1998]. However, pure titanium could leave metal debris in the tissue due to the higher tendency to produce wear in fretting conditions. Therefore numerous titanium alloys like Ti6Al4V with improved physical and mechanical properties have been developed.

The surface morphology, as well as manipulation with the physical state and chemical composition of implant surface may be significant for bone-implant integration. Surfaces are treated to facilitate an intimate contact between bone and implant. So, the tissue response to an implant involves physical factors, depending on implant design, surface topography, surface charge density, surface free energy and chemical factors associated with the composition of the materials. These substrate characteristics may directly influence cell adhesion, spreading and signaling, events that regulate a wide variety of biological functions [Sikavitsas et al., 2001; Sun et al., 2001; Ronold & Ellingsen(a), 2002; Ronold & Ellingsen(b), 2002; Ronold et al., 2003]. Numerous surface treatments including ion implantation [Beraceras et al., 2002, Tan et al., 2003; Assmann et al., 2007], coating [Vercaigne et al., 1998; Ong et al., 1997; Toth et al., 2002; Morra et al., 2003; Tian et al., 2004; Eisenbarth et al., 2007], shot blast [Darvell et al., 1995; Kawaura et al., 2002; Aparicio et al., 2003], machining [Sahin & Sur, 2004], plasma spray [Khor et al., 1999; Yang & Change, 2001], plasma nitrid [Galvanetto et al., 2002], nitrogen diffusion hardening [Venugopalan et al., 2000] are some of the relatively older techniques in the field of material processing which can be used to change implant’s surface topography. But perhaps the laser-assisted method has recently received more attention and has been successful in meeting the new objectives in this field which is mainly because of its wavelength selectivity, coherency, very low thermal or mechanical damage, high accuracy, control and less pollution during laser treatment process.

In fact, optical and kinetics of laser parameters like fluence and pulse number as well as surface physical parameters will affect this process [Gaggl et al., 2000; Wang et al., 2000; Fancsaly et al., 2002; Hollander et al., 2005; Hao(a) et al., 2005]. For example, surface modification of metals by CO2 [Tritca et al., 2001; Ghoor et al., 2001], HF [Deka et al., 1980; Khosroshahi, 2004], Nd:YAG [Peyer et al., 2000; Yang et al., 2004; Tritca et al., 2009], and diode lasers [Slocombe et al., 2000; Majumdar et al., 2005], titanium alloy by Nd:glass [Joob-Fancsaly et al., 2002], KrF [Deppe et al., 2005], XeCl [Tritca et al., 2005], diode lasers [Hao(b) et al., 2005] and However the recent studies on this subject using short Nd:YAG laser pulses have been performed as In vitro with some limited but informative results [Arisu et al., 2006; Turner et al., 2007; Mirhosseini et al., 2007]. Also recently a femtosecond laser was used for surface treatment of titanium in order to determine the potential of this technology for surface structuring of titanium implants [Vorobyev & Guo, 2007]. Thus, the success of uncemented orthopaedic implants depends largely upon the body's ability to synthesize new bone directly onto the surface of the device. This gap allows micromotion of the implant, which loosens the device and ultimately leads to implant failure. Some researchers have attempted to circumvent capsule formation by coating metal implants with the synthetic bone analogue, hydroxyapatite (HA). The synthesis and deposition of new bone onto an implant requires the attachment of osteoblast precursor cells to the implant surface, as well as the subsequent differentiation of these cells. Both of these processes are likely mediated by the integrin family of cell adhesion receptors. Thus, the main intension of this work is to extend the earlier research by carrying out some detailed In vitro and In vivo
experiments using a long pulse (200µs) Nd:YAG laser radiation on surface physico-chemical changes, surface wettability, corrosion resistance, microhardness and direct osteoblast cells adhesivity of Ti6Al4V with respect to possible orthopaedic applications. Finally, to assess and compare the effects of physico-chemical modifications made on laser treated surface (LTS).

2. Materials and methods

Sample preparation
Rectangular-shaped specimens with 20×10 mm dimensions and the thickness of 2 mm, were made from a medical grade Ti6Al4V (ASTM F136, Friadent, Mannheim- Germany- GmbH) with chemical formulation Ti(91.63%) Al(5.12%) V(3.25%). The samples were divided into three groups of untreated (7 samples), laser treated (14 samples). Prior to treatment, all samples were cleaned with 97% ethanol and subsequently been washed twice by distilled water in an ultrasonic bath (Mattachanna, Barcelona-Spain). A final rinse was done by de-ionized water at a neutral pH to ensure a clean surface is obtained. Finally, an optical microscope with magnification of x20 was used to ensure that no particles were left on the sample surface.

Experimental setup
Surface treatment was performed by Nd: YAG laser with 1.06µm wavelength, 200µs pulse duration and pulse energy of 50 J. The output beam was suitably imaged on to the target surface in a 500 µm spot diameter where it scanned the surface at a constant velocity using a motorized XYZ translator. All the experiments were carried out in air at pulse repetition frequency of 1Hz. In order to achieve the optimum surface treatment conditions, the melting, evaporation thresholds and variation of etch depth with fluence were evaluated. Etch depth per pulse variation as a function of laser fluence can be calculated from equation (1).

\[ X = a^{-1} \ln \left( \frac{F}{F_t} \right) \]  

(1)

Where, X is the etch depth, a is absorption coefficient and F_t is the threshold fluence. The samples were then sterilized by de-ionized water.

Surface roughness
The surface micro roughness (R_a) measurements were carried out using a non-contact laser profilemeter (NCLP) (Messtechnik, Germany) equipped with a micro focus sensor based on an auto focusing system. R_a is the arithmetical mean of the absolute values of the profile deviations from the mean line. Five two-dimensional NCLP profiles were obtained for each surface over a distance of 3.094 mm with a lateral resolution of 1µm using a Gaussian filter and an attenuation factor of 60% at a cut-off wavelength of 0.59 mm . The roughness parameters were calculated with the NCLP software similar to that described by Wieland et al. [Wieland et al., 2001].

Surface hardness
Surface microhardness test was carried out with 50 gram load in 10 seconds by a diamond squared pyramid tip (Celemx CMT, Automatic). Each related test was considered at 5 points and reported as an average. The Vickers diamond pyramid hardness number is the applied load divided by the surface area of the indentation (mm²) which could be calculated from below equation:
This equation could be re-written approximately as:

\[
VHN = 1.854 \left( \frac{F}{d^2} \right)
\]

Where \( F \) is load in Newton, \( d \) (mm) is the arithmetic mean of the two diagonals, \( d_1 \) and \( d_2 \) in mm and \( VHN \) is Vickers hardness. The Vickers diamond pyramid indenter is grounded in the form of a squared pyramid with an angle of 136° between faces. The depth of indentation is about 1/7 of the diagonal length. When calculating the Vickers hardness number, both diagonals of the indentation are measured and the mean of these values is used in the above formula with the load used to determine the value of \( VHN \).

**Corrosion tests**

The standard Tafel photodynamic polarization tests (EG&G, PARC 273) were carried out to study the corrosion behaviour of specimens in Hank’s salt balanced physiological solution at 37°C. The procedure for preparing the Hanks solution is schematically shown in The metal corrosion behaviour was studied by measuring the current and plotting the \( E \)-log\( I \) (Voltage – Current) diagram. The corrosion rate (milli per year (mpy)) was determined using equation:

\[
C.R. = 0.129 \left( \frac{M}{n} \right) \left( \frac{I_{corr}}{\rho} \right)
\]

Where \( M \) is the molecular weight, \( n \) is the charge, \( I_{corr} \) is the corrosion current and \( \rho \) is the density.

**Surface tension**

The surface energy of the samples were determined by measuring the contact angle (\( \theta \)) of test liquids (diiodo-Methane and water; Busscher) on the titanium plates using Kruss-G40-instrument (Germany). The geometric mean equation divides the surface energy into two components of dispersive and polar and when combined with Young's equation it yields:

\[
\gamma_{lv}(1 + \cos \theta) = 2 \left( \gamma_s^d \cdot \gamma_l^d \right)^{0.5} + 2 \left( \gamma_s^p \cdot \gamma_l^p \right)^{0.5}
\]

Equation (5) can be rearranged as by Ownes-Wendt-Kaeble's equation:

\[
\gamma_{lv}(1 + \cos \theta) \left( \frac{\gamma_l^d}{\gamma_s^d} \right)^{0.5} = \left( \gamma_s^p \right)^{0.5} \left( \frac{\left( \gamma_l^p \right)^{0.5}}{\left( \gamma_s^p \right)^{0.5}} \right) \left( \gamma_s^p \right)^{0.5}
\]

Where \( s \) and \( l \) represent solid and liquid surfaces respectively, \( \gamma_s^d \) stands for the dispersion component of the total surface energy (\( \gamma \)) and \( \gamma_s^p \) is the polar component.

### 2.1 In vivo test

**Anesthetization**

Before depilation of the operation site, the animal was completely anesthetized with midazolam (Dormicum®, Roche, Switzerland) 2.5 mg/Kg intravenously (IV). With any sign of recovery during operation, diluted fluanisone/fentanyl (Hypnorm®, India) was injected slowly until adequate effect was achieved, usually 0.2 ml at a time.
Animal implantation

One untreated sample and two LTS were implanted on femur bone of an eight months male goat weighing 30 Kg. Specimens were steam sterilized before implantation in an autoclave (Mattachnna, Barcelona-Spain). The steam sterilization was conducted under 132 °C, 2 bar and in 45 minutes. All the specimens were labeled by separate codes for further studies. The operation site was shaved and depilated with soft soap and ethanol before surgery; the site was also disinfected with 70% ethanol and was covered with a sterile blanket. In order to proceed with implantation, cortex bone was scraped by osteotom (Mattachnna, Barcelona-Spain) after cutting the limb from one-third end in lateral side and elevating it by a self-retaining retractor. Copious physiological saline solution irrigation was used during the implantation to prevent from overheating. To ensure a stable passive fixation of implants during the healing period, they were stabilized by size 4 and 8 titanium wires (Atila ortoped®, Tehran-Iran) without any external compression forces (Fig. 3).

Fig. 3. Placement of implants in the femur bone of the goat

Fig. 4. The X-ray of implants wired to the bone

Fig. 5. Implant removal from the femur bone of the goat: (a) before detachment of the wires, (b) the foot-print of the implants on the bone

After the operation the animal was protected from infection by proper prescribed uptake of Penicillin for first four days and Gentamicine for second four days. During the eight days of
recovery, the goat was administrated with multi-vitamins to help to regain its strength. During this period, the goat was kept in an isolated space under room temperature, ordinary humidity, lightening and air condition, and before it returns to its natural life environment, X-ray radiographs (Fig. 4) were taken in order to ensure that the implant has not been displaced during the maintenance period. It was observed that calus bone had grown in the vicinity of the implant. After five months the animal was sacrificed and the specimens were removed (Fig. 5).

The experiments had been approved by the Yazd School of Veterinary Science (Iran) and its animal research authority and conducted in accordance with the Animal Welfare Act of December 20th, 1974 and the Regulation on Animal Experimentation of January 15th, 1996. The explantation procedure was performed by first cutting the upper and lower section of femur bone using an electric saw and then the implant together with its surrounding tissues was placed in 4% formalin solution for pathological assessment and SEM.

**Cell analysis**

Osteoblast cells spreading (ie. lateral growth) on the six implants (three samples for imaging and three samples for coulter counter) was analyzed after removal by SEM(stero scan 360-cambridge) and their spreading condition in a specific area was studied using Image J Program software in three separate regions of each specimen at frequency of 10 cells per each region. The number of attached cells in 1 cm² area of each specimen was calculated by a coulter counter (Eppendorf, Germany) using enzyme detachment method and Trypsin-EDTA (0.025 V/V) in PBS media at pH = 7.5. The final amount of attached cell can be studied by plotting cell detachment rate versus time.

**Histopathology**

Surrounding tissues of specimens were retrieved and prepared for histological evaluation. They were fixed in 4% formalin solution (pH = 7.3), dehydrated in a graded series of ethanol (10%, 30%, 50%, 70% and 90%) and embedded in paraffin after decalcification. Then, 10 µm thick slices were prepared per specimen using sawing microtome technique. A qualitative evaluation of macrophage, osteoblast, osteoclast, PMN, giant cells, fibroblast, lymphocyte was carried out by Hematoxylin and Eosin stain and light microscopy (Zeiss, Gottingen-Germany). The light microscopy assessment consisted of a complete morphological description of the tissue response to the implants with different surface topography. Osteoblasts can be in two states; (a) active, forming bone matrix; (b) resting or bone-maintaining. Those make collagen, glycoproteins and proteoglycans of bone the matrix and control the deposition of mineral crystals on the fibrils. Osteoblast becomes an osteocyte by forming a matrix around itself and is buried. Lacunae empty of osteocytes indicate dead bone. Osteoclast, a large and multinucleated cell, with a pale acidophilic cytoplasm lies on the surface of bone, often an eaten-out hollow-Howship’s lacuna. Macrophages, are irregularly shaped cells that participate in phagocytosis.

**SEM of adhered cells**

After implants removal, all three group implants were rinsed twice with phosphate buffer saline (PBS) and then fixed with 2.5% glutaraldehyde for 60 minutes. After a final rinse with PBS, a contrast treatment in 1% osmium tetroxide (merck) was performed for 1 hour, followed by an extensive rinsing in PBS and dehydration through a graded series of ethanol from 30% to 90% as described in histology section. After free air drying, surfaces were thinly sputter coated with gold (CSD 050, with 40 mA about 7 min). Cell growth on implanted specimens and their spreading condition in a specific area was analyzed using Image J Program software in three separate regions of each specimen for 10 cells per each region.
**Statistical analysis**

All calculated data were analyzed by using a software program SPSS (SPSS Inc., version 9.0). The results of variance analysis were used to identify the differences between the cells spread area of the treated and cleaned un-treated samples ($p \leq 0.05$).

**3. Results**

**3.1 Characterization of surface topography**

*Optical and Mechanical Effects*

Fig. 6 indicates the variation of etch depth per pulse with the laser fluence where the metal absorption coefficient ($\alpha$) can be determined from the slope of the curve using the well known beer logarithmic equation (Eq. 1). From Equation 66 the values of $\alpha$ and $F_t$ were found $5 \times 10^3 \text{cm}^{-1}$ and 73 Jcm$^{-2}$, respectively. It is interesting to notice that, in our case, the interaction of laser radiation with metal surface can be divided into four different regions:

![Fig. 6. Variation of etch depth as a function of fluence](image-url)

In zone I (0-30 Jcm$^{-2}$) no morphological changes was observed but beyond that where zone II (30-70 Jcm$^{-2}$) commences, some minor topological alterations such as surface deformations were seen until it reached zone III (70-145 Jcm$^{-2}$) where the signs of melting were clearly observed and it gradually became stronger. This is consistent with the fact that the power density required for melting ($I_m$) most metals is in the order of $10^5 \text{ Wcm}^{-2}$ which in this case the corresponding range would be between 365-725 kWcm$^{-2}$. It is however, important to notice from metallurgical point of view that melting initially begins due to temperature rise of different elements at the surface. Hence, gradually these island-type molten centers are joined together producing a larger molten pool. Finally, zone IV (145 Jcm$^{-2}$-above) represents the onset of gradual thermal ablation (vis.vapourization) of Ti6Al4V at $\geq$145 Jcm$^{-2}$ with corresponding temperature of about 3280°C. Normally this process is accompanied by the presence of plume which basically is defined as ejection of material from the metal surface as gaseous and solid phase particles. Again this behaviour is expected to become dominant with vaporization intensity threshold of about $10^6 \text{ Wcm}^{-2}$. It is deduced from Fig. 7 that "Incubation" effect or period can be observed at lower fluences where more laser pulses may be needed in order to reach the ablation threshold. It may be worth while to notice that this
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Effect had also been observed and reported for polymers by other investigators [Srinivassan et al., 1990; Dyer & Karnakis, 1994]. Also to avoid possible errors in assessment and evaluation of interaction process a distinction must be made between the actual physical ablation and surface non-linear roughness (i.e. ripples) even occasionally with relatively high amplitudes which in our case this began at about 30 J cm\(^{-2}\). The etch depth saturation became particularly noticeable at higher fluences, which is mainly thought to be due to an intense interaction between the laser pulse and laser-induced vapor plume, hence causing some effective beam attenuation.

Fig. 7. Variation of etch depth as a function of laser pulse number at constant laser fluences.

Since in our experiment the pulse duration is much greater than the thermal relaxation time (i.e. \(\tau \approx 200 \mu s \gg \tau_r \approx \frac{\alpha^2}{4k} \approx 140 \text{ ns}\)) and that the optical absorption depth, \(\alpha^{-1}\), is much smaller than thermal diffusion depth, \(z_t\) (i.e. \(\alpha^{-1} \approx 2 \mu m \ll z_t \approx 2(\kappa \tau_p)^{1/2} \approx 7.5 \text{ mm}\)) thus, the temperature rise at the end of laser pulse on the surface is given by equation 7:

\[
T_f - T_i = \frac{(1-R)F}{\rho c \left(4k\tau_p\right)^{1/2}}
\]  

(7)

With [Ifflander, 2001]:
- \(T_f, T_i\) = final and initial surface temperature (\(^\circ\)C).
- \(R\) = surface reflection (0.6)
- \(C\) = specific heat capacity (0.52 J g\(^{-1}\)°C\(^{-1}\))
- \(\rho\) = density (4.51 g cm\(^{-3}\))
- \(k\) = diffusivity coefficient (0.07 cm\(^2\) s\(^{-1}\))
- \(\tau_p\) = pulse width (200 \(\mu s\))

Fig. 8 indicates the variation of Ti6Al4V surface temperature as a function of laser fluence. Now by assuming the melting and vaporization points of Ti6Al4V about 1668\(^\circ\)C and 3280\(^\circ\)C respectively and the melting region between (73-145) J cm\(^{-2}\), using Fig. 6, then it would be sensible to choose zone III as the treatment area (i.e. below ablation).

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Fig. 8. Plot of Ti6Al4V surface temperature versus laser fluence

Fig. 9. Surface morphology of Ti6Al4V treated at a) 100 Jcm$^{-2}$ b) 140 Jcm$^{-2}$ c) 210 Jcm$^{-2}$ d) Crack formation at Ti6Al4V surface after 10 pulses treated at 270 Jcm$^{-2}$
The comparison between morphologically different textures produced by laser at different energy density is shown in Fig. 9. Fig. 9a indicates the interaction effect and the pattern of dendritic melted zone at 100J/cm^2. It is known that as the growth rate of solid phase increases with time during solidification process, the morphology of liquid / solid interface changes from planar to dendritic structure. These random fluctuating dendritic features are in turn defined by dendritic tip radius and their spacing between them [Dreyfus, 1992]. When the laser fluence was increased to 140J/cm^2, the thermal ablation became the dominant mechanism where the irregularities and scratches due to machining and polishing process became smooth after direct laser surface thermal processing, Fig. 9b. Fig. 9c indicates the difference between the untreated and treated surface at 210J/cm^2 with formation of some grainy structure and cracks in cellular form in the central zone. Finally when the fluence was increased to 270J/cm^2 (Fig. 9d) these cracks became dominant and developed throughout the surface caused by residual mechanical stress originated from steep temperature gradients at the surface during the resolidification process.

Fig. 10 shows an example of the ablation site at fluence of ≥ 250 J/cm^2 (i.e. I >1mWcm^-2) with 10 pulses which has caused the metal eruption, cracks as well as surface melting. The spectra of colors, mainly blue, are probably because of high temperature plasma roughly defined by \( T_e = C \cdot (P \lambda\sqrt{\tau_p})^{1/2} \) where \( C \) is a material depending constant, \( T_e \) is electrons temperature, \( P \) and \( \lambda \) are the laser power and wavelength respectively [Majumdar & Manna, 2003]. Also, other factors like surface composition, solubility degree of alloys and base metal, thermal diffusion and rate of heating-cooling cycle may all have specific roles in crack formation which need a separate attention and analysis.

Fig. 10. Plasma -induced damage of titanium at 250 J/cm^2 with 10 pulses

Two morphologically different areas i.e. laser treated and untreated (Fig. 11) indicate that the inclusions are disappeared and the scratches due to machining and polishing are sealed due to direct laser surface heating. Ti6Al4V alloy is a (α+β) two-phase alloy with around 6wt% aluminum stabilizing the phase and about 4wt% vanadium stabilizing the β phase. At room temperature, the microstructure at equilibrium consists mainly of primary α phase (hcp) with some retained β phase (bcc). It is also well known that in laser surface melting, steep temperature gradient and thermal cycle leads to some micro structural changes in the heat affected zone within very short time. In particular, the α + β phase transformation
during rapid heating and decomposition of the $\beta$ phase during rapid cooling. The physical and mechanical properties of Ti6Al4V alloy are known to be sensitive to its microstructure. The Ti-$\beta$ phase has a diffusivity of two orders of magnitude higher than Ti-$\alpha$ phase and flow stress is strongly influenced by the ratio of these phases [Fan et al., 2005].

Fig. 11. Scanning electron micrographs of Ti6Al4V surface morphology for: (a) untreated, (b) laser treated at 140 Jcm$^{-2}$ indicating the disappearing of surface scratches by laser surface melting

**Surface Roughness**

In order to obtain a quantitative comparison between the original and treated surface, the arithmetic average of the absolute values of all points of profile ($R_a$) was calculated for all samples. A value of $7\pm0.02$ was obtained for laser treated surface at 140 Jcm$^{-2}$. Also the $R_a$ values for untreated and laser treated at 100 Jcm$^{-2}$ were $12.3\pm0.03$ and $14.2\pm0.29$ respectively. All the calculations were performed for $n=5$ and reported as a mean value of standard deviation (SD).

**Surface Hardness**

Fig. 12. Variation of surface hardness with fluence
The surface hardness measurements presented in Fig. 12 clearly indicate that microhardness of the metal increases with laser fluence. Again a non-linear behaviour was observed where initially the values of VHN were increased gradually up to about 100 Jcm\(^{-2}\). Afterwards a sharp increase occurred until the point of plateau was reached at 140 Jcm\(^{-2}\) which corresponds to roughly 50% improvement of surface hardness. The surface hardness was found to vary from 377 VHN for MTS to 850 VHN for LTS. The surface hardness results for all the specimens are illustrated in table 2.

| Sample     | Microhardness (HVN) |
|------------|---------------------|
| Untreated  | 394                 |
| 140 Jcm\(^{-2}\)| 850                 |

Table 2. Surface hardness tests before and after treatment

**EDX analysis**

The experimental results of EDX spectroscopy of the untreated and treated samples in the ambient condition is given in Fig. 13. The analysis exhibited K-\(\alpha\) lines for aluminium and titanium for both samples, though it was expected carbon to be detected too. It is seen from Fig. 14 that as the laser fluence was increased, the vanadium percent showed a decreasing trend until it reached to its maximum reduced value at about 145Jcm\(^{-2}\) where there onwards it was increased again. Perhaps from biomedical applications point of view a better and safer cell attachment and growth can be expected in above condition since surface toxicity has reduced even further. Table 3 indicates the differences in surface elements after treating by laser irradiation.

![EDX analysis of the implant surface](https://example.com/fig13.jpg)

Fig. 13. EDXA analysis of the implant surface, a) untreated b) treated at 100 Jcm\(^{-2}\) c) treated at 140 Jcm\(^{-2}\)
Fig. 14. Changes of Vanadium at the alloy surface as a function of fluence

| Element sample | %Al | %V | %Ti |
|----------------|-----|----|-----|
| Untreated      | 5.15| 3.25| 91.6|
| 140 Jcm^-2     | 3.96| 2.54| 93.5|

Table 3. Surface elements composition before and after treatment

**Corrosion test**

The comparison of these curves indicates a few important points: 1-a value of $1.77 \times 10^{-3}$ mpy for untreated sample (Fig. 15a), 2- the corrosion rate for laser treated specimen reduced to $0.46 \times 10^{-3}$ mpy at 140 Jcm^-2 (Fig. 15b), 3- $E_{corr}$ varied from -0.51 V to -0.21 V after the treatment at 140 Jcm^-2. This means that the laser treated specimen is placed at a higher position in the cathodic section of the curve hence releasing hydrogen easier and acts as an electron donor to electrolyte. Therefore, by smoothly reaching the passivation region, a more noble metal is expected to be achieved. The corrosion current ($I_{corr}$) was decreased from 2.54 μAcm^-2 to 0.66 μAcm^-2 after surface laser treatment which can imply a better corrosion resistance.

Fig. 15. Tafel potentiodynamic polarization curves of Ti6Al4V for: (a) untreated, (b) at 140 Jcm^-2
Surface tension
The change in surface wettability was studied by contact angle measurement for all specimens treated at 100 Jcm\(^{-2}\), 140 Jcm\(^{-2}\) (Fig. 16a). A smoother surface was achieved by laser radiation at 140 Jcm\(^{-2}\) which means a reduction in contact angle. This effectively implies an increase in degree of wettability of the metal surface. It is, however, important to note that a smoother surface and enhanced oxygen content, which depends on oxide layer thickness, can both help to reduce the contact angle. This is so because the surfaces with higher concentration of oxygen atoms and more incorporation of oxygen-base polar functionalities of surface exhibit higher wettability (ie. lower contact angle) hence an improvement of biocompatibility, though some believe that, hydrophilicity alone is an inadequate promoter of cell adhesion and retention [Baier et al., 1984]. As a result, it is emphasized that a better cell adhesion can be obtained for the specimens with apparently higher surface energy, rather than higher surface roughness.

According to primary melting centers topology, the surface roughness was increased slightly at 100 Jcm\(^{-2}\) (\(R_a=14.2 \pm 0.29\)). Thus an increase of contact angle occurs from 70° to 80° indicating a lower degree of wettability. Following the laser treatment at 140 Jcm\(^{-2}\) the contact angle reduced to 37° showing still a more acceptable hydrophilic behaviour. Also, variation of surface tension for all specimens was calculated by measured contact angle. It is known that as contact angle decreases, the related surface tension will be increased. Therefore, a value of 58mN/m was obtained for \(\gamma\) at 140 Jcm\(^{-2}\) which is considerably higher than 39mN/m of the untreated sample. The corresponding values of \(\gamma\) for 100 Jcm\(^{-2}\) were found as 31mN/m (Fig. 16b).

![Fig. 16. Variation of contact angle: (a) and surface tension, (b) with sample surface texture](image)

In vivo

Cell spreading analysis

The experimental results of bone cell growth are given in table 4. As it can be seen, cells spreading over the specimen surface is related to laser fluence and surface texture which was measured by Image J program software (IJP). The highest spreading area (488 \(\mu\)m\(^2\)) belongs to the LTS at 140 Jcm\(^{-2}\).

The SEM analysis of attached cells morphology (Fig. 17) indicates that the density of cell network is directly dependent on the laser beam fluence and surface topography. The smooth surface produced at 140 Jcm\(^{-2}\) not only caused a dense cell network but also resulted
in a wider area covered by a single cell spreading. Density of network is originated from monolayer attachment (cell-surface) change to multilayer (cell-surface & cell-cell). As it is seen no specific directional spread of attached cells was achieved in laser treated specimens.

| Row | Specimens       | Spread cell area (µm²) |
|-----|-----------------|------------------------|
| 1   | untreated       | 352 ± 6                |
| 2   | 100 (Jcm⁻²)     | 316 ± 10               |
| 3   | 140 (Jcm⁻²)     | 488 ± 8                |

Table 4. Bone cells spread over the surface of the implanted specimens (average of ten measurements in three separate regions)

Fig. 17. Scanning electron micrographs of attached cells on the surface for: (a) untreated, (b) at 100 Jcm⁻², (c) at 140 Jcm⁻²

Histopathology

Fig. 18. Light microscopy evaluation of bone tissue for: (a) untreated, (b) samples laser treated at 140 Jcm⁻²
When the implants were retrieved, no inflammatory reaction was observed inside or around the implants. Mineralized matrix deposition and bone cells were observed on the surface of implants which are formed during the five months implantation. This deposition was found all around of LTS (Fig. 18b) and bone formation was characterized by the occurrence of osteocyte embedded in the matrix. Also the above samples were surrounded by fibroblast and osteoblast cells and the untreated sample (Fig. 18a) showed not only less number of fibroblast cells, also contained osteoclast and polymorpho nuclear leukocytes (PMN).

As it is seen from Fig. 19 the bone tissue nutrition is carried out through the channel in LTS where as it was not observed in the case of untreated sample. No PMN, giant cells and osteoclast were seen in laser treated samples at 140 Jcm$^{-2}$. Also tissue healing was better conducted near mentioned implant rather than all the other evaluated specimens. Fibroblast and osteoblast cells were also numerous at 140Jcm$^{-2}$.

![Nutrition Channel](image)

Fig. 19. (a) Light microscopy evaluation of bone tissue for: (a) laser treated sample at 140 Jcm$^{-2}$, with a nutrition channel shown, and (b) untreated without the channel

In table 5, The symbols indicate the presence of 2-3 cells (+), 3-5 cells (++) more than 5 cells (+++) and lack of cells (-) respectively. No PMN, giant cells and osteoclast were seen in laser treated sample at 140 Jcm$^{-2}$. Also tissue healing was better conducted near mentioned implant rather than all other evaluated specimens. Fibroblast and osteoblast cells were also numerous in qualitative scales for 140Jcm$^{-2}$ case.

| Sample Cell | treated | 100 Jcm$^{-2}$ | 140 Jcm$^{-2}$ |
|-------------|---------|----------------|----------------|
| Fibroblast  | ++      | +++            | +++            |
| Osteoblast  | +       | ++             | +++            |
| Giant cell  | -       | -              | -              |
| Osteoclast  | +       | -              | -              |
| PMN         | +       | +              | -              |
| Lymphocyte  | ++      | +++            | ++             |
| Macrophage  | ++      | +++            | ++             |
| Healing     | +       | +              | ++             |

Table 5. Qualitative evaluation of histology results of bone tissue around the implants
4. Discussion

The successful incorporation of bone implants strongly depends on a firm longstanding adhesion of the tissue surrounding the implants. The cellular reaction is influenced by the properties of the bulk materials as well as the specifications of the surface, that is, the chemical composition and the topography [Birte et al., 2003; Sikavitsas et al., 2003; Fischer et al., 2004; Peto et al., 2002; Gyorgy et al., 2002]. When one is considering materials for application of orthopaedic implants, it is important to consider a number of factors, such as biocompatibility and surface wettability. The stable oxide layer formed on the implant sample is known to be responsible for the biocompatibility of metal implants. Hence, for a successful laser surface processing of materials some optical and physical parameters must be carefully optimized in order to achieve a desirable surface morphology. It is known that the modification efficiency and the quality is not only the laser fluence dependent but also depend on the spatial and temporal profile of laser beam. Generally the energy absorbed from the laser is assumed to be converted to thermal energy which causes melting, vaporization of the molten material, dissociation or ionization of vaporized material and shock waves in the vapour and the solid. The interaction process was shown to be dependent on the laser fluence, as well as physico-chemical and optical properties of material. Usually, the deposited energy of laser irradiation is converted in to heat on time scale shorter than the pulse duration or laser interaction time. The resulting temperature profile depends on the deposited energy profile and thermal diffusion rate during laser irradiation. Thermal conductivity, K is related to $k, \rho$ and c as follows:

$$K = k, \rho c$$

This gives a value of 16WC$^{-1}$m$^{-1}$ which is much larger than the optical absorption depth ($\approx 2\mu$m). Therefore, the heat source is essentially a surface source. As it was defined earlier the vertical distance ($z_t$) over which heat diffuses during the $\tau_p$ is about 7.5 mm wher $z_t$ determines the temperature profile. The condition $\alpha^{-1} << z_t$ is typically applicable for laser irradiation of metals. In the present study, a non-adiabatic thermal evaporation was considered as the dominant mechanism during the laser-material interaction process.

A numerical simulation is performed based on date given in before section in order to evaluate the variation of the surface temperature of Ti6Al4V during the heating and cooling cycles. The fundamental Fourier heat transform describing the surface temperature $T(x,t)$ is:

$$\rho c \frac{\partial T(x,t)}{\partial t} - k \nabla^2 T - Q(x,t) = 0$$

$$0 < x < \infty$$

where $\nabla$ is laplace transform and $Q$ is the heat source.

During heating ($t<\tau_p$), the solution is

$$T(x,t) = T_h(x,t) = \frac{I_{\alpha}}{K} \left[ \frac{4\alpha}{\pi} \right]^{1/2} \exp \left( \frac{x^2}{4\alpha t} \right) - xerfc \left( \frac{x}{(4\alpha t)^{1/2}} \right) + T_0$$

During cooling the temperature drops for all $t>\tau_p$ and
where \( i\text{erf}(x) \) is the integral of the complementary error function defined as

\[
\text{erfc}(x) = \int_{x}^{\infty} \text{erfc}(y) dy = \frac{1}{\sqrt{\pi}} e^{-x^2} - \text{erf}(x)
\]  

Equations (10) and (11) have been solved numerically using MATHCAD computer program.

Fig. 20 shows the calculated surface temperature variation with the time for the Ti6Al4V irradiated by the pulsed Nd:YAG laser. The following results can be calculated from the numerical simulation: (i) increasing the laser fluence, increasing the surface temperature, (ii) the numerical results indicate that the maximum temperature achieved at the end of the laser pulse in higher than experimental values of melting (\( \approx 1668^\circ\text{C} \)) and vaporization (3280\(^\circ\text{C}\)) temperatures, (iii) almost all the pulse energy at 70 J cm\(^{-2}\) is expended to raise the surface temperature to melting point at the end of its duration (i.e., 200 \( \mu \text{s} \)). (iv) consequently, at high fluences, a less time is required to reach the melting point and hence the rest of available time is spent for vaporization and some thermal damage. (v) at higher fluences the temperature gradient become steeper and hence the probability of an unstable front leading to dentritics or crack increases during the cooling cycle.

Therefore, selecting a correct energy density for surface treatment has direct influence on the physical properties such as surface microhardness, corrosion resistance, wettability and surface tension of Ti6Al4V. For example, it seems uniformly accepted that any contamination has unfavorable biological effects which may catalyze oxidation, hence it may enhance the dissolution of Ti ions from implant surface. With this view, it is suggested [Joob-Fancsaly, 2002] that the surface contamination decreases the surface energy of material which is responsible for the surface adsorption of proteins at molecular level. The contact angle decreased by about 50 percent between 100 J cm\(^{-2}\) and 140 J cm\(^{-2}\) when the surface became smoother. It is, however important to notice that the NCLP method cannot measure the topographic features smaller than 1\( \mu \text{m} \) which are often found on the roughened
implant surface due to its resolving power limitation. Also, a superior microhardness value was exhibited by the sample which can be attributed to grain refinement associated with laser melting and rapid solidification.

Generally a change in the corrosion potential ($E_{corr}$) indicates a microstructural modification in the metal. For this purpose the Tafel potentiodynamic polarization experiments were performed on Ti6Al4V in Hank’s salt balanced physiological solution for untreated, laser treated, see Fig. 15. Also, an increase of corrosion resistance by about 74% which is mainly thought to be due to removal of surface inclusions and surface micro-smoothing during the laser treatment possibly caused by the lateral flow of molten material due to surface tension. Practically this means that the laser treated specimen is placed at a higher position in the cathodic section of the curve hence releasing hydrogen easier and acts as an electron donor to electrolyte. Therefore, by smoothly reaching the passivation region, a more noble metal is expected to be achieved which should be more impermeable and resistant to corrosion. This effect was observed within zone III (Fig. 6), where the surface melting was set at about 70 Jcm$^{-2}$. Among the possible involved mechanisms controlling the hydrodynamics of the melt region is its displacement resulting from the local pressure applied to the liquid surface as a result of the evaporation process. In this range, surface porosities and irregularities of untreated sample became almost completely sealed, Fig. 9b, and the roughness was reduced. Treatment at fluence of 140 Jcm$^{-2}$ with 10 pulses produced optimum effects on the surface. However, at higher fluences in zone IV, even though melting and vaporization are both at work, the surface roughness mainly defined by grainy structure, meandering, cell boundaries, and the cracks were increased, Fig. 9d. This effect may be resulted from reduced thermal accumulation on the metal surface by blasting off the sealed layer hence, causing the increase of local roughness. The grains, surface cooling rate after laser treatment, heat transfer from lower or lateral metal layer and short time duration between heating and cooling cycle may all cause some changes in remaining $\beta$ phase shape. In addition to surface morphology, the properties of implant materials affect cellular behaviour such as wettability. The wettability of the surface plays an important role with respect to protein adsorption, cell attachment and spreading. It is known that surfaces with high surface free energy are to be more adhesive than those with a low surface free energy. In this study the values for surface free energy showed significant differences between laser treated, untreated sample.

It is also worth to notice that all the samples were treated in an ambient condition and were steam sterilized which would have a great influence on surface composition of Ti especially in TiO$_2$ formation. It is proved that the samples irradiated by laser beam during the treatment can cause oxygen diffusion through the molten materials and thus to oxidize the titanium [Perez del Pino et al., 2002]. Also the variation of surface oxidation layer thickness depends on steam sterilization process and the time of exposure to air [Sitting et al., 2000]. As the oxygen content of surface increases, the measured contact angle decreases. This is explained by the fact that surfaces with higher concentration of oxygen atoms and more incorporation of oxygen-base polar functionalities of surface exhibit higher wettability (lower contact angle). The local presence of the alloying elements Al and V within the passive oxide film is likely to influence the adsorption of proteins and their conformation on the surface which in turn is expected to modify cell-surface interaction. If cells are affected by the presence of hydroxides on the surface, then developing an understanding of the mechanisms that control this interaction could lead to the optimization of this parameter in
current and future metallic biomaterials [Bern et al., 2004]. Also, other factors like surface composition, solubility degree of alloys and base metal, thermal diffusion and heating/cooling rate may have a specific role in crack formation which needs a separate attention and analysis.

The interaction of living cells with foreign materials is complicated matter, but fundamental for biology medicine and is a key for understanding the biocompatibility. The initial cellular events which take place at the biomaterials interface mimic to a certain extent the natural adhesive interaction of cells with the extra cellular matrix (ECM). The osteoblasts, which play a principal role in bone formation, readily attach to the material surfaces via adsorbed protein layer consisting or RGD containing ligands like fibronectin, vitronectin or fibrinogen. Family of cell surface receptors that provide trans- membrane links between the ECM and the cytoskeleton. The focal point in laser treated surface at 140 Jcm$^{-2}$ should be approximately 10 nm which is convenient for bone cells to get close enough to be activated and attach to the surface in order to form an extra cellular matrix. In this state, bone cells will spread over the smooth surface much more easily and fluently.

Our study showed that surface micro grooves can affect the orientation guidance of bone cells i.e. the deeper grooves were more effective in guiding the cells as it was evaluated by SEM. However, we did not conduct or evaluate systematically the exact effects of grooves depth and size on cell orientation, but our preliminary results were similar to those reported by Xiong & Yang, 2003. It seems, however, that laser treated surface did not regulate the cells shape exactly similar to other investigations. The finding of most research works that the osteoblast cells grow slightly better on the rougher surface indicate the fact the surface topography indeed affects the osteoblast cell proliferation. However, our results showed that laser treated samples at 140 Jcm$^{-2}$ produced a smoother and higher wettability characteristics than the mechanically roughe nd surface. This finding agrees with Hao$^{(a)}$ et al., 2005 and Mirhosseini et al., 2007 works where they found similar results with Ti6Al4V using diode laser. It is, believed that oxygen content of material surface can contribute to the improvement of the wettability characteristics in laser surface modification. It is also equally important from our point of view to carefully distinguish and discuss the difference between the mechanism of cell attachment on a rough surface and cell adhesivity on a smooth surface and their impacts on biomedical engineering [Hao$^{(a)}$ et al., 2005, Hao$^{(b)}$ et al., 2005].

5. Conclusion

This study was focused on the topographic effects of Ti6Al4V produced by laser radiation on goat bone cell adhesion. The results showed a common feature reported in the previous studies on a variety of cell types and substrates ie, topographic features strongly affects the cell guidance. We found a significant difference when comparing the cells behaviour on unevenly micro grooves and smooth surfaces. This study shows that a smooth surface can exhibit more cell adhesivity compared with micro grooved surface due to its increase of surface tension and reduction of contact angle and probably the presence of oxygen content at the surface. The test confirmed that the highest number of cells are attached to the LTS at 140 Jcm$^{-2}$. It is also concluded from the SEM, contact angle measurements and preliminary in vitro and in vivo tests that Nd:YAG laser can induce a desirable surface modification on Ti6Al4V alloy for cell adhesivity and that a noble and biocompatible Ti alloy with better
physico–chemical properties can be obtained under suitably defined optical condition. Finally, it is suggested that more detailed experiments are required and would be useful to distinguish and clarify the difference between attachment on the rough surface via physical anchoring and adhesion on smooth surface by chemical binding. Also, the relation between the grooves size and their orientation must be studied more carefully with respect to cell attachment and their reliability as well as endurance.

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