THE EFFECT OF TITANIUM SURFACE TOPOGRAPHY ON BIOCOMPATIBILITY, HEMOCOMPATIBILITY, AND BACTERIAL BEHAVIOR

by

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May the force be with all the scientists.

ACADEMIC ETHICS AND INTEGRITY STATEMENT

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ABSTRACT

THE EFFECT OF TITANIUM SURFACE TOPOGRAPHY ON BIOCOMPATIBILITY, HEMOCOMPATIBILITY, AND BACTERIAL BEHAVIOR

Titanium and Ti alloys are widely used in several biomedical applications including cardiovascular stents, shoulder, hip replacement and dentistry. Ti and its alloys are preferred biomaterials, due to their mechanical, physical and biological characteristics. Ti is a biocompatible material with high corrosion resistance and several methods are used to process the material in addition to various alloying options. Although, Ti is biocompatible by its nature, cell- material interactions can be improved by surface modification. It is known from the literature that the interactions between material surface and cells, bacteria and proteins are affected by the surface topography. The characteristics of the surface; topography, roughness, surface free energy and wettability alter the cellular response. In this thesis, four different Ti surfaces; polished, sandblasted, laser processed line and mesh structured, were examined from the aspects of material surface characterization, biocompatibility, hemacompatibility, and biofilm formation. All 4 surfaces showed hydrophilic characteristics. However, sandblasted and line structured surfaces are more hydrophilic than polished and mesh structured surfaces. Although higher platelet activation was observed on polished surface, it is shown that all 4 groups are blood compatible in accordance with ISO10993-4. When the adhesion and viability of fibroblasts were examined on the surfaces, no significant difference was observed. Therefore, the difference of biocompatibility on the polished, sandblasted, line and mesh structured surfaces are shown to be insignificant. For the bacterial adhesion, *S. aureus* bacteria showed higher tendency for polished surface and *E. coli* bacteria showed higher tendency for the line structured, sandblasted and mesh structured specimens in descending order. In this thesis, it is shown that by changing surface topography physically, cellular response to the material can be altered.

Keywords: Titanium, Laser Processing, Roughness, Biocompatibility, Hemacompatibility.

ÖZET

TİTANYUM YÜZEY TOPOGRAFİSİNİN BİYOUYUMLULUK, KAN UYUMLULUĞU, VE BAKTERİ DAVRANIŞINA OLAN ETKİSİ

Titanyum ve alaşımları kardiyovasküler stentler, omur ve kalça implantları ve dişilik gibi birçok biyomedikal uygulamada kullanılmaktadır. Titanyum ve alaşımları mekanik, fiziksel ve biyolojik özelliklerinden dolayı tercih edilen biyomalzemelerdir. Titanyum korozyon rezistansı yüksek, biyouyumlu ve çeşitli alaşım seçeneklerine ek olarak yüzeyi farklı metotlarla işlenebilen bir malzemedir. Titanyum biyouyumlu bir malzeme olmasına rağmen hücre ve malzeme etkileşimleri yüzey modifikasyonları ile geliştirilebilmektedir. Literatüre göre malzeme ile hücre, bakteri ve protein etkileşimleri yüzey topografilerinden etkilenmektedir. Yüzey topografisi, pürüzlülüğü, serbest yüzey enerjisi ve ıslanabilirliği hücre tepkisini değiştirmektedir. Bu tez çalışmasında, parlatılmış, zımparalanmış, lazer ile çizgi ve ızgara şeklinde işlenmiş yüzeyler, biyouyumluluk, kan uyumluluğu, yüzey karakterizasyonu ve biyofilm oluşumu açılarından incelenmiştir. Tüm yüzeyler hidrofilik özellik göstermiştir. Fakat, zımparalanmış ve çizgi şeklinde işlenmiş yüzeylerin, parlatılmış ve ızgara şeklinde işlenmiş yüzeylerden daha hidrofilik olduğu görülmüştür. Parlatılmış yüzeylerde platelet aktivasyonu daha yüksek olmasına rağmen, tüm grupların ISO10993-4 standartlarına göre kan uyumlu olduğu görülmüştür. Fibroblast hücrelerinin yüzey tutunması ve canlılığı incelendiğinde dört yüzey arasında istatistiksel olarak anlamlı bir fark görülmemiştir. Bu sebeple, parlatılmış, zımparalanmış, lazer ile çizgi ve ızgara şeklınde işlenmiş yüzeylerin biyouyumluluklarınının birbirinden farklı olmadığı sonucuna ulaşılmıştır. Bakteri tutunumu incelendiğinde *S. aureus* bakterilerinin parlatılmış ve *E. coli* bakterilerinin azalan sırada çizgi şeklinde işlenmiş, zımparalanmış ve ızgara şeklinde işlenmiş yüzeylere tutunma eğilimi gözlemlenmiştir. Bu tez çalışmasında yüzey topografisini fiziksel olarak değiştirerek, hücrelerin malzemeye tepkisinin değiştirilebildiği gösterilmiştir.

Anahtar Sözcükler: Titanyum, Lazer, Pürüzlülük, Biyouyumluluk, Kan Uyumluluğu.

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LIST OF ABBREVIATIONS

Ti	Titanium
CP	Commercially Pure
MSC	Mesenchymal Stem Cell
ECM	Extracellular Matrix
BMP-2	Bone Morphogenic Protein 2
TGF-β	Transforming Growth Factor-β
AMP	Antimicrobial Peptide
PLA	Poly(lactic acid)
CaP	Calcium Phosphates
CPC	Calcium phosphates cements
HA	Hydroxyapatite
SEM	Scanning Electron Microscopy
WCA	Water Contact Angle
ALP	Alkaline Phosphatase
DMEM	Dulbeccoâs Modified Eagle Medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate Buffered Saline
OD	Optical Density
FBS	Fetal Bovine Serum
RBC	Red Blood Cell
PRP	Platelet Rich Plasma
UFG	Ultrafine-Grain
TNTA	Titanium dioxide Nanotube Arrays
UV	Ultraviolet

1. INTRODUCTION

1.1 Motivation

Titanium (Ti) and its alloys are widely used in biomedical applications, such as neurosurgery, shoulder, elbow, hip and knee replacements, and dentistry. It is a preferred material because of its high biocompatibility. In addition to its biocompatibility, it has other characteristics and advantages, which make Ti a preferred biomaterial, such as its high corrosion resistance, low elastic modulus, low specific gravity, and fatigue resistance and having several available methods to process the material [1, 2, 3, 4]. It is known fact that both bulk of the material and surface of the material have influence on the biological performance of the material and specifically, the surface topography plays important role in cellular response [5]. Structures with higher roughness in micron-scale are more suitable than flat surfaces in terms of having more surface area to interact with the cells leading to adherence [6]. Also, nanostructures on the surface of the material can affect adsorption of proteins on the surface and in that way alter the cellular cascades in addition to have direct interaction with cell receptors [7, 8]. The impact of surface topography on the biological response of host which involve; protein and biomolecule adsorption, thrombosis, [9] endothelial cell attachment, cellular differentiation etc. [10, 11] required to be evaluated for each surface model of biomaterials separately.

There are various Ti alloys that are being used in medical applications, wide range of compositions are being developed with the aim of maximizing advantages for the application. Many research studies are conducted to disclose the interactions in between material to host biological system [12]. The studies include but not limited with Ti and Ti alloy-based biomaterials.

For instance, it is shown that nitrogen-doped titanium affect blood clot formation and platelet adhesion [13], research elucidates that change in surface roughness of

Ti and Ti-based alloys alter macrophage polarization, osteoclast genesis and osteoblast differentiation [14], alterations in surface roughness of stainless steel affect Mesenchymal Stem Cell (MSC) differentiation [15], surface roughness of Zirconia has significant effect on osteoblast responses [16], surface roughness of Silicon has variable impact on macrophage polarization [17]. It is possible to alter cellular response by physical modifications as much as chemical modifications. In this thesis the effect of surface roughness is addressed, and the laser processing is used as a method to alter the surface properties. Laser processing is chosen in this thesis due to its advantages of providing repeatable and precise results as well as area controlled processing [18].

It is an established fact that surface topography is as determinative as the bulk material composition on protein adsorption, cellular response to the material, bacterial adhesion on the material [19].
As already been stated, Ti and Ti alloys are known to be highly biocompatible [20]. Biocompatibility is generally defined as âthe ability of a material to perform with an appropriate host response in a specific application.â [21]. In the enlightenment of this statement biocompatibility of Ti implants should be examined in more detailed.

As known from the literature, surface topography affect host response significantly [18]. This is because, change in surface topography alter wettability and surface energy and in this way the molecular interactions [22]. This chain of reactions which result in change in surface energy affect cellular adhesion [23]. Although the interaction is proven, it is not possible to derive definite formula that indicates increase in roughness or hydrophilicity corresponds to increase or decrease in adhesion of certain cells or expression of certain genes etc. This is because this relations donât reflect to directly proportional pattern mathematically. In general hydrophilic surfaces are expected to promote cellular adhesion but the opposite results are also reside in literature [23, 24]. Therefore, for unique surface designs, it is required to examine the cellular response. In this thesis the in order to evaluate cellular response, L929 cells were employed.

There have been several in vitro studies conducted to examine cell to Ti surface interaction, cell response to various modification with the employment of MSCs, os-

teoblasts, fibroblasts [25]. However, the results of the in vitro studies may not comply completely with the in vivo studies. This is because biological dynamics in in vivo significantly different from the in vitro experimental set up, due to its multidimensionality in the reactions [26]. To exemplify when the cellular response of osteoblast cells to topographical changes of Ti is examined in vitro, this will be the result of solely interactions between Ti surface and osteoblast.

However, when this examination is held in in vivo then the osteogenic cells will not be in direct contact with Ti. Instead, the blood cells will be in direct contact with material to form blood clot in the wound site. The cells will be interacting with blood-modified Ti surface [27].

Therefore, in this research the blood compatibility is also studied by examining platelet adherence on the material and hemolysis rate on different surfaces.

The necessity of processing the material comes with the idea of advancing strategies to add different biological features to the originally inert material while employing suitable mechanical property. Even though Titanium and Titanium alloys have several advantages, there are certain topics to examine and improve regarding the use of these materials to engineer implant and biomedical devices. One of those topics is the biological complications which may involve inflammatory factors because of bacterial invasion on the surface of the material.

Since the Ti has no intrinsic antibacterial activity, various surface modification strategies including coating with antibacterial metals, have been applied to endow antibacterial property of the material [28]. There are several approaches in this regard employing, inorganic antiseptics, loading of biochemical chemical molecules to the surface and altering surface topography [29]. Considering the accelerated elevation in antibiotic resistance, the physical strategies have started to draw more attention compared to biochemical strategies. Another downside of using natural or designated antibacterial agents is that the addition of those molecules not only affect bacterial cells but also affect host cells. That effect may compromise the biocompatibility, hemocom-

patibility or osteogenic property of the material [12]. It has been shown that tailored nano topography on the Ti surface changed the response of *Staphylococcus aureus* which is known to be relevant for orthopedic and other medical device related infections, *Escheria coli* and other bacteria such as *S. aureus*, Streptococcus mutants in a favorable way [30]

On this basis, in this thesis the chemical and physical characteristics of the laser processed surfaces are analyzed. The topography-dependent inhibitory effect on bacterial adhesion, the influence of topographies with different roughness on cellular attachment and metabolism of fibroblast cells, the blood material interactions are investigated. The results are compared with the literature studies investigating the topography effect on surface characteristics and the interaction between cells, blood, and bacteria with the employment of different metallic and non-metallic biomaterials.

1.2 Objectives

Although the general properties of Ti are well known and it is a biomaterial that is being used for several decades, the effects of surface topography and roughness changes on hemocompatibility and bacterial behaviour remained unclear. In the literature, it is given that by only changing surface pattern and surface roughness, the cellular responses change [8, 14]. It is not mandatory to provide chemical change on material for different cellular responses such as cell adherence, cell proliferation, expressed genes, activated pathways, released cytokines and chemokines. Although titanium (Ti) is convenient to be used in biomedical implants because of its cytocompatibility in addition to its ideal physical characteristics as mentioned earlier, the surface alterations will alter cellular behavior. Therefore, the modified surface require investigation of hemacompability and bacterial behavior. As explained in more detail, for the surface modification, laser processing method is chosen specifically since it enables to alter the surface in a controlled way.

So, the results of this thesis will help to fill the critical void in our understanding

of the link between surface topography and the reaction blood cells and bacteria. In this way we will be able to reveal dynamics of that relation and utilize the knowledge in designing biomaterials. In addition, we will be able to manipulate better cellular responses to the biomaterials.

Our main goal in this thesis is examining the alterations of biocompatibility, hemacompatibility and bacterial behaviour with respect to change in topography and roughness of laser processed Titanium surfaces.
he objectives to achieve main goals of the study will be as following;
1. Use laser processing to develop Ti surfaces that will differ in topography and roughness promoting biocompatibility, hemacompatibility.
2. To examine the surface modification in terms of morphological and chemical characterizations.
3. To evaluate biocompatibility of the surface with L929 in terms of adherence proliferation and viability and to evaluate effect of topographical change on cellular behaviour with respect to BS EN ISO 10993-5.
4. To examine effects of surface topography on hemacompatibility with respect to BS EN ISO 10993-4.
5. To conduct live infection model with S. aureus and E. coli test viability and biofilm formation.

2. BACKGROUND

2.1 From Biomaterials to Bio-Instructive Materials

Traditionally, until 1990s the expectation from biomaterials was to be able to replace damaged tissue in long term. However, with the advancement in tissue engineering this perspective changed into more instructive expectations in terms of manipulating biological responses to the material. Biomaterials now are expected to be more interactive with the host tissue, specifically implants are being designed to promote the desired biological responses in every scale, rather than just to be replacement [31].

In the earlier generations of biomaterials, the primary concern was to obtain bioinert materials to diminish immune response. As the field evolved, the bio-instructive biomaterials gained interest to elicit specific biological functions. It is acknowledged that cells respond diversely to physical, mechanical, and chemical stimuli within their micro niche [32]. This specific awareness rises the questions on acquisition of the best strategy for designing the biomaterial to achieve the most desirable cellular response.

In terms of physical aspects, it is essential to evaluate the extracellular matrix (ECM), which is a dynamic, three-dimensional scaffold for the cellular constituents. The cytoskeleton of the cells is connected to ECM via integrin adhesion receptors [33]. The behavior and function of the cells are altered with the changes in constituents and physico-dynamics of ECM. It was shown in the literature that not only the migration of specific cell types depends on the physical properties of underlying matrix but also the differentiation of stem cells is significantly affected by these properties [34]. Besides affecting the stem cell fate, mechanical feature also alters cell proliferation, self-renewal and even senescence [35]. To this respect, various approaches have been employed to manipulate different lines of cells in desirable ways by altering stiffness, roughness, and elasticity. Nevertheless, there is not only one golden ratio for those properties to provide desired differentiation, proliferation, and spread of cells due to variations of natural

ECM. The architecture of ECM is specific to tissue where it locates, for instance natural and desirable micro-niche for cortical bone is dense whereas the ECM of trabecular bone is more porous. Therefore, for planning physical alterations to design biomaterials, it is important to consider the natural tissue structure and the environment that the biomaterial will be interact within [36]. Designing electroconductive biomaterials is another way to guide cellular behavior. Although pathways enabling the phenomena is poorly understood, electrical stimulation is an effective way to control stem cell fate [37]. There are many strategies are being utilized to design electroconductive biomaterials via manipulations in charge density and wettability of the material [38].

In terms of biochemical aspects, there are many strategies employed for the delivery of specific signal molecules such as Bone Morphogenic Protein 2 (BMP-2) and other members of transforming growth factor-β superfamily (TGF-β superfamily) which are known to enhance bone and cartilage formation are administered on biomaterials to accelerate tissue regeneration and stem cell differentiation in musculoskeletal tissue [39, 40]. The molecules can be embedded on the polymeric biomaterials or can be immobilized onto the surface of biomaterials.

In addition to protein administration, gene delivery is an alternative way to manipulate cell fate. It is possible to incorporate viral and non-viral gene delivery and RNA delivery to enhance regeneration in tissue of interest [41].

Although, improving regeneration and controlling cellular fate intensified, the modulation of immune response has never been marginalized so far. Therefore, more and more new techniques have been utilized in this respect. For instance, protein delivery techniques are being employed to deliver cytokines to reduce immunological rejection. It has been observed that the cytokines are able to change macrophage phenotype and affect mesenchymal stem cells and osteoprogenitor cells. Therefore, there have been numerous research studies conducted to resolve the dynamics and interactions in between cytokines to different cell types in addition to cell-to-cell interactions after the cells are exposed to cytokines [42, 43].

There are many aspects of designing a bio-instructive material. As it is possible and practiced controlling the behavior eukaryotic cells, it is also possible to control behavior of prokaryotic cells which consist of infection causing microorganisms. Infection is one of the main implant failure reasons that has never been eliminated from the equation. There are various approaches to diminish the micro organismal bioactivity. Bacteria are responsive to biomaterial surface topography and the electroconductivity as cells. Biomaterials are also functionalized with antimicrobial peptides (AMPs), silver and gold nanoparticles, and antibiotics to avoid microbial colonization [44].

Although the technical strategies to functionalize biomaterials are increasing consistently, each one of those strategies have their very own limitations. They are not suitable to be used in every material, some are applicable in polymers (Polydimethylsiloxane (PDMS), chitosan, Polylactic acid (PLA) etc.) some are applicable in metallic biomaterials (Titanium and Titanium-based alloys (Ti-6Al-4V), stainless steel (316L), tantalum, cobalt chromium alloys (CoCrMo) etc.) and some are applicable in bio ceramics (Calcium phosphates (CaPs), Calcium phosphates cements (CPCs), Hydroxyapatite (HA) etc.). The biomaterials have intrinsic properties due to their bulk material as their physical and chemical properties due to their surface. Therefore, both bulk material and the modification are significantly affecting the biological system and must be chosen in accordance with the tissue in which biomaterial will be interacting with

2.2 Titanium and Titanium Alloys as Biomaterial

The type of biomaterial should be chosen properly, depending on aim of medical application. For the orthopedic musculoskeletal applications metallic biomaterials have been used since 19th century. Metallic implants become reasonable and popular choice in bone repair due to their superior physical properties such as high tensile strength. Having close Youngâs modulus to the ceramics while having acceptable fatigue life; the greatest advantage that the metals provide as biomaterial is being adjustable. This means, their wear rate, corrosion resistance, fatigue life, other mechanical properties and even their biocompatibility can be altered and improved by forming alloy [45].

Titanium is one of those metals which gives opportunity to form alloy with different elements of choice in various combinations and attain or improve certain properties. Titanium changes its crystallographic form depending on temperature and microstructure that significantly affect mechanical properties. Titanium alloys can be examined under the following categories: α alloys, near α alloys, α and β alloys, near β alloys, and β alloys. Al, V, Nb, Mo, Zr, and Ta are the mainly used alloying elements for titanium. Each category and alloying elements alter the characteristics of the material [45]. Titanium and Titanium alloys are extensively used biomaterials in several biomedical applications because of their excellent combination of biocompatibility, corrosion resistance, fatigue strength, low modulus, light weight, being modifiable both in surface with various methods and in bulk material via element compositions [45]. These characteristics provide unique advantages to Ti and Ti alloys to be used in dental implants, artificial joints, bone screw, cardiovascular stents, lead wires and spinal trauma fixation devices etc [46, 47]. As the area of utilization enhanced for Ti in the biomedical field, the idea of modifying the material, gained specific attention within the last few decades.

2.3 The Topography of Titanium

There are many Ti alloys that have been employed in the research studies to improve performance of the material from both physical and biological perspective.

In addition to the studies on the bulk material composition, the surface enhancement strategies substantially advanced as the material surface to cell interaction dynamics have been illuminated. The accumulation of knowledge regarding response of wide range of protein, blood composition, endothelial cells, mesenchymal stem cells, osteoblasts, and various bacteria strongly depend on the material surface, has significantly accelerated [30, 48, 49]. This fact is valid but not limited to Ti and Ti alloy-based biomaterials, there are many bioinspired surface topography that are being employed to modify material surfaces such as shark skin, bone surface, lotus leaf, inner surface

of blood vessels etc [50, 51].

Although, it is possible to form various kinds of patterns with the use of laser, bioinspired structures are preferred due to being naturally suitable for those cells to adhere and interact with. Since the Titanium implants are mostly used in orthopedic surgeries they are supposed to be integrated with the bone tissue. When this fact is considered with advantages of bioinspired surfaces, several strategies of modifying Ti surface in a way to converge bone surface characteristics, has risen.

In that aspect; biochemical, chemical and physical modifications are applicable. From the biochemical modification perspectives, surface can gain antibiotic property by depositing antibiotic agents or cellular response in the means of osteogenesis can be enhanced via immobilization of collagen, various peptides or bone morphogenic proteins etc [52, 53, 54]. Furthermore, Ti material surface can be enriched with deposition of different nanoparticles to gain antibacterial property and upgrade material to tissue integration [43]. Despite the fact that many of the proposed nanoparticles or biomolecules regardless of being designed or natural, enhance cellular response in terms of adherence, proliferation, and differentiation in addition to diminish bacterial activity; there are certain drawbacks for clinical use of this methodologies. This is due to complications to project and control long term release of the bound molecules. Those complications prompt the researchers to examine the effect and utilization of physical modifications on the material surface. It is known that physical characteristics of the material surface affect adsorption of proteins, precipitation of minerals, cellular behavior of both mammalian cells and bacteria [55]. In the context of alterations in cellular behavior, the material surface topography influences the process both direct via cell to material interaction and indirect via deposited extra-cellular matrix proteins and minerals [56]. Various research prove that by only changing material surface topography the cellular morphology, functionalization, differentiation, bacterial adhesion can be controlled [57, 58, 59]. Considering the impact of surface topography, although there are several available methods to process and modify the Ti based materials, it is important to use one that is not causing any remaining residue on the surface of the material because of above mentioned properties may be affected beside possible inter-

ruption of interaction between cells and implant surface specificall [48]. Laser provides repeatable and more precise results than other processing techniques such as micro-Arc oxidation or anodization [49].

Studies that utilize micro-Arc oxidation to process Titanium surface and form porous structure show that it is possible to adjust pore size by changing exposure time. However, neither the pores can be distributed through surface homogeneously nor the pore size can be adjusted specifically, the size range is quite wide such as from 4 nm to 14 nm for one experimental group [50].

For the anodization technique, it is possible to arrange size of nanotubes with the changes in applied voltage and exposure time. However, the similar problems stand such as having 10 nm range rather than being specific. Also, both techniques are not as convenient as laser to create patterns with specific and precise shape.
In this perspective, laser-processing rises as a good option to make modification on the surface of Ti based materials. Also, one other very important advantage of laser is being able to process large scale in a short period of time [60].

Laser processing is a widely used method due to its capability to progress accurately and rapidly on both surfaces of organic and inorganic materials. The fundamental advantages of lasers are controlling the frequency of the light, having wide range of frequencies, being able to focus on narrow areas, having high energy density, controlling reaction time. Laser treatments are pulsed from picoseconds to 100 nanoseconds pulse times and continuous wave that have interaction duration less than 1 microsecond [61].

Working principle of laser processing is grounded on focusing an intense laser beam on a specific area where the laser beam evaporates the material. The schematic representation of laser processing of Ti is shown in Figure 2.1. Adjustment on the resolution of structures can be achieved by controlling the pulses. Short pulses create higher spatial resolutions and increased accuracy of control [62] Micro-seconds laser pulses are shown to be adjustable in the high ablation rate while compromising mini-

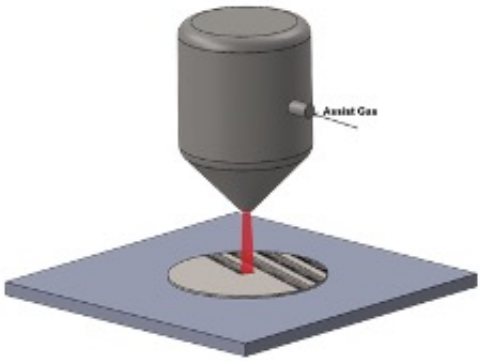


Figure 2.1 Schematic representation of laser surface processing.

mum from quality of micro-processing [62]. Types and specifities of laser processing on Titanium surface examined in many research including excimer and Nd:YAG [63, 64].

When the Nd:YAG laser processing is used on Ti surface with various adjustment including different wavelength, pulse duration and energy density of laser beam, it was shown that energy absorbed by material from laser beam is partially converted to thermal energy. This results in adverse reactions on irradiated area, including cracking and periodic microstructures and nanostructures on near and farther periphery, respectively [64].

Morphological observations on laser processed Ti surfaces indicates that the alteration on surface mainly depends on energy density, power density of peak, duration and number of pulses and wavelength [64]. These interactions are visualized and represented in Figure 2.2. As seen in the Figure 2.2. long pulse laser beam cause a damage on the surface and since the energy of the beam is partially converted in the heat energy, heat affected zone is formed in the processed material. The heat results in melting zone and micro cracking. These side effects can be overcome with the use of ultrashort pulse laser beam which can give more precise results for the laser treatment.

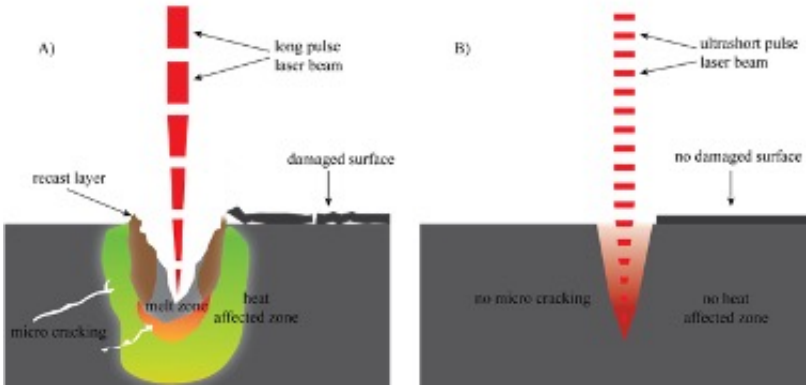


Figure 2.2 The schematic representation of the effect of A) long pulse laser beam and B) ultrashort pulse laser beam.

2.4 Effects of Titanium Topography on Cellular Behavior

Surface characteristics such as roughness, wettability, and surface energy are known to effect cell response to material as already mentioned above [51, 65]. These characteristics affect not only cellular behavior and protein adsorption on the surface but also they affect each other as a chain reaction. The change of the surface topography alters surface free energy and wettability of the surface. The schematic representation of the interactions are given in Figure 2.3. The variations of surface free energy and wettability affect the chemical forces in between material and biochemical molecules including proteins in surrounding host tissue. When the proteins are adhered on the surface, they form a layer and interact with the membrane proteins of the host cells [12].

The effect of surface characteristics on cellular behavior involve but not limited to derivatives of mesenchymal stem cells, osteoblasts, endothelial cells, and immune cells since cells are responsive against physical stimuli. The main regulator of the interactions in between cells and material surfaces are the integrin adhesion receptors. Integrins are found in the form of heterodimers consisting of α and β subunits which are able to selectively bind to specific amino acid sequences [66]. Integrins significantly affect host response to the material since they have various important roles in the path-

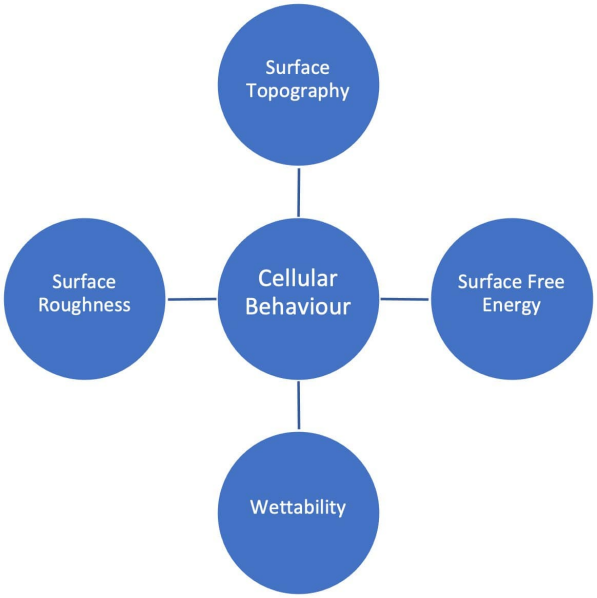


Figure 2.3 The schematic representation of the surface characteristics affecting cellular behaviour.

ways controlling development, regeneration, gene expression, cell survival, migration and differentiation of cells. Integrins are capable of regulating the cellular response in many ways since there are many types of integrins which bind to different molecules and activate various cascades controlling from blood coagulation to macrophage activation [67].

Surface of Ti can trigger pro- and anti-inflammatory immune response [52, 53, 45, 68, 69]. On this basis, desired properties of an implant are increasing osteogenic differentiation of osteoblast, increasing ALP activity, enhancing the interaction between surface and cells, creating anti-inflammatory response rather than pro-inflammatory response in terms of immune cell phenotype differentiation, especially macrophages, and cytokine releases. In the aspect of topographical effect on cellular response, porous structures are better than flat surfaces in terms of having more surface area to interact with the cells leading to adherence. Pore size also affects osteogenesis and cell adherence, and it has been observed that porous structures are better to create pro-inflammatory response since cellular interaction between macrophages will be lowered leading to suppression of E-cadherin expression which results in β-catenin release from membrane to cytoplasm and eventually inducing anti-inflammatory (M2) phenotype of

macrophages [60, 70]. Surface wettability is another significant factor in cell to surface interactions, higher surface wettability gives rise to enhanced interaction. Additionally, the titanium-oxide film formation on the surface of the material is important for better osseointegration, also provides corrosion resistance to the implant by protecting it against the biological fluids [69, 71]. All of those interactions are combined and lead to a better tissue material integration as a result.

2.5 Effects of Titanium Topography on Bacterial Behavior

Although Ti has unique combination of desired characteristics for biomedical use, it has no intrinsic antibacterial property. Therefore, this is one of the main objectives to improve. Researchers are conducting several studies with different methodologies as mentioned while explaining biochemical and chemical modifications in section 2.1. Surface characteristics also affect bacterial response to the material, and more importantly this bacterial response affects cellular adherence to the implant. Eukaryotic cells which are the host cells for implantation case always compete with prokaryotic cells are the bacteria that may cause infection, in order to achieve colonization on the surface. Bacteria has specific advantages on this regard, being motile and being able to form biofilm [72]. In order to overcome this complication, several strategies have been employed including deposition of antibacterial agents onto the surface of the material [25]. However, this approach most likely to bring new obstacles in terms of the effect of antibacterial agents on different cell types which means potential side effects [73, 74].

Therefore, developing surface topography model which will diminish bacterial adhesion and proliferation while enhancing cellular adherence and tissue integration concept gained more attention. There are many suggested surface topography models that prevent antibacterial adhesion and cause decrease in the bacterial proliferation [75, 76, 77]. Especially nanotopographical surfaces are promising, nanoflower texture which is obtained by Hydrothermal treatment of etched titanium technique shows antibacterial property even against methicillin resistant *S. aureus* [76]. Additionally,

nanosheet formation on the surface again has been proven to provide antibacterial property to the material and bacterial adhesion is affected by the width of the nanotopographical structures [77].

Biofilms are heterogenous structures including bacterial cells and their secretion of exopolysaccharides rich extracellular matrix that protect the bacteria from host immune system [78]. Biofilms are harder to eliminate in comparison to bacteria in planktonic state and commonly seen in implant infections by *Staphylococcus aureus* [79]. Biofilms can be both antibiotic resistance and antibiotic tolerant. Antibiotic resistance is usually gained by horizontal gene transfer or mutation and it indicates rise in the minimum inhibitory concentration of antibiotic. Antibiotic tolerance, on the other hand, refers to ability of bacteria to survive in the presence of antibiotics by altering their phenotypic state [80]. Although the mechanism of biofilm to gain antibiotic resistance, is not fully illuminated, it is thought that many mechanisms interact together. This hypothesis also supported by the fact that biofilms are heterogenous structures and not necessarily formed by physiologically identical cells, there are usually different subpopulations within biofilm [81]. Antibiotic tolerance depends on extracellular matrix, slow rate of growth, preventing the antibiotics to penetrate biofilms and altering metabolism. Antibiotic resistance of the biofilm depends on matrix structure, the features of subpopulations in biofilm, mutation and gene transfer [80]. *E. coli* is a gram negative bacterium and known to one of the most common infection causing bacteria. Gram negative bacteria have thinner peptidoglycan layer compared to gram positive bacteria. However, they have a protective outer shell. *S. aureus* is a gram positive bacteria with thick peptidoglycan cell wall. [79].

2.6 Effects of Titanium Topography on Hemocompatibility

Titanium is commonly used in implantation and therefore it is known as blood-contacting biomaterial. As already mentioned above, immune cells reside in blood composition are affected by the surface topography of Ti and their genetic expression profile changes this results in the phenotype change of the immune cells. That phe-

notypically altered immune cells interact and affect differentiation of bone precursor cells [60, 70]. In addition to this significant cascade, the first blood contact is very important for the biological response. This is because, when the biomaterial first come in contact with the blood, blood proteins will be adsorbed onto the surface and govern further biological response [70]. Difference in surface topography affects platelet adherence and it may cause hemolysis [82, 83].

3. MATERIALS AND METHODS

3.1 Preparation of Titanium Substrates

1 mm thick Titanium plates (CP â GR2) were polished with silicon carbide polishing papers (P600, P1000, P2000, P2500) and alumina powders (1 mm and 5 mm). Then samples were cut to in circles (15 mm of diameter) with modulated microsecond pulsed fiber laser (PALSS laser, 400K) operating at 1080 nm.

3.2 Surface Modifications

Ti samples are cleaned with absolute ethanol. Then the surfaces were treated with laser to produce 70 mm of spot diameter over the surface and samples were scanned in both x and y axis with the speed of 5 mm/s. During the process, pulse length is fixed to 50 ms, operation frequency was fixed to 4 kHz and peak power was set to 95.6 W. (PALSS laser, 400K) One set of Ti specimen were kept as polished and one other set of Ti specimen was sandblasted. The laser treatment were processed in order to obtain two distinct topographies. Line structured Ti specimens were processed to be homogenous surfaces having 18.8 μm width and 1 μm length hemicycle-like patterns. On the other hand mesh structured specimens have composite surface texture since there are regions on their surfaces where laser didnât process, where laser processed once and where laser processed twice. The schematic representations of the surface topographies are given in Figure 3.1.

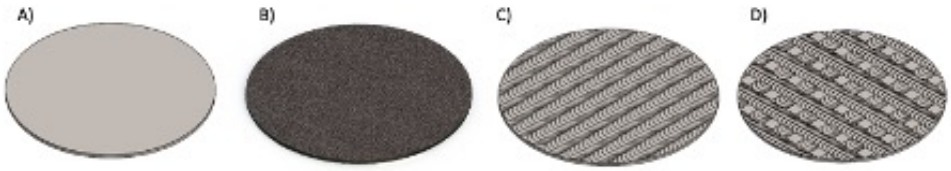


Figure 3.1 Schematic representation of Ti specimens with A) Polished, B) Sandblasted, C) Line structured and D) Mesh structured surfaces.

3.3 Characterization of the Titanium Surface

3.3.1 Investigation of Ti Surface Topography

Surface morphology and structures on polished, sandblasted, line and mesh laser structured Ti specimens were examined with the use of Scanning Electron Microscopy (SEM) (GAIA 3, Tescan, Czech Republic). Through imaging the settings will be; 4.99 mm of working distance. The images were taken under the magnifications 100x, 500x, 5000x, 10000x [84].

3.3.2 Determination of Hydrophilicity of Ti Surface

In order to assess wettability of the specimens water contact angles were analyzed for polished, sandblasted, line and mesh laser structured Ti specimens. Water Contact Angles of polished, sandblasted, line and mesh laser structured Ti specimens were measured with the use of Contact Angle Measurement Instrument (Iolin Scientific, Attension Theta, Finland) 10 μL of deionized water droplets are dropped onto the surface of each group and angle was measured (n=6) [58].

3.4 Evaluation of Biocompatibility of the Material

3.4.1 Cell Culture Studies

Mouse fibroblast cells L929 (American Type of Culture Collection (ATCC), CCL 1) (passage number 9) is employed in this thesis to evaluate biocompatibility. Polished, sandblasted, line and mesh laser structured Ti specimens were cultured with complete DMEM medium containing 10% FBS and 1% penicillin-streptomycin at 37 °C, 5% CO_2 [84].

3.4.2 MTT Cytotoxicity Assay

In order to assess cell viability, MTT assay was performed on the polished, sandblasted, line and mesh surfaces. The Ti samples were sterilized with 70% EtOH and 30 minutes of UV treatment. L929 cells were cultivated on each sample group as $1x10^4$ cells per Ti disk. MTT assay was performed on 24 h. The medium was aspirated and 5 mg/mL MTT solution added onto the wells and incubated for 3 hours. After the incubation 1 mL of DMSO was added onto the wells and OD was measured at 570 nm and 750 nm wavelength with the use of microplate reader (iMark Microplate Absorbance Reader, BioRAD Laboratories, CA, US) [84].

3.4.3 Investigation of Cellular Morphology

L929 Fibroblast cells ($1x10^4$) were seeded on polished, sandblasted, line and mesh structured Ti specimens in 24 well plates. In order to examine cellular attachment the cells were fixed with 4% Glutaraldehyde at 24 h. After that the glutaraldehyde solution was discarded and PBS was added to the wells prior to gradual dehydration with the use of 30%, 50%, 75%, 90%, 100% ethanol for 15 minutes of incubation each. The SEM images were taken with the use of the same set up 4.99 mm of working distance under 100x, 500x, 5000x, 10000x magnifications.

3.5 Investigation of Bacterial Behavior on the Material Surface

3.5.1 Analysis of Biofilm Formation on Ti Surface

3.5.1.1 Qualitative Examination of Biofilm Formation.

Bacteria *S. Aureus* ATCC6538 and E.coli were grown in LB medium overnight and resuspended at concentration of 1x10^7 cells/mL. 1 mL of bacteria suspension was added on each 24 well-plate containing Ti substrates. Three different plates were prepared as described for different incubation periods which are 30 minutes, 4 hours and 24 hours at 37 ï°C. Once the incubation duration was completed, supernatant were discarded and samples were washed with sterile PBS twice. The samples were fixed with 2.5% glutaraldehyde aqueous solution for 30 minutes in the dark.
After that the glutaraldehyde solution was discarded and PBS was added to the wells prior to gradual dehydration with the use of 30%, 50%, 75%, 90%, 100% ethanol for 15 minutes of incubation each. The SEM images were taken with the same set up of 4.99 mm of working distance under 100x, 500x, 5000x, 10000x magnifications.

3.5.1.2 Quantitative Examination of Biofilm Formation.

To measure metabolic activity of biofilm on Ti surfaces, the same culture conditions and protocols that are used in Qualitative Examination of Biofilm Formation (section 3.5.1.1.) is followed for incubation, only for 24 hours. When the incubation is completed, 5mg/mL MTT solution is prepared in sterile PBS. After the medium is aspirated the MTT solution is added into the wells and plate is incubated in 37 °C for 2.5 hours in dark. After the incubation 1 mL of DMSO added onto each well and solutions were transferred into the 96-well plate. OD is measured at 570 nm with the use of microplate reader (iMark Microplate Absorbance Reader, BioRAD Laboratories, CA, US) [83].

3.5.2 Examination of Hemacompatibility of the Material

For the following experiments ethics committee approval is taken from Bogazici University Sciences and Engineering Research Ethics Committee. E-84391427-050.01.04-1048

3.5.2.1 Analysis of Hemolysis Rate.

Whole blood is centrifuged at 1500 rpm and supernatant is removed. The precipitated red blood cells are washed with PBS. The red blood cells (RBC) are mixed with PBS to obtain 2.0% suspension. The suspension was incubated on the material for 1 hour at 37 °C. After the incubation the plate was centrifuged at 3000 rpm for 5 minutes and solutions were transferred into the 96-well plate. OD of supernatant is determined at 450 nm with the microplate reader (iMark Microplate Absorbance Reader, BioRAD Laboratories, CA, US) [83]. For the OD measurements;
Positive control is prepared as 2.0% RBC in ultra pure water.
Negative control is prepared as 2.0% RBC in PBS.
Hemolysis ratio is calculated in accordance with Eq. 3.1,

$$\frac{Absorbance\,of\,Experimental\,Group - Absorbance\,of\,Negative\,Control}{Absorbance\,of\,Positive\,Control - Absorbance\,of\,Negative\,Control} \times 100 \quad (3.1)$$

3.5.2.2 Platelet Adhesion.

20 mL of whole human blood was collected in sterile tube and centrifuged at 1000 g for 10 minutes. The RBC precipitated and upper layer was transferred into a new sterile tube, the platelets were mixed with the serum. 200 uL of platelet rich plasma is dropped on each sample and incubated for 2 hours at 37 °C, 5% CO2. Then the samples are washed with PBS and adherent platelets are fixed with 2.5% glutaraldehyde. After that the glutaraldehyde solution was discarded and PBS was added to the wells prior to gradual dehydration with the use of 30%, 50%, 75%, 90%,

100% ethanol for 15 minutes of incubation each and SEM (GAIA 3, Tescan Czech Republic) images were taken [83].

4. RESULTS

4.1 Material Characterization

4.1.1 Morphology of Titanium Surface Topography

Images of polished, sandblasted, line and mesh structured Ti specimens were taken with SEM to examine topography. The images are represented in Figure 4.1 Polished surfaces are the smooth surfaces lacking grooves, peaks and other kinds of irregularities on the surface. Sandblasted surfaces show the highest roughness having abundant micro-grooves without an ordered pattern. Line structured surfaces are also rough surfaces and they represent a regular pattern provided by laser processing. Lastly, mesh structured surfaces represent more complex surface structure since they contain three different regions; regions that are not processed by laser and stayed as polished, regions that are processed by laser once therefore showing characteristics of line structured specimens and regions that are processed by laser twice, perpendicularly.

4.1.2 Water Contact Angle Measurements

In order to assess wettability of the surfaces water contact angles of Ti surfaces were measured. Water contact angles for Polished, Sand Blasted, Line and Mesh structured titanium surfaces were measured and the results are represented in Figure 4.2 The images are given in Figure 4.2 A - D and the comparable data is represented in Figure 4.2.E with standard deviations. Results show that the sandblasted and line structured specimens have the lowest water contact angle which are 82.4 ± 5.7 and 83.7 ± 3.4 respectively. Water contact angle of polished and mesh structured surfaces, which are 86.6 ± 4.7 and 87.9 ± 4.8 respectively, are significantly higher than water contact angles of sandblasted and line structured groups.

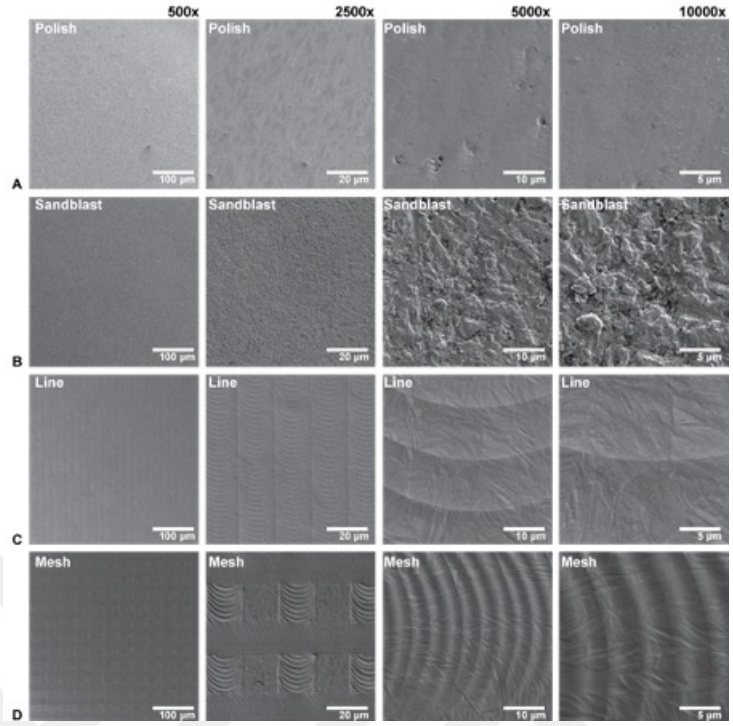


Figure 4.1 SEM images of A) Polished, B) Sandblasted, C) Line and D) Mesh structured surfaces.

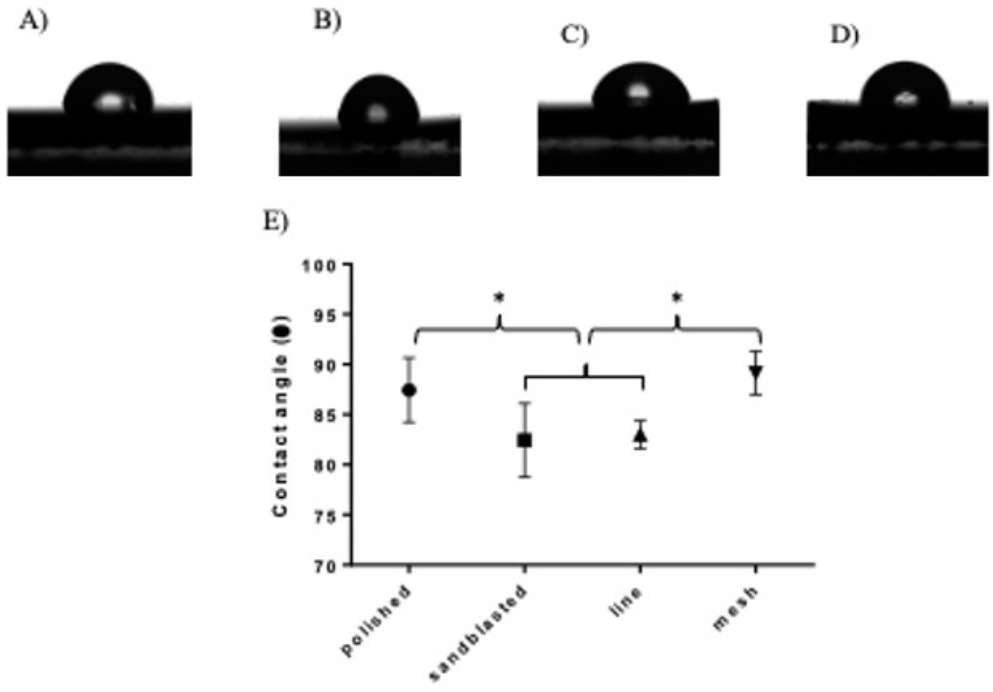


Figure 4.2 Water contact angle images of Ti substrates which were processed with different treatments. (A) Polished, (B) Sand Blasted, (C) Line Structured, (D) Mesh Structured. The results of the water contact angle measurements are given with standart deviations (E) (*$p<0.05$, $n=6$).

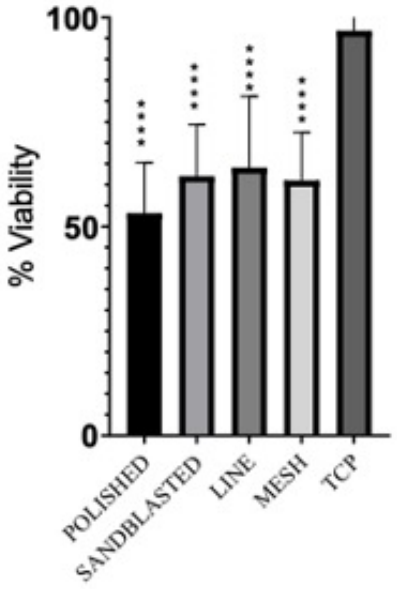


Figure 4.3 MTT cytotoxity assay with L929 cell line on polished, sandblasted, line and mesh structured surfaces and TCP as control at 24 h. Significant differences are shown on the graph (****: $p<0.0001$ comparison in between TCP and Ti groups).

4.2 Evaluation of Biocompatibility

4.2.1 MTT Cytotoxicity Assay

The effect of surface topography to biocompatibility of the Ti specimens with polished, sandblasted, line and mesh structured surfaces were investigated by employing MTT cytotoxicity assay. L929 cells were seeded on the samples in 24-well plates and incubated for 24 hours in appropriate conditions described in section 3.4. Results are represented in Figure 4.3. L929 viability is shown to be higher on TCP compared to Ti specimens with polished, sandblasted, line and mesh structured surfaces; whereas no significant difference on viability of cells is observed in between Ti groups. The viability percentage on polished surface is 53.2 ± 12.0, sandblasted surface is 62.1 ± 12.3, line structured surface is 64.1 ± 16.9 and mesh structured surface is 61.0 ± 11.5.

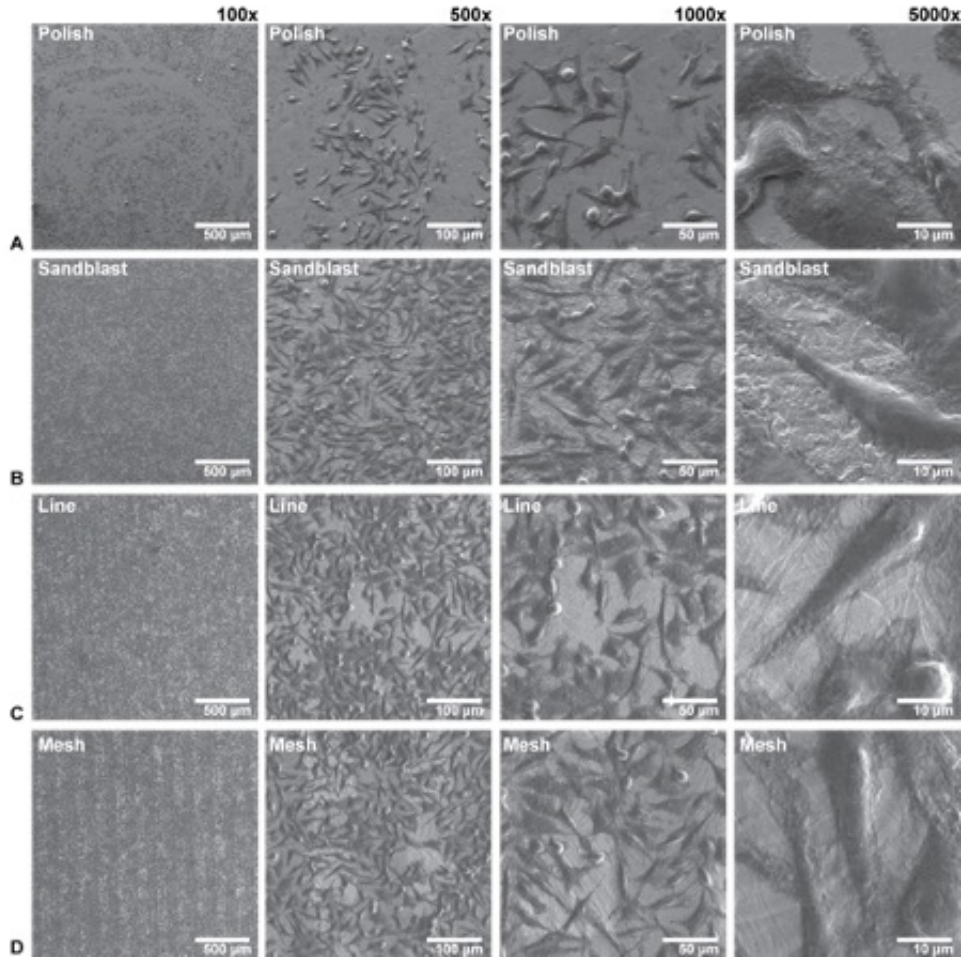


Figure 4.4 SEM images of the L929 cells on A) Polished, B) Sandblasted, C) Line and D) Mesh structured surfaces.

4.2.2 Evaluation of Cellular Morphology

In order to evaluate cell to material and cell to cell interaction on different topographies, the images were taken via SEM on each topography. The adherence of L929 cells were observed at 24 h, images are represented in Figure 4.4. As seen in Figure 4.4, although there are abundant L929 cells on each of the surfaces, higher number of cells is observed on sandblasted, line and mesh structured surfaces in comparison to polished surface.

4.3 Evaluation of Bacterial Response to the Topography

4.3.1 Examination of Metabolic Activity of Biofilm

To assess the relation in between metabolismic response of gram negative and gram positive bacteria to different topographies and roughness both *E. coli* and *S. aureus* bacteria were cultivated on Ti disks for 24 h in accordance with the culture conditions which are described in section 3.5. After 24 h of incubation, the MTT assay was employed to quantify metabolic activity and the results for *E. coli* and *S. aureus* are represented in Figure 4.5. and Figure 4.6 respectively. As shown in Figure 4.5 the highest metabolic activity of *E. coli* is observed on line structured surfaces with 155.3 ± 21.3 and followed by sandblasted surface with 131.6 ± 24.0 and mesh structured surfaces 130.7 ± 18.3. Among Ti groups metabolic activity of *E. coli* is shown to be the lowest on polished surfaces with 88.4 ± 14.3. Also, there is no significant difference observed in between polished Ti and TCP groups. Figure 4.6 represents the metabolic activity of *S. aureus*, the highest activity is observed on polished surfaces with 116 percent. Although the metabolic activity of *S. aureus* on polished surfaces are significantly higher than all the other groups, no significant difference is observed in between the remaning groups; sandblasted which has 99.4 ± 8.7, line 89.0 ± 11.2 and mesh structured Ti surfaces which has 97.3 ± 11.4 and TCP.

4.3.2 Qualitative Observation of Bacterial Adhesion on Ti

In order to observe bacterial adhesion on different topographies both *E. coli* and *S. aureus* bacteria were cultivated with the same culture conditions as explained in section 3.5. The bacteria were fixed at 24 h and images were taken via SEM. The images for *E. coli* and *S. aureus* are represented in Figure 4.7 and 4.8, respectively.

According to SEM images on Figure 4.7, *E. coli* adhesion is observed on all 4 specimens. However, on sandblasted surfaces more bacterial aggregations and coloniza-

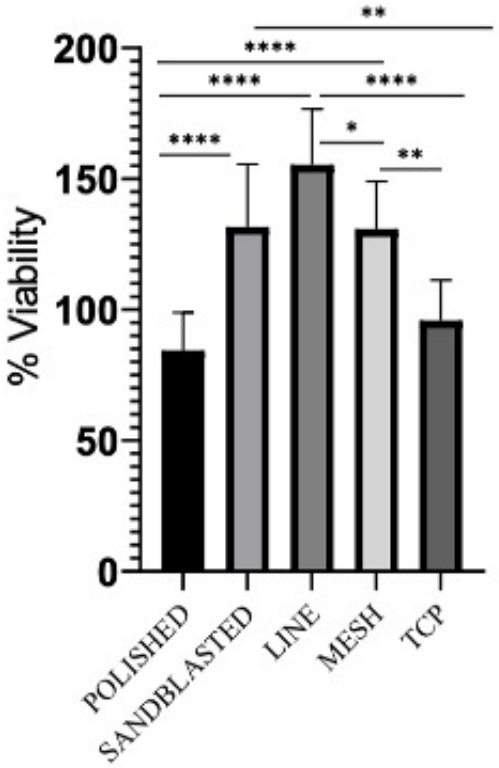


Figure 4.5 Assessment of viability of *E. coli* bacteria via MTT assay on polished, sandblasted, line and mesh structured surface topographies and TCP (**** $p < 0.0001$ in comparison with polished and Ti surfaces, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ and n=3).

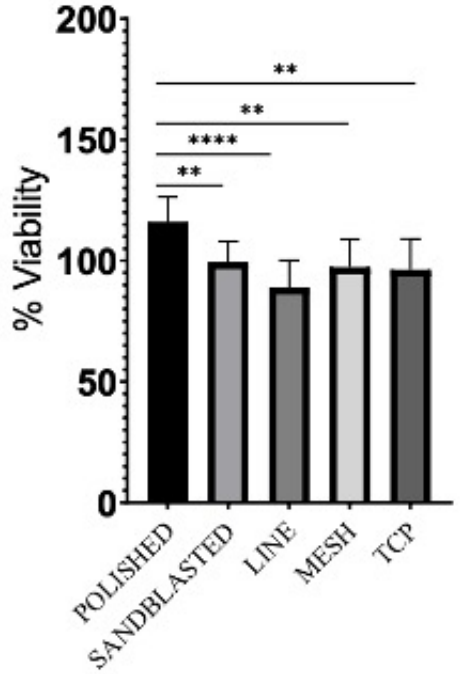


Figure 4.6 Assessment of viability of *S. aureus* bacteria via MTT assay on polished, sandblasted, line and mesh structured surface topographies and TCP (**** $p < 0.0001$ in comparison with polished and Ti surfaces, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ and n=3).

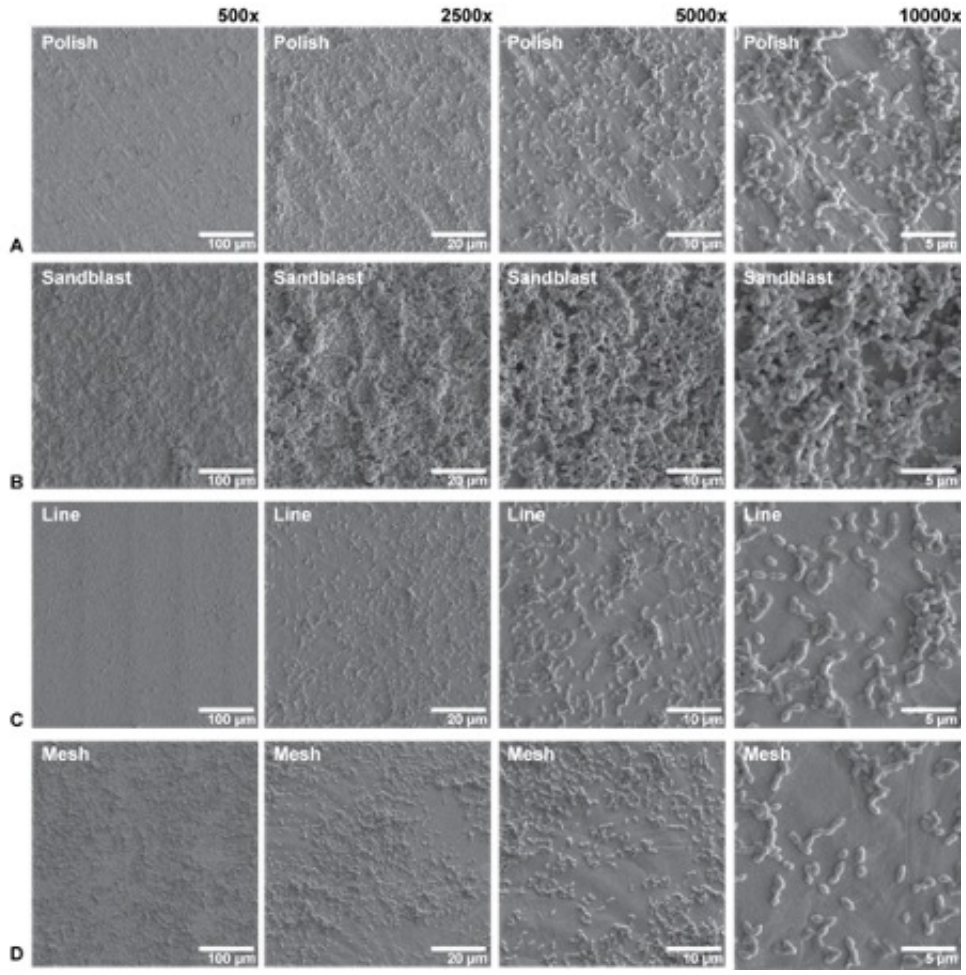


Figure 4.7 SEM images of the *E. coli* on A) Polished, B) Sandblasted, C) Line and D) Mesh structured surfaces.

tions are observed in comparison to polished, line and mesh structured surfaces. As seen in Figure 4.8, more aggregation and multi-layered adhesion of *S. aureus* is more obviously observed on polished surfaces. Although, *S. aureus* successfully adhered on all 4 surfaces, line structured surface represents significantly less colonization when compared to polished, sandblasted and mesh structured groups.

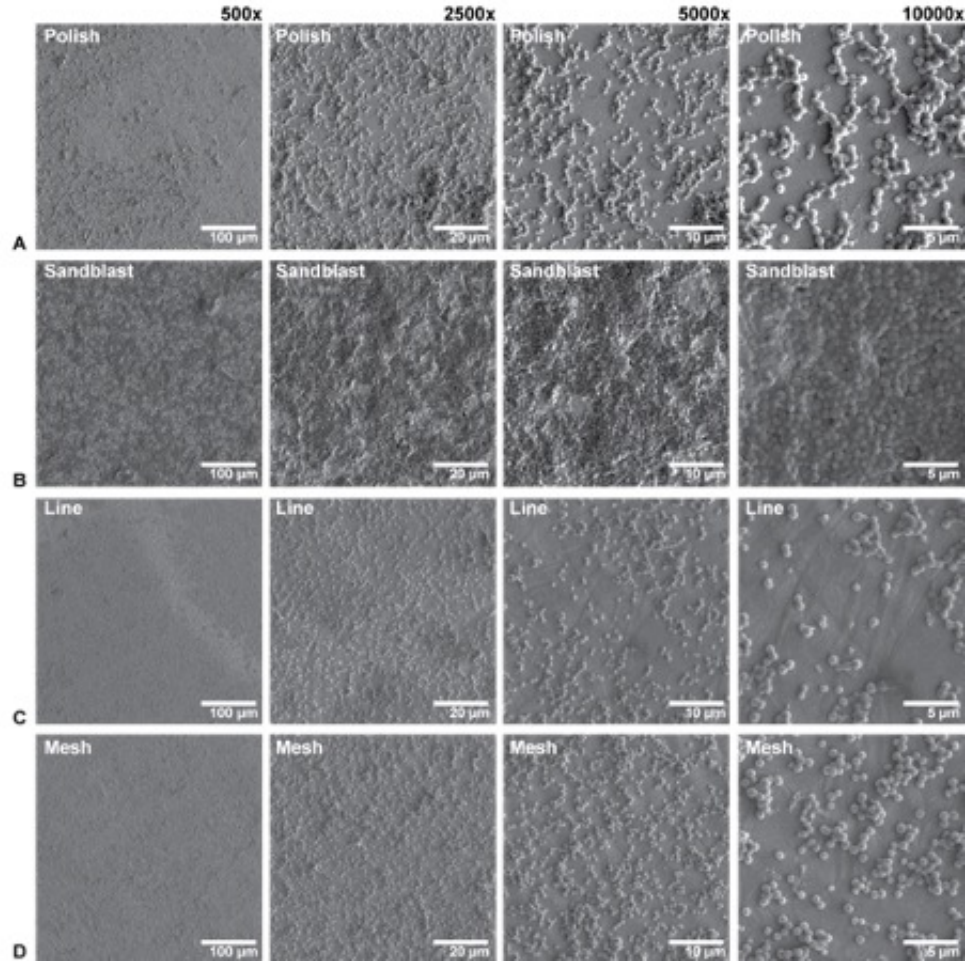


Figure 4.8 SEM images of the *S. aureus* on A) Polished, B) Sandblasted, C) Line and D) Mesh structured surfaces.

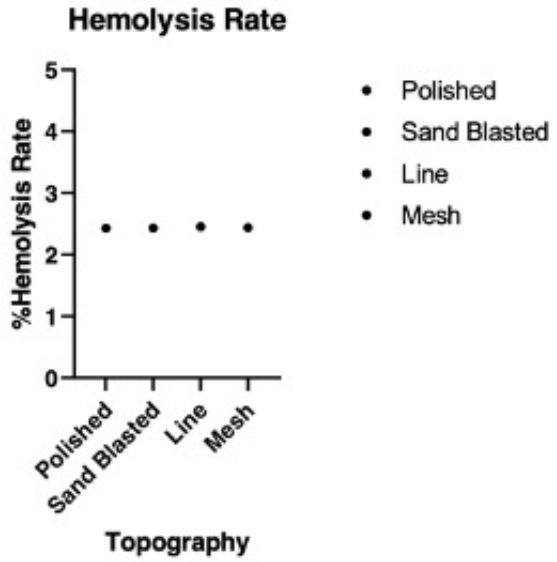


Figure 4.9 The resulting hemolysis rate of Red Blood Cells interactions with varying roughness.

4.4 Investigation of the Effect of Topographical Change to Hemocompatibility of Ti

4.4.1 Analysis of Hemolysis Rate

To evaluate how the topographical change affect hemocompatibility of the Titanium samples with polished, sandblasted, line and mesh structured surfaces, the hemolysis rate is analyzed individually. The RBCs were isolated and cultivated on Ti disk for 1 hour under the culture conditions described in section 3.6.1. The hemolysis rate was calculated with the given formula for each topography individually, the results are given in Figure 4.9. The result of hemolysis rate analysis for polished surfaces is 2.42 percent, for sandblasted surfaces 2.43 percent, for line structured surfaces 2.45 percent and mesh structured surfaces 2.43 percent. Therefore the hemolysis rate results for the Ti specimens are not significantly different from each other as also seen in Figure 4.9.

4.4.2 Examination of Platelet Adhesion

In order to examine the effect of topographical change on hemacompatibility and provide insight on biological response to the topographies in regenerative perspective, the platelet adhesion is investigated. As described in section 3.6.2. Platelet Rich Plasma (PRP) was prepared from fresh human whole blood. Following that the PRP was incubated in proper conditions for 1 hour and fixated afterwards to take SEM images. The SEM images are represented in Figure 4.10. As SEM images represents, platelets are adhered on each of the surfaces. However, as seen in 4.10.A platelets show higher tendency to form aggregation on polished surfaces compared to other groups. Mesh structured specimens show the least platelet adhesion especially on the regions with higher roughness due to being processed by laser twice.

4.5 Statistical Analysis

To analyze statistical difference in between experimental groups, one-way ANOVA with post-hoc Tukeyâs test were used in this thesis, $p<0.05$ was accepted to statistically meaningful difference.

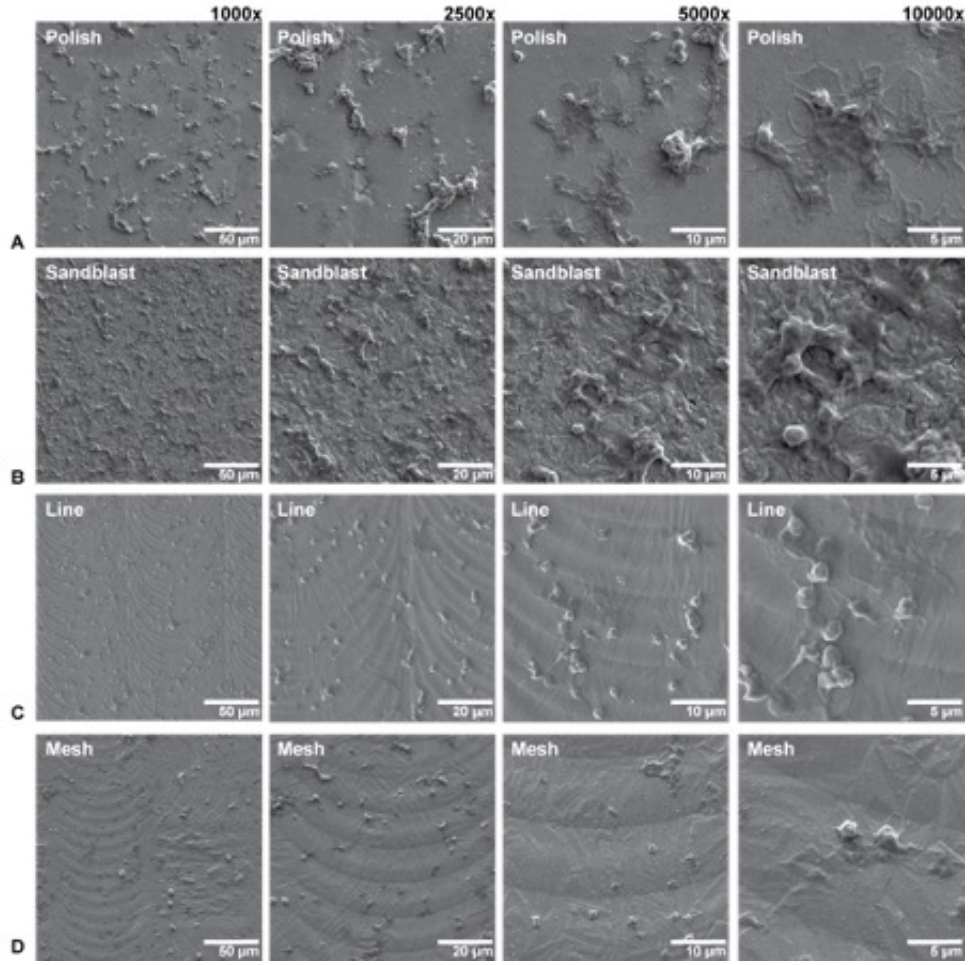


Figure 4.10 SEM images of the platelet on A) Polished, B) Sandblasted, C) Line and D) Mesh structured surfaces.

5. DISCUSSION

5.1 Material Characterization

The surface topography of cp-Ti was examined by SEM. SEM images of the material shows polished, sandblasted, line and mesh structured surface topographies. Polished surfaces are used in this thesis as control while evaluating the effect of change in surface roughness to the interaction of material with the host body as well as its own characteristic properties. The sandblasted surfaces are the surfaces with highest roughness employed in this thesis. As seen in Figure 4.1 the sandblasted Ti samples have cavities which are smaller than 1μm and ridges heterogeneously spread on the surface lacking controlled pattern. Laser processed line structured samples have crescent shaped regular patterns with 18.80 μm width aligned side by side. The mesh structured samples have more complex surface topography as depicted in the figure. On the surface of mesh samples, there are 3 different surface structures since there are regions that are not processed which corresponds to polished surface, there are regions laser treated once therefore corresponds to line structured surface, and there are regions that laser processed twice therefore having higher roughness than the other regions on the surface. Hence, mesh structured surfaces have more composite topography than other samples employed in the thesis.

The alterations in the surface topography cause change in other surface characteristics since the physico-chemical characteristics are dependent on each other. Surface topography of the material affects surface free energy which determines potential interaction between material and surrounding molecules and affect wettability. The surfaces with water contact angle lower than 90° are considered as hydrophilic and the surfaces with water contact angle higher than 90° are considered as hydrophobic. The wettability of the surface affect the molecular interactions in between material and biochemical molecules in surrounding tissue [85]. The wettability of the different surface topogra-

phies were measured with water contact angle in this thesis. As the results indicate in Figure 4.2. all four topographies examined in the extent of this research have slightly hydrophilic surfaces. cp-Ti polished surfaces and mesh structures have closer water contact angle to each other, which are 86.6 ± 4.7 and 87.9 ± 4.8 respectively and higher than the sandblasted surfaces and line structures which are 82.4 ± 5.7 and 83.7 ± 3.4. respectively. Having closer WCA results is expected in between polished and mesh structures since mesh structures consist polished regions on the surface. Lower water contact angle degrees of sandblasted and line structured samples, mean the sandblasted and line structured surfaces are more hydrophilic. This results support the previous studies showing the increase in surface roughness is positively correlated with the hydrophilicity. As stated in the literature, it is known and expected to observe increase in hydrophilicity as the surface roughness of the material is increased. However, the roughness and wettability are not linearly correlated [86]. Liu et al. has shown the effect of enhanced roughness on wettability with ZrO_2 surfaces, as the surface roughness increased with grit blasting the water contact angle of the specimen decrease. Showing that the relation between surface roughness and wettability of the material is not specific to Ti materials [87]. Moreover, this relation is not only limited with the metallic biomaterial, Feng et al. conducted to disclose surface roughness and wettability of dentin. Research presented the wettability of dentin samples treated with the laser ablation of ultrashort pulsed laser, significantly increased [88]. In another article the wettability change of Ultrafine-Grain (UFG) Ti and Cp-Ti were examined. Both UFG Ti and Cp Ti were treated with sandblasting and acid etching and results showed that water contact angle of the specimens are dependent on the roughness of corresponding specimen. However, in this study the wettability of the materials were not improved with the increase in roughness [88]. The reason for the difference in the relation between wettability and roughness in this study with the presented results might be the use of acid etching method. Acid etching is a technique widely used for obtaining topographical modifications on the materials but it may also affect chemical composition on the surface [89]. Therefore, there might be chemical alteration on the surface due to acid etching and since the chemical composition also affect wettability, those interactions might have interfered the observation of direct effect of surface roughness on wettability.

5.2 Biocompatibility

In order to evaluate the biocompatibility of the surface topographies L929 mouse fibroblast cells were incubated on the polished, sandblasted, line and mesh structured surfaces for 24 hours and MTT assay was employed to quantify cell viability. MTT is widely used method to determine the rate of mitochondrial activity. The measurement is also considered as reflecting the number of viable cells [90, 91]. As demonstrated in Figure 4.3. there is no significant difference in between Ti specimens. Although, higher metabolic activity meaning higher number of viable cells seems to be detected on the sandblasted and line structured surfaces which are the specimens with the highest hydrophilicity and roughness. Statistically; polished, sandblasted, line and mesh structured cp-Ti gave the same results in terms of the evaluation of L929 viability on the surfaces. Additionally, the results are supported with the SEM images that were taken on 24 h as seen in Figure 4.4. Even though L929 cells present higher tendency to adhere on sandblasted, line and mesh structured surfaces compared to polished surfaces, high number of adhered cells are observed in all 4 specimen surfaces. These results are not contradictory to the literature on the basis of surface roughness effect on the cellular behavior in terms of adherence and viability. The relation between material roughness and cellular adherence is neither limited to titanium as a biomaterial nor limited with the endothelial cells. Khrunyk et al. exhibits the increase in adhesion and viability of human bone marrow derived mesenchymal stem cell line (SCP-1 cells) and Osteogenic Saos-2 cells after the hydrophilicty is increased with O2 plasma treatment on Ti-6Al-4V and TNZTS alloys [92]. In the results of the research it is shown that with the plasma treatment hydrophilicity increase and this increase reflect into cellular behavior as increase in cell viability on the surface. However, there is no significant difference on the cellular viability on untreated TNZTS and Ti-6Al-4V surfaces although their water contact angle therefore the hydrophilicity are different [92]. The degree of difference of roughness and wettability is a key factor on the translation of these material characteristics into the material-cell interaction.

There are other important factors affecting the observation of cell adhesion and viability on the material. The chemical composition is also changed while pro-

cessing the surface then this might also alter cellular response. For instance, Liu et al. showed biocompatibility of Strontium-Modified Ti surface, although wettability of the experimental groups are significantly different, difference in average adhesion does not correspond to hydrophilicity of the surface directly [93]. Yu et al. examined the response of MC3T3 cells to the 3D printed Ti-6Al-4V implant. Results shows that higher roughness increase cell proliferation starting from day 3. However, in this research the correlation between roughness, hydrophilicity and cellular response is not directly supported because hydrophilicity of the 3D printed specimens change due to chemical treatment that they were exposed to [94]. Another study examined the effect of surface characteristics of TiAl6V4 alloy on biocompatibility of the material with osteoblast cells. Results showed that the roughness change in the range of 2.81-16.68 μm which are obtained with different surface treatment namely, mechanical polishing, acid treatment, sandblasting, mechanical polishing and acid treatment, sandblasting and acid treatment and electro-chemical treatment didnât change the osteoblast cell attachment which was quantified by MTT assay, neither in 3h nor in 3 days of duration [95]. Therefore, according to the results of this study, the roughness and wettability alterations within this range donât significantly affect the L929 cell viability on the surface.

5.3 Hemacompatibility

In order to evaluate the hemacompatibility of the polished, sandblasted, line and mesh structured Ti surfaces, hemolysis rate and platelet adherence were examined in this study. To assess material surface to red blood cell interaction, hemolysis assay was performed following ISO 10993-4:2009. According to ISO-10993-4 standard, in order to meet the requirement, measured hemolysis rate should be smaller than 5%. If the hemolysis rate is higher than 5% than the material is accepted to have hemolysis effect. As seen in Figure 4.9 all 4 surfaces have hemolysis rate smaller than 5%. Therefore, it can be concluded as polished, sandblasted, line and mesh structured cp-Ti specimens donât cause hemolysis and there is no significant difference observed between those surfaces in the aspect of hemolysis.

To examine platelet adhesion and activation, platelet rich plasma (PRP) was prepared from whole fresh human blood. The adhesion and activation of the platelets are seen in SEM images Figure 4.10. As presented in Figure 4.10 there are high number of platelets adhered on the each of the surfaces. However, the mesh structure which provide areas having different roughness to the platelet clearly shows that the platelet adherence decrease as the roughness increase. The other factor causing this result is the hydrophilicity because when the hydrophilicity increases the non-specific binding decrease due to the absorbed water and hydroxyl groups on the surfaces [96]. In addition to diminished platelet adherence, platelet activation is also lower on more hydrophilic surfaces. Hence, as already seen in hemolysis rate results all 4 specimens are blood compatible. However, as indicated on the surface of polished Ti, the adhered platelets show more extended pseudopodia and exhibit more cluster structures in comparison to sandblasted and line structured specimens. The extended pseudopodia is a cue for platelet activation. Gong et al. showed similar results indicating the higher the roughness the less adherence and activation of platelet on the material surfaces are conducted with the employment of titanium dioxide nanotube arrays (TNTAs) [83]. Therefore, the results are in accordance with the literature showing hydrophobicity on the surfaces promote platelet activation. Additionally, all four topographies examined, are shown to be blood compatible within the limitations of the.thesis.

5.4 Bacterial Behavior

Bacterial adhesion to the biomaterial surface is a highly complex process and governed by many mechanisms depending on both material and the bacteria itself. The surface topography specifically, roughness, wettability, porosity, polarity, surface charge and surface energy and the characteristics of the bacteria namely, being gram positive or gram negative and the structure of cell wall factor are all regulate the adhesion [97]. For the biofilm formation assessment at 24 h of cultivation, MTT assay is employed. Results showed that *E. coli* biofilm formation significantly promoted on the sandblasted and line structured surfaces. The biofilm formation by *E. coli* is the highest on the line structured surface followed by sandblasted and mesh structured surfaces. The biofilm

formation on the polished surface is significantly lower than the other Ti specimens. That data express that *E. coli* have tendency to adhere and form biofilm on the surfaces with higher roughness and hydrophilicity. Cell surface of *E. coli* is also hydrophilic according to the research which examined the characteristics of the surface of different *E. coli* strains which were cultured on various conditions [98]. Bayoudh et al. shows that the more hydrophobic is the bacteria cell surface the more that bacteria adhere onto the hydrophobic surface and vice versa. Although, adhesion density results in the study at the first hour didnât give the directly correlational outcome for hydrophilic strain, measurements at the 2 hours time-point showed significant difference in this research [99]. From that aspect, represented findings on high *E. coli* biofilm adhesion on more hydrophilic surfaces are expected. At 24 hours time-point also SEM images were taken for all 4 specimens to observe biofilm formation, qualitatively. As seen in Figure 4.7 there are *E. coli* bacteria adhered on all 4 surfaces. It is hard to deduce a comment such as there are significantly less colonization on polished surfaces from the image. However, denser aggregations are observed on sandblasted and line structured surfaces rather than small groups.

When the biofilm formation of *S. aureus* is examined, viability is significantly higher on polished surfaces compared to other Ti specimens sandblasted, line and mesh structured surfaces and TCP as seen in Figure 4.6. In contrast to *E. coli*, *S. aureus* show more tendency to adhere and colonize on more hydrophobic surfaces. This results are expected when the cell surface composition and characteristics of *S. aureus* are considered. *S. aureus* bacteria have hydrophobic cell surfaces [100]. Therefore as observed before, they have higher tendency for hydrophobic surfaces [101]. Braem et al. shows that decrease in roughness of Ti surface results in decrease in bacterial adhesion and viability on the surface. Also, research elucidated that if the hydrophilicity of the surface is altered the bacterial colonization change, eventhough the roughness or porosity of the surface remain the same. For *S. aureus* if the hydrophilicity of the surface increases the bacteria mass on the surface decreases [102]. Vishnu et al. examined the effect of alterations on the Ti surface via changing time and temperature parameters of hydrothermal treatment to bacterial adhesion. Hydrothermal treatment cause decrease in water contact angle meaning increase in hydrophilicity. The *S. aureus* bac-

teria significantly decreased in nanoflower structures which have higher hydrophilicty and roughness [76]. Jaggessar et al. presented a research investigating the surface topography effect of Ti-6Al-4V alloy on *S. aureus* adhesion, showed that *S. aureus* have less tendency to adhere and colonize on textured surfaces [103]. These findings which are now supported with the results of this study are significant in the aspect of representing designed topographies to the literature diminishing initial adhesion and colonization of gram-positive bacteria. Since gram-positive bacteria are proven harder to be eliminated [104].

5.5 Conclusion

Ti has preferred for its mechanical characteristics in addition to being biocompatible, easily modifiable biomaterial. Therefore, it is utilized in various biomedical applications for several decades. Hence, numerous research studies were conducted to improve preferred characteristics of the Ti by developing new alloys and modifying surface. Mainly, the interactions in between Ti and host cells are controlled in a way to improve material-host tissue integration while diminishing bacterial adhesion on the surface of the material.

In this thesis, laser processing as a physical surface modification was utilized to avoid chemical residue interference. Polished, sandblasted, line and mesh structured surfaces are examined and shown to be hydrophilic. Moreover, the higher roughness is correlated with the increase in wettability according to the results of this research, sandblasted and line structured surfaces are more hydrophilic compared to polished and mesh structured surfaces. Additionally, all four groups are shown to be blood compatible and only polished surface caused higher platelet activation in comparison to other groups. The viability and adherence of fibroblasts on the surfaces were not significantly different from each other. When the biofilm formation was examined, gram negative *E.coli* bacteria showed least tendency for polished surface while gram positive *S. aureus* bacteria showed the highest. In the enlightenment of these results, it is concluded that the change in surface roughness of Ti, alters wettability, cellular responses,

and bacterial behavior on the surface. Therefore, biomaterial tissue integration can be improved with physical surface modifications.

5.6 Future Studies

To investigate the host response to Ti specimens with the polished, sandblasted, line and mesh structured surfaces, roughness will be analyzed. Additionally, in order to assess the effect of the topographies on osteogenesis, cell culture studies will be conducted with osteoblast cells and osteogenic gene expression of the cells will be analyzed. Also, immunomodulation is another important aspect to examine. Therefore, the pro- and anti- inflammatory responses of immune cells, specifically macrophages will be analyzed.

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