AMINO ACID CONJUGATED SELF-ASSEMBLED MOLECULES MODIFIED TITANIUM SURFACES

by

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AMINO ACID CONJUGATED SELF-ASSEMBLED MOLECULES MODIFIED TITANIUM SURFACES

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ACADEMIC ETHICS AND INTEGRITY STATEMENT

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ABSTRACT

AMINO ACID CONJUGATED SELF-ASSEMBLED MOLECULES MODIFIED TITANIUM SURFACES

The goal of this thesis is to increase biocompatibility of titanium (Ti) surfaces, which is an extensively used biomaterial in medicine, by an easy and timesaving surface modification procedure. For this purpose, 3-aminopropyltriethoxysilane (APTES) molecule was conjugated by 3 different amino acids (histidine, leucine, and tryptophan). Newly synthesized molecules were used to form amino acid conjugated self-assembled monolayers on titanium surface. After modification of the surfaces by each of the amido amino acids, histidine and leucine amino acids were chosen to make up hydrophilic and hydrophobic regions on the surface and they were mixed with changing concentrations (v/v, 80:20, 50:50, 20:80). X-ray photoelectron spectroscopy (XPS) analysis and water contact angle measurements were performed for the characterization studies of all of the modified surfaces. Human osteoblast cells culture studies were performed on the surfaces, which were modified by this method aimed at improving the biocompatibility of titanium, in order to investigate cell viability on the modified surfaces.

Keywords:: Titanium, Surface Modification, Self-assembled Molecules (SAMs), Amino Acids, Biocompatibility

ÖZET

AMİNO ASİT KONJUGE KENDİLİĞİNDEN DÜZENLENEN MOLEKÜLLER İLE MODİFİYE EDİLMİŞ TİTANYUM YÜZEYLER

Bu tez çalışmasında, kemik hasarlarının tedavisinde sık kullanılan bir malzeme olan titanyumun (Ti), kolay ve zaman tasarrufu sağlayan bir yöntem ile yüzeyi modifiye edilerek biyouyumluluğunun artırılması amaçlanmıştır. Titanyum yüzeylerin modifikasyonu için 3-aminopropiltrietoksisilan molekülü 3 farklı çeşit aminoasitle (histidin, lözin, triptofan) konjuge edilmiştir. Yeni sentezlenen bu amido amino asitlerle yapılan modifikasyonlar ile yüzeylerde kendiliğinden yönlenebilen tek tabakalar oluşturulmuştur. 3 farklı aminoasitle modifikasyonun ardından, yüzeylerde hidrofilik ve hidrofobik bölgeler yaratmak amacıyla 2 amino asit seçilmiş (histidin ve lözin) ve bu aminoasitler farklı derişimlerde (v/v, 80:20, 50:50, 20:80) karıştırılarak modifikasyonlar gerçekleştirilmiştir. Yüzeylerin karakterizasyonu için X ışını spektrofotometresi (XPS) ve su temas açısı ölçümleri yapılmıştır. Titanyumun biyouyumluluğunu artırması hedeflenen bu yöntemle oluşturulan yüzeylerde hücre davranışını incelemek amacıyla; bu yüzeylerin üzerinde insan osteoblast hücreleri kültür edilmiştir.

Anahtar Sözcükler: Titanyum, Yüzey Modikasyonu, Kendiliğinden Düzenlenen Moleküller, Amino Asitler, Biyouyumluluk

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LIST OF ABBREVIATIONS

APTES	(3-Aminopropyl)triethoxysilane
Bt	Benzotriazole
Cbz-AA-Bt	Carbobenzyloxy amino acid benzotriazole
CMCs	Ceramic-matrix composites
DI	Deionized water
DCP	Dicalcium phosphates
DMEM	Dulbeccoâs Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
Et_3N	Triethyl amine
HA	Hydroxyapatite
His	Histidine
Leu	Leucine
MMCs	Metal-matrix composites
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
PMCs	Polymer-matrix composites
RGD	Arginine-Glycine-Aspartic acid
PDMS	Polydimethylsiloxane
SAM	Self assembled monolayers
TCP	Tricalcium phosphate
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
Trp	Tryptophan
UV	Ultraviolet
WCA	Water contact angle
XPS	X-ray photoelectron microscopy

1. INTRODUCTION

1.1 Motivation

Bone grafting is a method used for the treatment of large bone defects. It can be carried out by organic materials such as autograft, allograft, and xenograft or by artificial bone substitutes like ceramic, polymeric and metallic biomaterials. Among all these materials, titanium has an important role in bone treatments because of its mechanic properties such as durability, resistance to corrosion and low elastic modulus. Besides, it is bioinert, highly biocompatible and it has osseointegration ability [7, 8].

Biomaterials are implanted in the body, so the interaction between a biomaterial and the physical environment in the body is crucial. Body's response to a biomaterial is controlled by nano-micron scale [9]. Previous studies have shown the impact of surface properties on cell differentiation, attachment and proliferation [8, 10]. It is therefore important to create a tissue-like environment for osteogenesis of cells surrounding the biomaterial.

Various chemical and physical modification methods have been used in order to increase osseointegration and bioactivity of the titanium implants. Plasma spraying [11, 12], acid etching [13, 14], anodization [15], grit-blasting [16, 17] and self-assembled monolayer [18] are some of the modification techniques implemented on Ti surfaces. Self-assembled monolayers are an ensemble of organic molecules. They bind to a surface and spontaneously form a one molecule thick layer in a self-organized way. They are amphiphilic; composed of a hydrophobic functional group, a hydrophilic head, which forms covalent bonds on the surface, and an alkyl chain. Functional group of self-assembled monolayers' arranges the characteristics of the surfaces. It forms areas that have different hydrophilicity and roughness, which lead to different cellular responses and dictates the fate of surrounding cells [19].

Effects of surface chemistry on cell behavior directed scientists' interest towards surface engineering applications [2]. Investigation of adsorption of biomolecules on these surfaces is valuable because it regulates the interaction of cells with the surface. In this study, titanium surfaces were modified by newly synthesized amido amino acid self-assembled molecules (SAMs). Amido amino acid SAMs were synthesized by reacting 3-aminopropyltriethoxysilane with 3 different amino acids (histidine, leucine and tryptophan). Amino acids are complex molecules in their nature and their side chains have different properties. So it is important to inspect cell differentiation, viability and proliferation on these titanium surfaces, which were modified with amino acid conjugated SAMs, which pose amino acids as a functional group. Response of osteoblasts on such surfaces was investigated in this thesis.

1.2 Objectives

Due to insufficient amounts of organic bone substitutes for bone grafting, artificial implants should be produced for treatment of large bone defects. Recently, titanium (Ti) has been one of the most commonly used materials for bone implants. Therefore, investigation of biocompatibility of Ti implants and construction of tissue-like surfaces on Ti implants are important for bone-tissue engineering applications [20, 21]. For this purpose, Ti surfaces were modified by histidine, leucine and tryptophan amino acid conjugated self-assembled molecules. These molecules were synthesized by 3-aminopropyltriethoxysilane and amino acids. Newly synthesized amido amino acid based biomolecules have the ability to form self-assembled monolayers on Ti surfaces. On the surfaces modified with novel amido amino acid conjugated SAMs, behavior of osteoblast cells were investigated.

The aims of this thesis are listed below:

- Synthesizing amino acid conjugated self-assembled molecules to create different functionality on the Ti surfaces,

- Evaluating the biocompatibility of surface modified Ti substrates,
- Observing the effects of surface modification with hydrophobic/hydrophilic amido amino acid mixtures on osteoblast cells.

1.3 Outline

This thesis gives background information about bone tissue, biomaterials used in bone tissue engineering, surface modification of titanium and SAMs in Chapter 2. Experimental procedures are described in Chapter 3. Chapter 4 presents the results and in the last chapter (Chapter 5), results are interpreted in order to reach a conclusion.

2. BACKGROUND

2.1 Bone Tissue: Structure and Function

Skeletal system is the mechanical framework of human body. Main component of the skeletal system is the bone tissue [22]. Bone tissue, which has its own blood vessels, is a living and growing tissue. Living cells and blood vessels help bone tissue to repair and grow itself. Bone tissue is mainly composed of collagen protein. Besides protein fibers like collagen, bone tissue also contains matrix proteins as glycoproteins and proteoglycans, water and mineral salts as calcium and phosphorus. Minerals provide strength and it hardens bone tissue [23, 24].

Mainly, bone tissue comprises 2 types of osseous tissue which are cortical bone and trabecular bone (cancellous bone). While the outer layer of the bone tissue consists of cortical bone, which is a compact tissue; inner layer of the bone is made up by trabecular bone, which is a spongy structure. Cortical bone, which makes up 80% of total bone mass, contains multiple canals called Haversian canals that are surrounded by blood vessels and nerve cells. Haversian canals are made up multiple layers of lamellae around it and this system is called Haversian system or osteon [24, 25]. Osteons are microscopic columns and they contain osteoblasts and osteocytes around Haversian canals. The outer surface of cortical bone is covered by periosteum. Between inner and outer layers of bone tissue there is a structure called endosteum which is assigned to be boundary between those two layers. Inner part of the bone tissue is called trabecular bone. It has a porous structure and less density than cortical bone. Trabecula (lattice-shaped units) is the smallest functional unit of trabecular bone, found near the joints, in the end of the bones. Also, bone marrow and adipose tissue is located in hematopoietic space surrounding trabecular bone [26, 27].

Bone is a dynamic tissue. There are different cell types in bone tissue such as osteocytes, osteoclasts, bone lining cells and osteoblasts. These cells provide an

important feature to the bone tissue: bone remodeling, which is a significant process for bone healing. Although whole functions of these cells have not been unraveled, it has been known that osteoblasts have role in bone formation and osteoclasts in bone resorption. Osteoclasts are multinuclear cells; they are associated with bone resorption. Mainly, osteoclasts are found on the surface of bone tissue and injured/old bone. They are derived from hematopoietic stem cells and do not have the ability to divide, which means they are terminally differentiated. The balance between bone resorption by osteoclast and bone formation by osteoblasts provides the reshaping of bone [28, 29]. If this mechanism is disrupted for any reason, it will lead to bone diseases like osteoporosis. Pre-osteoblasts, which originate from mesenchymal stem cells, are precursors (progenitor) of osteoblast cells. Osteoblasts are found in growing parts of the bone tissue because they have a bone forming function [30]. They have large amount of rough endoplasmic reticulum and Golgi apparatus organelles and they secrete collagen proteins, osteonectin, bone sialoprotein and osteocalcin, which are major bone extracellular matrix proteins. Together with these proteins, proteoglycans as decorin and biglycan form organic matrix that will undergo the mineralization step. Afterwards, calcium ions are trapped in the matrix and the matrix is calcified leading some osteoblasts to differentiate into osteocytes [23, 30, 1].

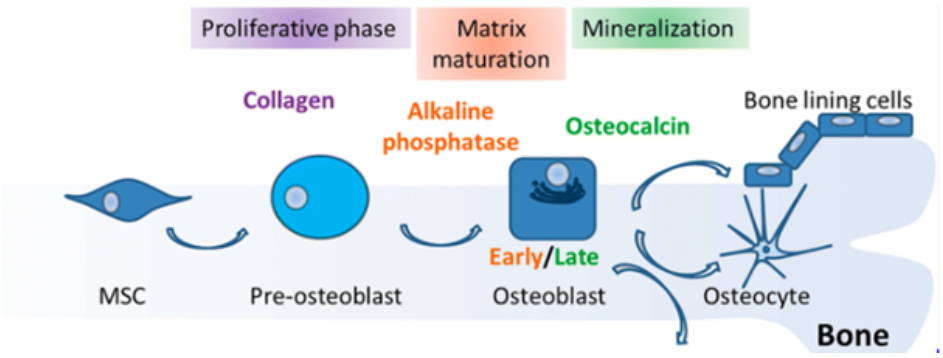

Figure 2.1 Maturation of Osteocytes [1].

Mature osteoblasts undergo differentiation and form osteocytes. During this process, quantity of some organelles as rough endoplasmic reticulum and Golgi apparatus decline, eventually leading to a reduction in protein synthesis. The most abundant cell types in bone tissue are osteocytes. They are the primary cell type for bone tissue and they make up 90-95% of the cells composing bone tissue [1]. They are found both in

trabecular bone and cortical bone. Osteocytes have several functions in bone tissue such as retaining mineral concentration of matrix, performing as mechanosensors and having a role in bone remodeling. Apoptosis of osteocytes is relevant to bone resorption and it has been known that they are engulfed by osteoclast cells when they are in apoptotic state [31, 32]. The last cell type found in bone tissue is bone lining cells, which are found on the bone surface. Their function is not fully understood, their role in osteoclast differentiation and blocking of osteoclast and bone matrix direct interaction have been shown [33, 34].

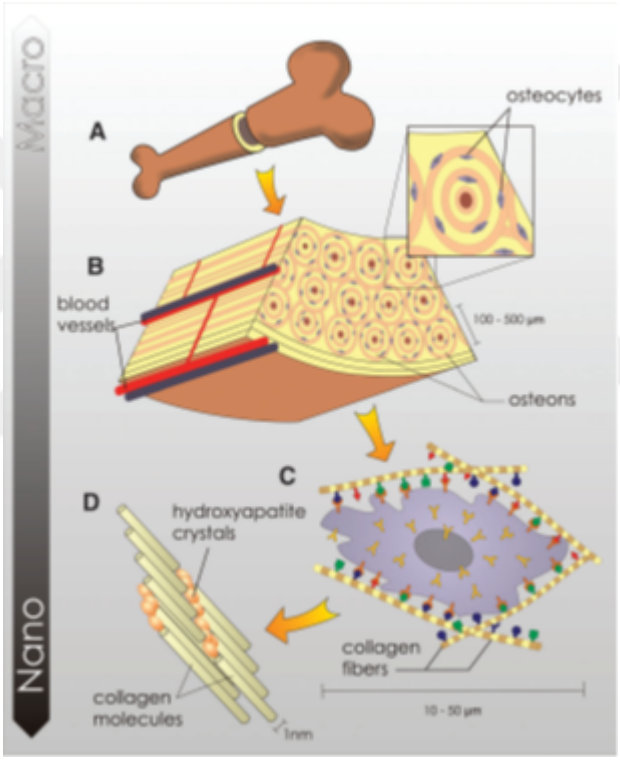

Figure 2.2 Structure of Bone Tissue. A: Outer layer. B: Osteons. C: Cells with Cell Membrane Receptors. D: Structure of Extracellular Matrix (ECM) [2].

2.2 Bone Defects and Treatments

Bone defects may derive from skeletal complications such as tumors, infection or bone resection caused by any other traumatic event. In modern medicine, there are many different ways of treating bone defects. Still, especially large bone defects necessitate bone grafting, which is a method involving replacement of missing bone by biological or synthetic materials. The main parameter that determines the success of

any transplantation operation is its effect on achieving remodeling of bone tissue and supporting formation of new bone. Any graft that is placed in the human body should be osteoinductive, osteoconductive, and osteogenic [35]. If it is osteoinductive, it can stimulate bone formation; if it is osteoconductive, it may promote immature bone cells into osteocytes; and if it is osteogenic, it may provide the living cells from the donor, to survive and proliferate after the operation [36].

There are various bone substitutes used in practice: autografts, allografts, xenografts and other organic or synthetic bone substitutes [37]. Autograft is the procedure in which bone that is to be grafted to a patient is obtained from the same patient. Due to the fact that it has less risk of rejection and developing an immune response, autologous bone graft is the most preferred method. Nevertheless, the biggest drawback of autografts is the need for one extra site of surgery; therefore it may not be favored. In allotransplantation, bone graft donor and recipient are different individuals of the same species. Allograft transplantation has various advantages. They have osteoconductive and osteoinductive features. Also, structure of the transplanted bone is comparable to that of the patient. However, it is risky due to the possibility of disease transmission and rejection of the transplant [38]. Another therapeutic option is xenotransplantation. It is an interspecies transplantation: animal organs, tissues or cells are transplanted in human body. Xenotransplantation presents some benefits. Source of xenotransplantation products is not restricted. Furthermore, it allows comprehensive screening of donor animals prior to transplantation for the purpose of reducing immunogenic concerns. As in allograft transplantation, xenotransplantation has some hurdles that prevent the success of a transplantation procedure to be successful. Transmission of pathogens, e.g. viruses, is the major burden because it may spread to other people around the recipient. Also, there is a rejection possibility [39, 40].

In addition to biological bone substitutes, biomaterials are used for replacement or repairing of damaged tissue or organ [38].

2.3 Biomaterials

Biomaterials are materials of synthetic or natural origin that are implanted in the body for treatment. They have been used in the field of medicine since the prehistoric age and have been developed further especially after World War I. These early biomaterials mostly consisted of metals, such as titanium and stainless steel; also polymers and nylon. After the late 1940s and the early 1950s, their usage became widespread [41, 42]. The scientific field of biomaterials is multidisciplinary, combining synthetic biology, computational biology, systems biology; and its growth helped save the lives of millions. Today, biomaterials are being used for key applications in the skeletal system, cardiovascular system, ophthalmology, as well as many other operations. Heart valve prostheses, stents, joint replacement prostheses, bone fixation plates, dental implants and intraocular lenses are among the most common applications of biomaterials. Also, parts of a medical device that interacts with the body such as catheters, sutures and blood oxygenators, can also be considered biomaterials [38].

Success of a biomaterial is primarily determined by the host response. This includes protein and cellular responses of an organism to the material implanted. If a biomaterial is incompatible with the body, it leads to host response, such as inflammation, infection, pain, swelling, tumor formation, blood clotting, and activates the immune system [38]. The implanted biomaterials were expected to be bio-inert, so that they would not trigger any host response [43]. Biomaterials most commonly comprise of polymers, bioceramics, biocomposites and metals. Each has different characteristics, leading them to have certain advantages/disadvantages [43, 44].

2.3.1 Polymeric Biomaterials

Polymeric biomaterials consist of natural and synthetic polymers. They are biocompatible, biodegradable in the body and support cell growth. Collagen, hyaluranic acid and chitosan fall under natural polymers, while polyethylene glycol, polyglycolide, silicones and poly (methyl methacrylate) are examples of synthetic polymers. Biopoly-

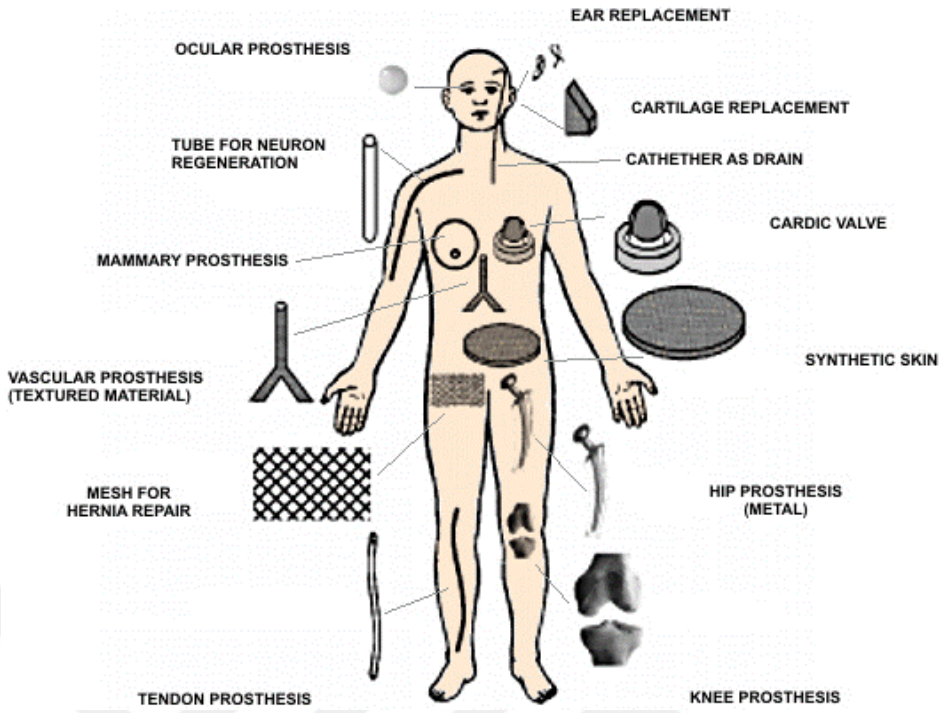

Figure 2.3 Examples of Biomaterials [3].

mers are osteoconductive and they are easy to manufacture and modify. However, they have some disadvantages, such as difficulty of sterilization and absorption of water or other proteins from the blood. Some example uses of biopolymers can be listed as catheters, dialysis devices, acetabular hip and knee joints (polyethylene), sutures and vascular grafts (polypropylene) and contact lenses [8, 35, 38, 44].

2.3.2 Ceramic Biomaterials

Bioceramics are mostly used as biomaterial in dental and orthopedic implants. They are very biocompatible, anticorrosive, and highly inert and they are not immunogenic. Furthermore, they are capable of binding directly to the bone and they can induce appropriate biological response. Because of these features and their similarity to mineral phase of bone tissue, they are preferred in orthopedic applications [38]. Zirconium dioxide (ZrO_2), aluminum oxide (Al_2O_3), calcium phosphates, dicalcium phosphates (DCP) calcium sulphate ($CaSO_4$), calcium carbonate ($CaCO_3$), hydroxyapatite (HA) and glass-ceramics are examples of ceramic biomaterials. Zirconia

and aluminum biomaterials are bioinert and they are non-toxic, however, they do not form bonds with bone tissue. HA (sintered at low temperature), tricalcium phosphate (TCP), calcium sulphate ($CaSO_4$) and calcium carbonate ($CaCO_3$) are biodegradable bioceramics and this is their principal advantage. Biodegradable bioceramics are broken down, resorbed and replaced by the body, ending up with the own tissue of the host. During this process it is important that resorbed materials could be metabolized in the body in normal metabolic pathways. HA (sintered at high temperature), bioglasses and glass-ceramics are bioactive bioceramics. They lead to low mechanical stress and they can form chemical bonds with tissue. Ceramic implants are wear resistant, biocompatible and resistant to compression. These properties grow the use of bioceramics in biomaterial field [45]. However, they also possess disadvantages, such as brittleness, weakness in tension and difficulty in fabricating and shaping [24].

2.3.3 Composite Biomaterials

Biocomposites are composed of at least two different materials that have distinct physical and chemical properties. Basically, they are comprised of two phases which are called matrix and reinforcement. Matrix of a biocomposite is bulk and continuous while reinforcement is discontinuous and distributed in the composite. Interface is the surface between these two phases [46]. Bone, dentin and cartilage tissues also can be considered biocomposites [47]. Both of the two phases of a biocomposite should be compatible with the body and the interface should be resistant to degradation by the body [48]. Biocomposites allow scientists to design for specific applications through choice of materials and their ratios. That is, it is possible to choose/combine the distinct properties of materials used in a biocomposite and to improve the performance of a biomaterial. Features of a biocomposite material are mainly regulated by the reinforcement phase. Matrix binds reinforcement and mechanically supports it. Biocomposites can be classified according to incorporation of reinforcement: fiber-reinforced and particle-reinforced composites or they can be classified depending on the matrix material: polymer matrix composites (PMCs), ceramic-matrix composites (CMCs), or metal-matrix composites (MMCs) [46]. Dental filling composites and orthopedic im-

plants with porous surfaces are some of the biocomposite material applications [47]. Biocomposites are biocompatible, bioinert, resistant to corrosion and compression [45]. Also, they may present characteristics that none of the components have [49]. Their principal disadvantages are their difficulty to produce and reproduce [45].

2.3.4 Metallic Biomaterials

Use of metallic biomaterials had started as early as 200 A.D. First examples of metallic biomaterials were sutures used in dental applications [41]. After British Surgeon Joseph Lister spearheaded the use of antiseptic techniques in operations, applications of metals as a biomaterial have increased [50]. Recently, many metals such as Ti and its alloys, cobalt-chromium (Co-Cr) alloys, gold (Au), silver (Ag), copper (Cu), palladium (Pd) and stainless steel has been used for many biomedical operations including orthopedics (total hip joint, bone plates, screws), dentistry (tooth implants, dental fillings), reconstructive surgery (maxillary and orbital floor reconstruction) and cardiovascular surgery (artificial heart valves, vascular stents). Metallic biomaterials make up 70% of implants. They are widely preferred in load bearing situations due to their strength, durability, resistance to fracture and high ductility. Additionally, metallic biomaterials have fatigue resistance, good electrical conductivity and it is simple to fabricate and sterilize them. Major disadvantages of metallic biomaterials are that they have high elastic modulus, they are subject to corrosion in body environment and they may cause metal ion toxicity [51, 52].

Stainless steel, Co-Cr and Ti and its alloys are predominantly used metallic biomaterials [52, 53]. Co-Cr alloys have relatively higher wear resistance; while stainless steel has the highest ductility and Ti is the most biocompatible in this group. Furthermore, Ti presents the highest corrosion resistance and lowest stiffness among them [54]. Stiffness is an important parameter of a biomaterial because high stiffness may lead to bone resorption and loosening of the implant by a phenomenon called stress-shielding [47, 55].

2.4 Titanium as a Biomaterial and Surface Modification of Titanium Biomaterials

Titanium is a transition element which was discovered near the end of the 18th century. Ti and its alloys are of great interest in biomedical applications because they combine outstanding properties such as bioinertness, biocompatibility, resistance to corrosion, low elastic modulus, low density, high strength and ability to integrate with bone (osseointegration) and other tissues [55, 56].

Mechanical properties of titanium are considered an advantage for hard tissue operations such as joint and bone replacement and dental implants. In addition to good mechanical properties, osseointegration capability is an important parameter to be a successful implant and it is established that surface structure of a biomaterial plays a role in osseointegration [57]. If surface of the implanted material is not osteoinductive and osteoconductive and does not allow integrating with neighboring bone tissue, implant will loosen and there will be a fibrous tissue between the implant and bone tissue [55].

Topography, roughness and chemistry of the surface of an implant have a vital role in osseointegration because they moderate biological response. Physical structure at the contact area of an implanted material with tissue provides an environment for biomolecules, like proteins and cells, to integrate with the surface. Interaction of the body with a biomaterial that has a modified surface can have a positive effect. Surface engineering showed that biocompatibility and bioactivity of implanted material can be improved by using different techniques for modification [4, 58].

Different modification techniques can provide chemically or physically remodeled molecules on the surface, or they may cover the surface of a biomaterial with a different material. Modification techniques achieve biocompatibility through morphological, biochemical and topographical alterations on the surfaces [4, 58]. Some of these modification techniques can be listed as plasma treatment and deposition [59],

photolithography [60], sol-gel deposition [61], silanization and Langmuir-Blodgett deposition [62], grit blasting [16, 17], acid etching [13], immobilization of biomolecules [63], and self-assembly [18].

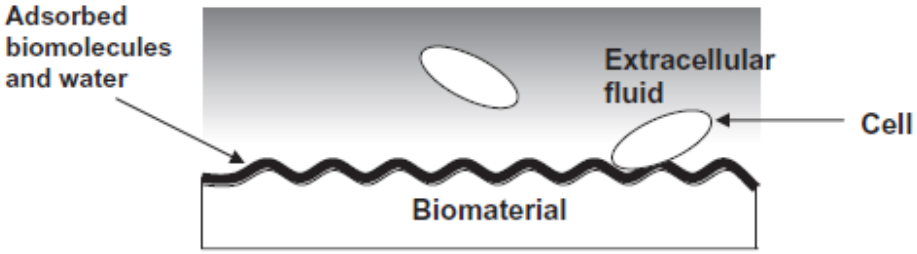

Figure 1. Interaction at the biomaterial surface.

Figure 2.4 Biomaterial-tissue interface [4].

Self assembly is a process in which components in a disordered configuration form an organized structure. It is spontaneous ordered arrangement of molecules and small particles. When the constitutive elements are molecules, it is called molecular self assembly. In self assembly, active surfactants spontaneously adsorb on surfaces and form a monomolecular organic film. SAMs have a terminal functional group which is exposed to SAM-gas/liquid interface; a hydrocarbon segment that are generally alkyl chains and a head group bonding to specific substrate sites. The structure is stabilized by intermolecular van der Waals interaction of the alkyl chain. Self-assembled monolayers are amphiphilic, having both hydrophilic (polar) and hydrophobic (nonpolar) parts. Their formation is achieved by introducing a hydroxylated surface (TiO_2, SiO_2) with a solution and enabling a reaction on the surface with alkylsilane derivatives. Terminal functional groups of self-assembled monolayers (-OH, -CH_3, -COOH, -NH_2, -SO_3H) elicit chemical and physical diversity on the surfaces. SAMs are preferred not only because they are stable, inexpensive and easy to prepare but also they enable the use of many different molecules in order to form a surface with desired properties [64, 5].

A wide variety of physical or chemical modification techniques have been implemented on Ti surfaces. Use of SAMs on Ti biomaterial has improved interaction of the material with tissues by increasing the stability and functionality of the Ti surface.

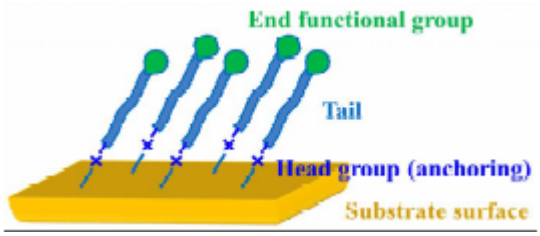

Figure 2.5 General structure of Self Assembled Monolayers [5].

SAMs help to arrange some of the surface features such as wettability and corrosion inhibition. Surface wettability, which is the measure of surface hydrophilicity, is an important parameter on cell behavior and host response. Protein adsorption on biomaterial surface also has an important role in host response. When a biomaterial is implanted, water molecules are the first to arrive at the biomaterial's surface. Proteins reach the surface after water molecules and their adsorption is affected by the presence of water molecules, because water molecules may create a shell effect. This shell affects attachment of many other biomolecules besides proteins. Therefore, cell attachment on implant surface is directed by biomolecules adsorbed on. Protein adsorption increases on poorly wettable surfaces and decreases on SAMs, owing to their -OH termination which is hydrophilic [5, 65]. It was shown that moderately wettable surfaces are the best option for cell growth and biocompatibility [66].

Aforementioned, titanium is a biocompatible material, which owes its biocompatibility to self-organized titanium oxide (TiO2) structure on the surface. Interaction and transition between titanium and physiological environment is critical because cell behavior in the vicinity of implanted material is mediated by organic (collagen proteins, osteonectin, bone sialoprotein, osteocalcin, dentin matrix protein) and inorganic components (hydroxyapatite, calcium and phosphate salts). These components and implant surface dictates cell fate by regulating protein adsorption on implant surface. Also, it has been shown that specific amino acid sequences, such as Arg-Gly-Asp (RGD), in ECM proteins have a role in osteoblast adhesion [67, 68, 69, 70]. Recently, studies have demonstrated relevance of hydrophilic titanium surfaces with increased osseointegration and enhanced osteoblast adhesion and proliferation [71, 72, 73, 74]. In this study, titanium, which is one of the mostly used biomaterial in hard tissue re-

placement, was used. Three different amino acid conjugated self-assembled monolayers (Histidine-SAM, Leucine-SAM, Tryptophan-SAM) were used to create surfaces having different hydrophilicity. Amino acids are complex structures and their side chains present them different properties in terms of polarity, hydrophilicity/hydrophobicity and acidity.

It was aimed to modify Ti surfaces in a simple way and to see impacts of surface features on cellular responses of osteoblasts.

3. METHODS

3.1 Surface Modification

3.1.1 Synthesis of Amino Acid Conjugated Self Assembled Molecules

Synthesis steps for amino acid conjugated self assembled molecules (His-SAM, Leu-SAM and Trp-SAM) is illustrated in Figure 3.1.

Firstly, 1 equivalent of amino acid solution and 2 equivalents NaOH were mixed in 50 mL water and the solution was cooled. Afterwards, the solution was supplemented with dioxan solution which contains Cbz-Cl (1.2 eq). Addition was done in a drop wise manner. Cbz molecule functions as protective for the amine group. Solution was mixed for 16 hours at room temperature. Vacuum was used to evaporate dioxane. Then, water addition and ethyl acetate extraction was done in order to get rid of excess amount of Cbz-Cl. HCl (1 M) was added to set the pH at 2.0 and the solution and ethyl acetate was used for another extraction. Anhydrous Na_2SO_4 was used to dry ethyl acetate layers and vacuum was applied to remove ethyl acetate and therefore to collect Cbz-amino acids (Cbz-His-OH, Cbz-Leu-OH, Cbz-Trp-OH).

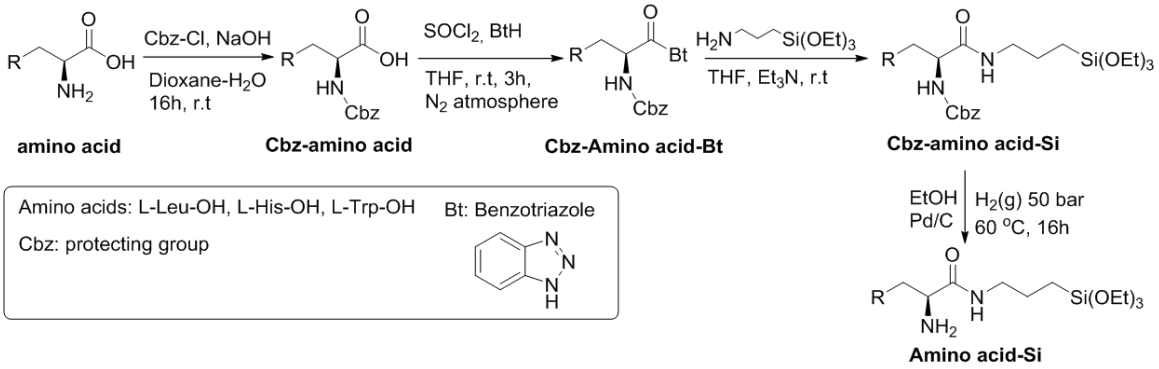

Figure 3.1 Synthesis of amido amino acid conjugated SAMs [6].

1.2 equivalent of $SOCl_2$ was supplemented and mixture was mixed for 30 minutes. Stirring continued for 3 hours under nitrogen atmosphere after white precipitation was detected. Filtration was implemented to remove white precipitation. Remaining mixture was mixed in ethyl acetate after vacuum was applied to get rid of THF. Extraction of the solution in ethyl acetate was carried out with 20% Na_2CO_3 aqueous solution. After solvent was eliminated, Cbz-AA-Bt was attained.

1 equivalent of APTES (3-(aminopropyl)triethoxysilane) and 2 equivalents of triethyl amine (Et_3N) was supplemented in the reaction mixture under nitrogen atmosphere. The course of the reaction was followed and Thin Layer Chromatography (TLC) was used to display the end of the reaction. When it was decided that the reaction is over, reaction mixture was dissolved in ethyl acetate. Extraction of the solution was carried out with 20% Na_2CO_3 aqueous solution in order to eliminate Bt. Drying of the organic layer with anhydrous Na_2SO_4 was followed by filtration. Eventually, Cbz-AA-Si molecules were obtained after evaporation the solution in a rotary evaporator. Deprotected amino acid conjugated SAMs were collected after the removal of protective Cbz group under H_2 atmosphere (in EtOH, at 60°C)

3.1.2 Cleaning of the Ti Surfaces

Ti coated glass slides that will be modified firstly were cleaned by dipping 30 minutes in HNO_3 solution (10%) for 30 minutes at 80°C. Then, they were washed by de-ionized (DI) water and dried in oven. Ti coated glass slides were treated by oxygen plasma (March Plasma Systems, PM-100) for 10 minutes and thus, hydroxyl groups were created on the surfaces and they were activated. Plasma conditions were 200 mT and 50 sccm oxygen flow. At the end of the cleaning procedure, Ti surfaces were ready for modification.

3.1.3 Preparation of the SAM Solutions

In order to optimize the self-assembled monolayer formation, dipping time (1-24h) and concentration of the solutions were changed. All solutions used in modifications were prepared by dissolving SAMs in absolute ethanol.

SAM solutions were prepared with different concentrations (1, 2, 5, 10, 20 mM). Solutions were prepared with one of 3 different amido amino acid conjugated self-assembled molecules (histidine, leucine, and tryptophan) in absolute ethanol.

3.1.4 Chemical Modification of Ti Surfaces by SAMs

Modification was performed at room temperature by dipping the samples in the solutions containing SAMs in absolute ethanol. 10 mM solutions were prepared with chosen amido amino acids. After dipping 24 hours, samples were incubated at 120°C for 5 minutes. Then, they were washed by absolute ethanol and DI water. At the end of this step, samples were sterilized. For this purpose, they were washed by 70% ethanol and incubated under UV for 30 minutes.

Effect of amido amino acids on surface hydrophilicty/hydrophobicity was determined by water contact angle measurements. 2 of the amido amino acids were selected (histidine and leucine) according to the water contact angle measurement results, and their mixtures with ranging concentrations were prepared for modification studies (v/v, 100:0, 80:20, 50:50, 20:80, 0:100).

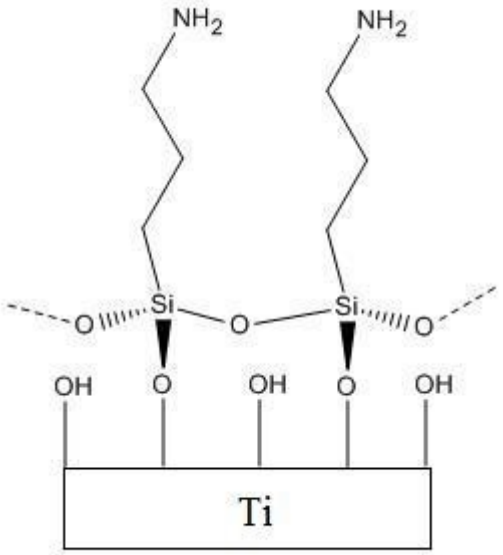

Figure 3.2 APTES modified Ti.

3.2 Characterization Studies

3.2.1 Characterization of SAMs by ^{1}H-NMR

^{1}H-NMR characterization of newly synthesized amino acid conjugated self-assembled monolayers and all by-products were done by ^{1}H-NMR spectroscopy at Eskişehir Technical University (Brueker, 500 MHz, Germany). ^{1}H-NMR spectra (^{1}H-500 MHz) were taken in $CDCl_3$ or DMSO-*d6* with respect to internal standard tetramethylsilane (TMS).

3.2.2 Characterization of the Surfaces

3.2.2.1 Characterization of SAMs by Ellipsometry. Optic thicknesses of the modified surfaces were measured by auto-nulling elliopsemetry (Auto-Nulling Ellipsometer, Nanofilm EP3, Germany). All measurements were taken under green laser beam at 658 nm with incidence angle of 65°. In film thickness analysis one spot auto-nulling, which can integrate on 50×50 µm representative area of the specimen

corresponding to convenient algorithm, was implemented.

In single layer thickness analysis, 4-phase model that composed of Ti/TiO_2/organic layer/air was assumed. Designed surfaces are organic layers that consist of C-bonds on substrates. Because there is a relationship between thickness and refractive index for very thin (<10 nm) films, it should be noted that refractive index of the upper layers could be estimated and therefore their thickness can be determined. In optic model, refractive indexes of Ti, TiO_2, organic layer (His-SAM, Leu-SAM, and Trp-SAM) and air were estimated as 2.24, 2.493, 1.460 and 1.000, respectively.

3.2.2.2 Water Contact Angle Measurements. Static water contact angle measurements of modified Ti surfaces, which are bare Ti, activated Ti, His-SAM, Leu-SAM, and Trp-SAM, were measured by contact angle goniometer (Model DSA 100, Krüss, Germany) by sessile drop method. Calculations were taken in 1 minute with 1µl droplet profile placed on the surfaces and the results were calculated by the software of the equipment. Every sample was measured for 3 different times and results are the average value of these measurements.

3.2.2.3 X-Ray Photoelectron Spectroscopy (XPS). Chemical composition of modified Ti surfaces was determined by XPS (ThermoScientific K-Alpha X-ray Photoelectron Spectrometer, Germany). Monochromated aluminum Kα radiation at 72 W was used during the experimentation with the spot size of 400 µm. 90° angle was used along with a 128-channel detector.

3.2.3 Cell Culture Studies

Cell culture studies were performed with human osteoblast cells (ATCC-CRL-11372, Rockville, MD, USA) in 24-well plates. After modification, Ti surfaces were placed in the plates. 20000 cells per well seeded on the Ti surfaces in 500 µl DMEM-

F12 medium.

3.2.3.1 Viability Test. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on 1^{st} and 4^{th} days. For this purpose, 40 µl (5mg/ml) of the MTT solution were added in the wells. After 3 hours of incubation medium containing MTT solution was discarded. 150 µl of acidic ispropanol solution were added and plates were mildly shaken for 5 minutes. Afterwards, solution were transferred to 96-well plates and measurements were taken by microplate reader at 570 nm and 750 nm (as control) [75].

For the statistical tests, 1^{st} and 4^{th} day data were compared among themselves, taking bare Ti group as control. Analysis of variance (ANOVA), followed by t-tests against control group with Bonferroni correction were applied.

4. RESULTS

4.1 Characterization of Amino Acid Conjugated Self Assembled Molecules by ^{1}H-NMR

Amino acid conjugated self-assembled molecules were used to modify various surfaces such as PDMS, Si wafer and Au in our laboratory. In her Master of Science thesis, Öztürk. Ö., modified PDMS substrates with amino acid conjugated self-assembled molecules (Histidine, Leucine and Tryptophan) in order to mimic the chemistry of the cartilage tissue microenvironment [76]. In another Master of Science thesis by Aktaş B., Si wafers were modified Histidine and Leucine SAMs, to increase the biocompatibility [77]. Si wafer and Au surfaces were modified to manipulate and change the adsorption of the proteins (Albumin, Fibrinogen and Immunoglobulin G) on these surfaces using amino acid conjugated SAMs by Eren S. [78]. Particularly, to show that, those amino acid conjugated SAMs (Histidine, Leucine and Tryptophan) could be also used for the modification of Ti surfaces to evaluate the osteoblastic cell behavior.

4.1.1 His-Si SAM

Histidine amino acid conjugated self-assembled molecules, were characterized by using ^{1}H-NMR spectroscopy (Brueker, 500 MHz, Germany) in $CDCl_3$ or DMSO-d6. Tetramethysilane was used as internal standard. ^{1}H-NMR (500 MHz, $CDCl_3$) δ = 10.2 (broad, s, 1H, NH imidazole), 7.55 (s, 1H, Ar-H imidazole), 6.92 (s, 1H, Ar-H imidazole), 6.80 (s, 1H, amide NH), 4.55-4.45 (m, 1H, -CH-NH_2), 4.23 (broad, s, 2H, -NH_2), 3.85 (q, J= 6.90 Hz, 6H, OCH_2CH_3), 3.30-3.20 (m, 2H, -CONH-CH_2-), 3.12 (broad, s, 1H, CH-CH_2-imidazole), 2.98 (dd, J= 14,16, 5.00 Hz, 1H, CH-CH_2-imidazole), 1.60-1.46 (m, 2H, -CH_2-CH_2-CH_2-), 1.24 (t, J= 8.27 Hz, 9H, OCH_2CH_3), 0.50-0.40 [m, 2H, -CH_2-Si(OEt_3)] ppm (Figure 4.1).

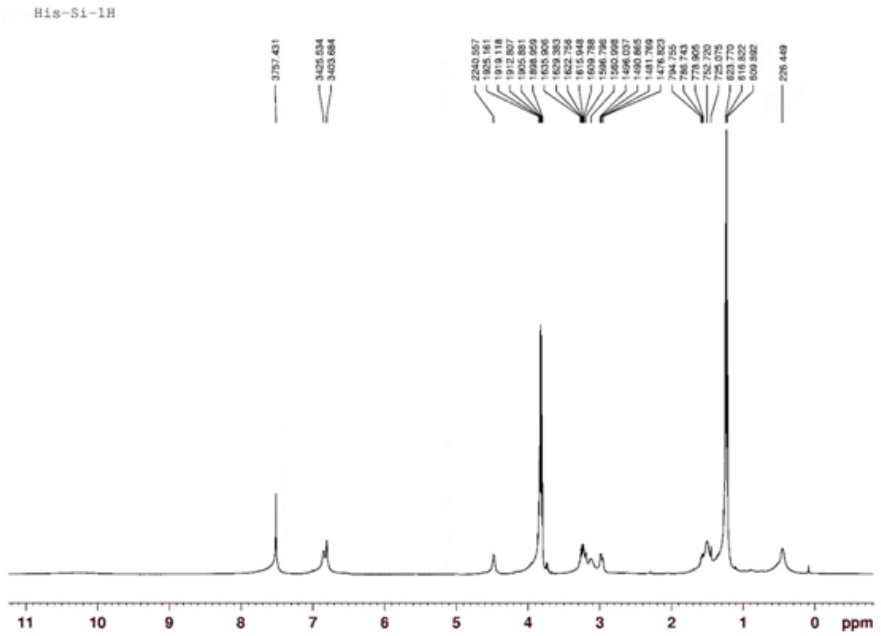

Figure 4.1 ^{1}H-NMR spectrum of Histidine conjugated self assembled molecule.

4.1.2 Leu-Si SAM

Leucine amino acid conjugated self-assembled molecules were characterized by using ^{1}H-NMR spectroscopy (Brueker, 500 MHz, Germany) in $CDCl_3$ or DMSO-d6. Tetramethysilane was used as internal standard. ^{1}H-NMR (500 MHz, $CDCl_3$) $\delta = 7.24$ (s, 1H, amide NH-), 4.25 (broad, s, 2H, -NH_2), 4.15 (dd, J= 8.29, 4.88 Hz, 1H, –CH-NH_2), 3.85 (q, J= 6.98 Hz, 6H, OCH_2CH_3), 3.28 (dt, J= 11.92, 6.35 Hz, 2H, -CONH-CH_2CH_2-), 1.70-1.60 (m, 2H, -CH_2-CH_2-CH_2-), 1.54 (p, J= 8.40 Hz, 2H, -CH_2-CH_2-CH_2-), 1.24 (t, J= 6.98 Hz, 9H, OCH_2CH_3), 0.98-0.92 (m, 6H, -$CH(CH_3)_2$), 0.64 [t, J= 8.00 Hz, 2H, -CH_2-$Si(OEt_3)$] ppm (Figure 4.2).

4.1.3 Trp-Si SAM

Tryptophan amino acid conjugated self-assembled molecules, were characterized by using ^{1}H-NMR spectroscopy (Brueker, 500 MHz, Germany) in $CDCl_3$ or DMSO-d6. Tetramethysilane was used as internal standard. ^{1}H-NMR ($CDCl_3$): $\delta = 11.00$ (s, 1H, NH-indole), 8.00 (s, 1H, NH), 7.60-7.50 (m, 1H, indole-H), 7.30 (d, 1H, J=7.80 Hz,

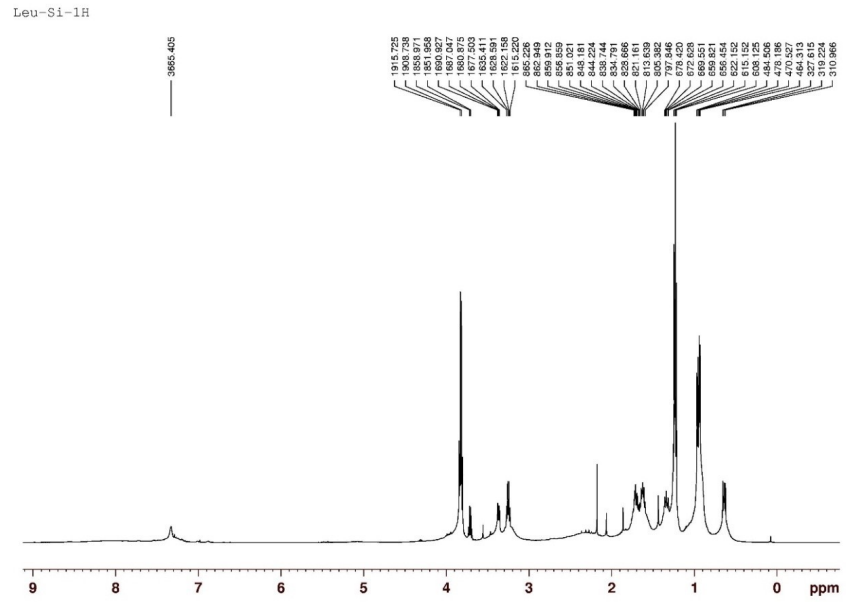

Figure 4.2 ^{1}H-NMR spectrum of Leucine conjugated self assembled molecule.

indole-H), 7.20 (s, 1H, Trp-H), 7.10-6.90 (m, 2H, Trp-H), 3.50 (s, 9H, $(OCH_3)_3$), 3.30-2.90 (m, 3H, NH-CH_2), 2.80-2.60 (m, 2H, -CH_2-), 1.80-1.40 (m, 2H, -CH_2-), 0.80-0.50 (m, 2H, Si-CH_2-) ppm (Figure 4.3).

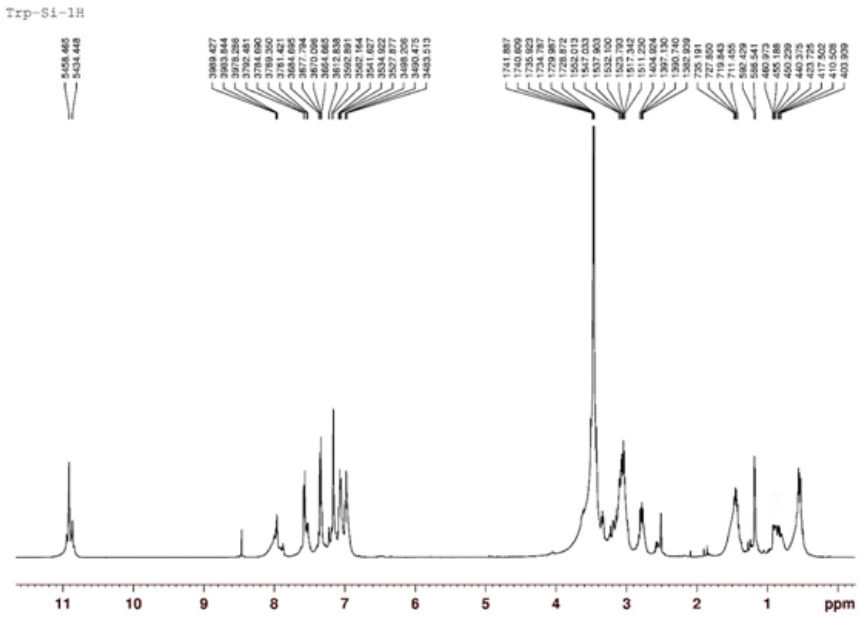

Figure 4.3 ^{1}H-NMR spectrum of Tryptophan conjugated self assembled molecule.

4.2 Surface Characterization

4.2.1 Surface Thickness Measurements by Ellipsometry

Ellipsometry was used to measure SAM thicknesses of the modified surfaces (Auto-Nulling Ellipsometry, Nanofilm EP3, Germany). Thickness of His-SAM, Leu-SAM, and Trp-SAM modified surfaces were measured as 1.8 ± 0.3, 1.8 ± 0.2, 2.2 ± 0.4 nm, respectively.

4.2.2 Water Contact Angle Measurements

Hydrophilic/hydrophobic feature of bare Ti, activated Ti and modified Ti surfaces were determined by contact angle goniometer. Contact angle values of Bare Ti, activated Ti, His-SAM, Leu-SAM, and Trp-SAM modified surfaces are $60.2^\circ \pm 3.8^\circ$, $9.9^\circ \pm 3.3^\circ$, $32.28^\circ \pm 2.34^\circ$, $68.05^\circ \pm 2.84^\circ$, $65.97^\circ \pm 3.47^\circ$, respectively.

Table 4.1
Contact Angle Measurements of Ti Surfaces.

Amino acid-SAM*	WCA (°)
Bare Ti Surface	60.2 ± 3.8
Activated Ti Surface	9.9 ± 3.3
Histidine	32.28 ± 2.34
Leucine	68.05 ± 2.84
Tryptophan	65.97 ± 3.47

*: 10 mM / 24 h dipping time

After the result given in Table 4.1 were obtained, contact angle measurements were executed for the surfaces modified with mixtures of His-SAM and Leu-SAM with ranging percentages (v/v, 80:20, 50:50, and 20:80). Results are given in Table 4.2.

Table 4.2
Contact angle measurements of Ti surfaces modified by ranging concentrations of His-SAM and Leu-SAM.

Amino acid-SAM*	WCA (°)
His/Leu 20:80	65.99 ± 4.81
His/Leu 50:50	55.85 ± 2.82
His/Leu 80:20	39.1 ± 6.92

*: 10 mM / 24 h dipping time

4.2.3 X-ray Photoelectron Spectroscopy (XPS) Analysis

4.2.3.1 XPS Analysis of Bare Titanium. In order to acquire information about the chemical bonds of the molecules on modified and unmodified surfaces, X-Ray Photoelectron Spectroscopy (XPS, Thermo Scientific K-alpha, Germany) at Advanced Technologies R&D Center in Bogazici University were used. Measurements were taken via monochromatic Al Kα X-radiation ($h\nu = 1486.6$ eV) source. XPS spectrums of the samples were analyzed and characteristic of Si2p, Ti2p, C1s, N1s, and O1s areas were investigated for each of the samples (His-SAM, Leu-SAM, Trp-SAM).

In Figure 4.4 survey spectrum of bare titanium is given. C1s, N1s, Ti2p and O1s peaks are shown in XPS survey of bare Ti. In C1s core level analysis, C-Ti, C-C and C-O peaks were found at 284.58, 286.08 and 288.08 eV, respectively. In O1s level Ti-O, C-O and C=O peaks were found at 529.68, 530.98 and 532.38 eV, respectively.

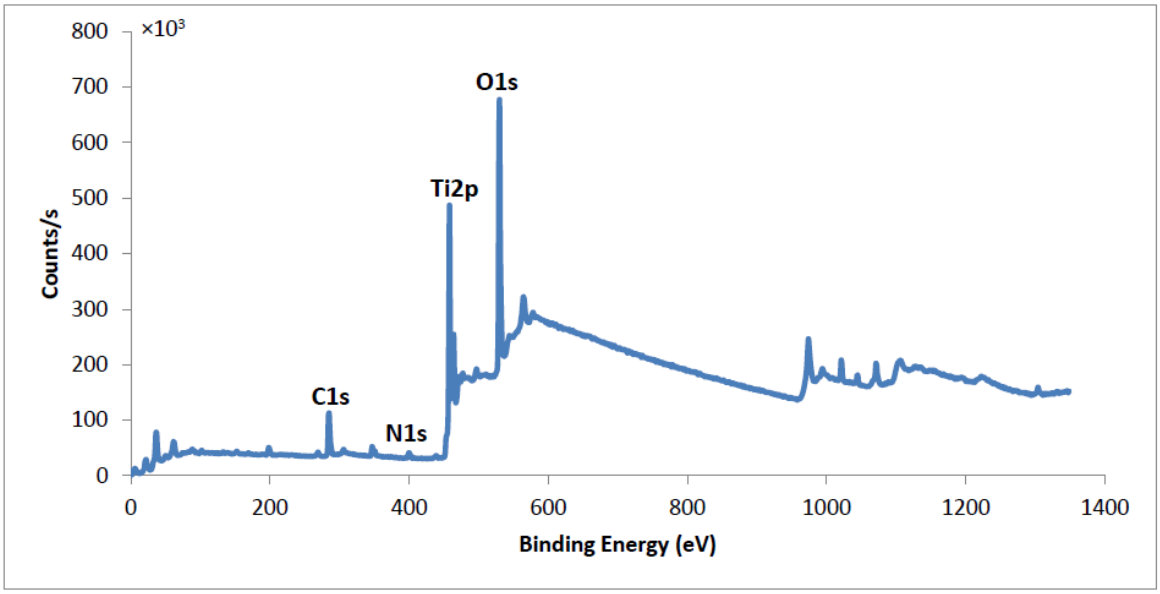

Figure 4.4 XPS Survey Spectrum of Bare Ti.

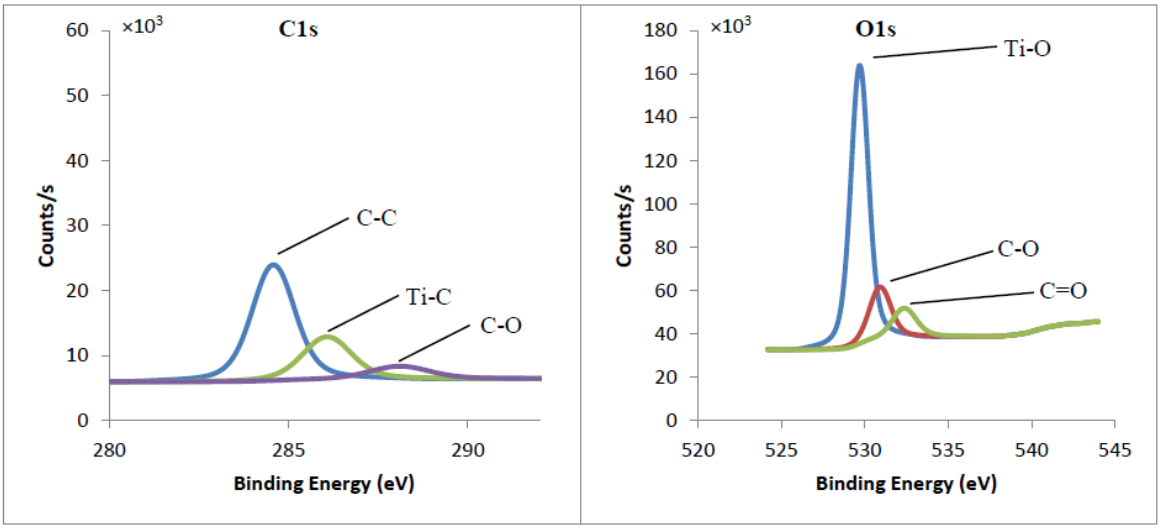

Figure 4.5 High-resolution C1s and O1s Peaks of Bare Ti.

4.2.3.2 XPS Analysis of His-SAM Modified Titanium. In Figure 4.6 survey spectrum of titanium modified by His-SAM is given. It is clear that after modification, levels of C1s and N1s increased while Ti2p and O1s level decreased with respect to bare Ti peaks.

In C1s core level analysis, C-C/C=C, C-Si, C-N, C=N and C=O peaks were found at 284.38, 284.68, 285.18, 286.08 and 287.58 eV, respectively. In O1s level Ti-O, C-O, Si-O and C=O peaks were found at 529.78, 531.18, 531.98 and 533.18 eV, respectively. In N1s core level analysis binding energies at 398.48, 399.38 and 399.68 eV correspond with N-H, -C=NH and R-CN peaks, respectively. In Si2p analysis, O-Si-C peak was found at 102.28 eV.

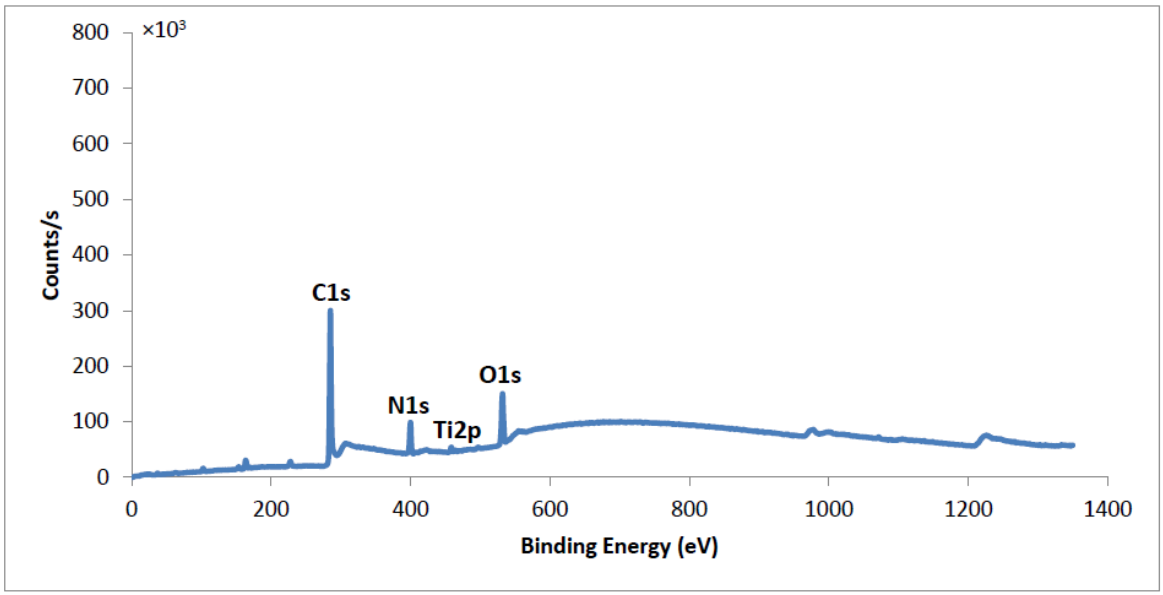

Figure 4.6 XPS Survey Spectrum of His-SAM Modified Ti.

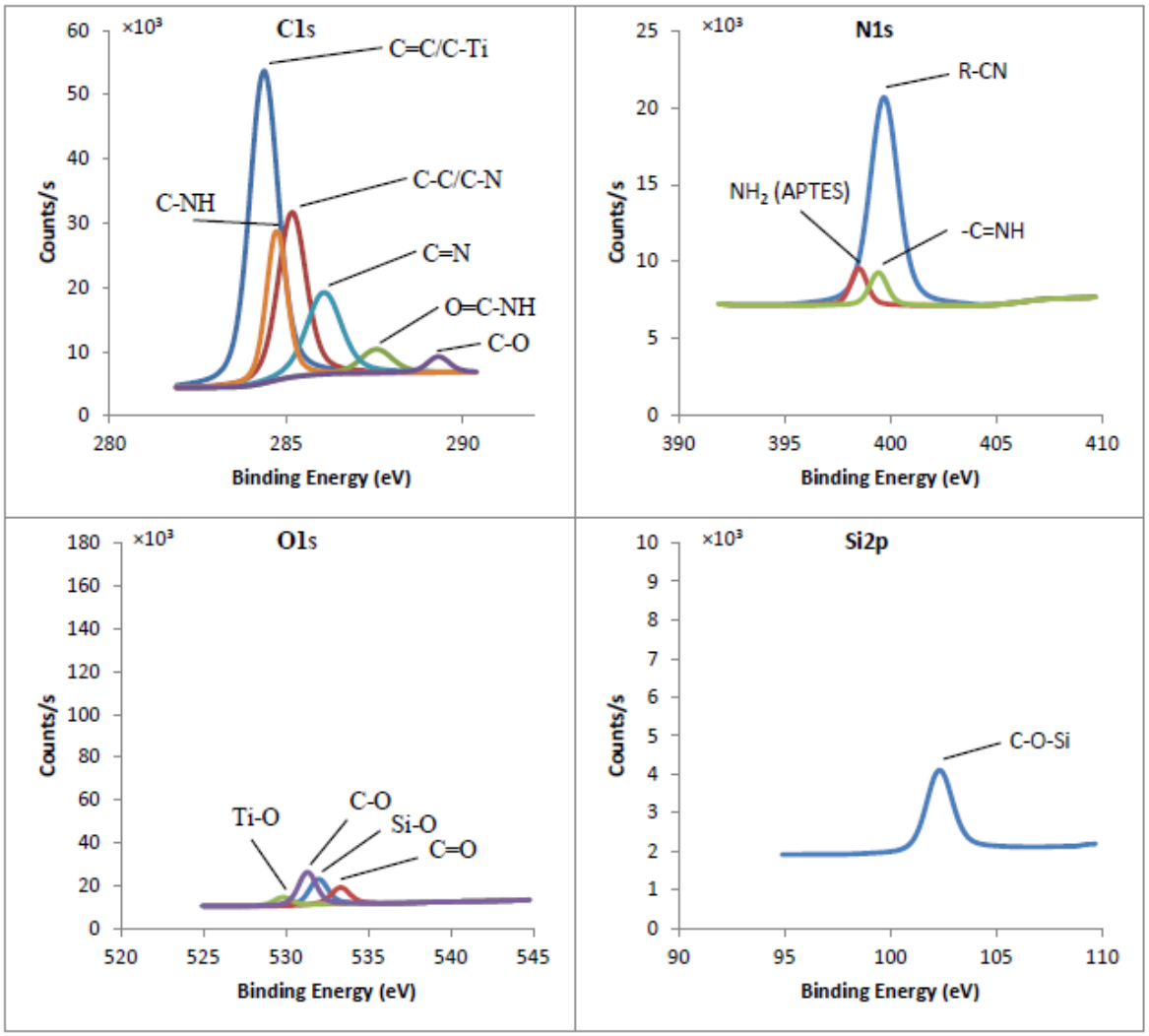

Figure 4.7 High-resolution C1s, N1s, O1s, and Si2p Peaks of His-SAM Modified Ti.

4.2.3.3 XPS Analysis of Leu-SAM Modified Titanium. In Figure 4.8 survey spectrum of titanium modified by Leu-SAM is given. It is obvious that levels of C1s and N1s increased while Ti2p decreased after modification.

In C1s core level analysis, C-C, C-N, C-Si and C=O peaks were found at 284.18, 285.08, 285.98, 287.48 and 288.98 eV, respectively. In O1s level Ti-O, C-O, Si-O and C=O peaks were found at 529.88, 531.18, 531.88 and 532.78 eV, respectively. In N1s core level analysis binding energies at 398.18, and 399.28 V correspond with N-H, and R-CN peaks, respectively. In Si2p analysis, O-Si-C bond was found at 101.58 eV.

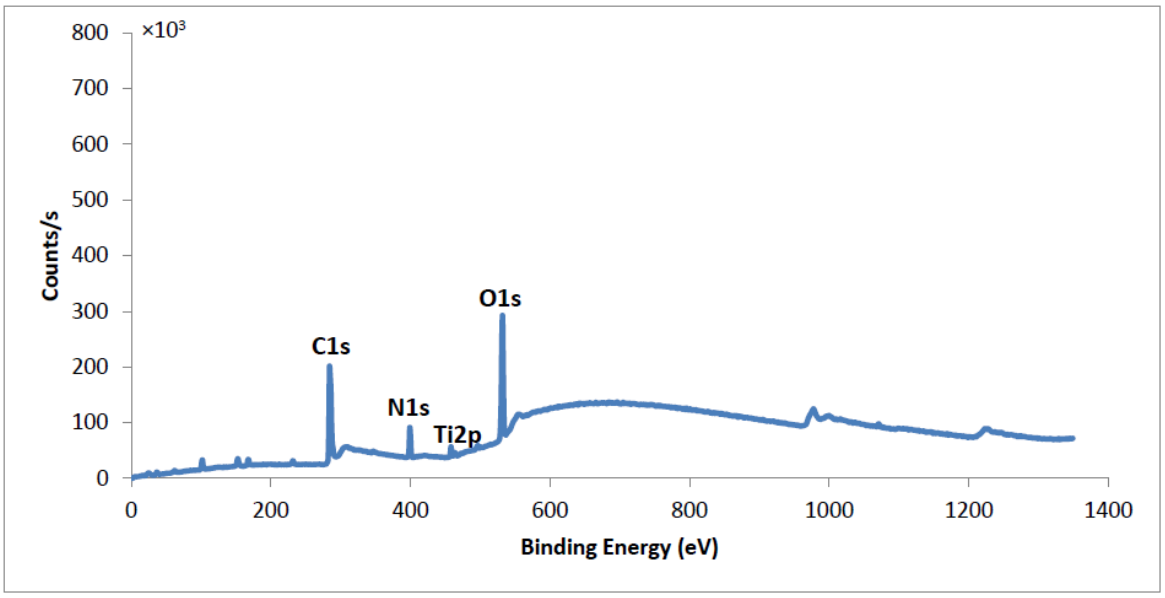

Figure 4.8 XPS Survey Spectrum of Leu-SAM Modified Ti.

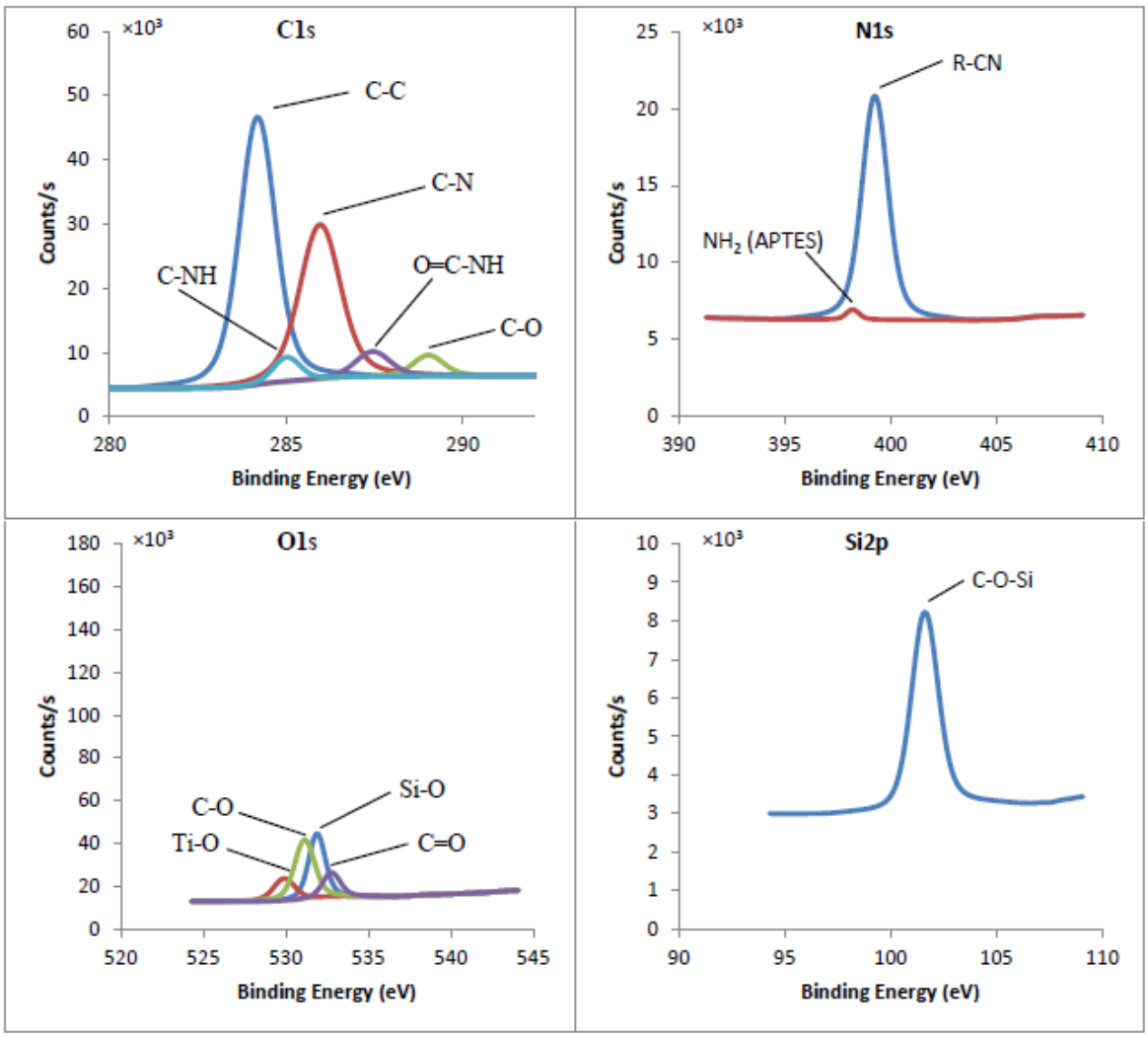

Figure 4.9 High-resolution C1s, N1s, O1s, and Si2p Peaks of Leu-SAM Modified Ti.

4.2.3.4 XPS Analysis of Trp-SAM Modified Titanium. In Figure 4.10 survey spectrum of titanium modified by Trp-SAM is given. It is obvious that levels of C1s and N1s increased while O2s and Ti2p peaks decreased after modification.

In C1s core level analysis, C-Si, C-C/C=C, C-N and C=O peaks were found at 284.08, 284.68, 285.68 and 287.38 eV, respectively. In O1s level Si-O, C-O, Ti-O, and C=O peaks were found at 529.58, 530.48, 531.28 and 532.08 eV, respectively. In N1s core level analysis binding energies at 398.58, 399.28, and 399.38 V correspond with N-H, R-CN, and N-H (ring) peaks, respectively. In Si2p analysis, O-Si-C bond was found at 102.08 eV.

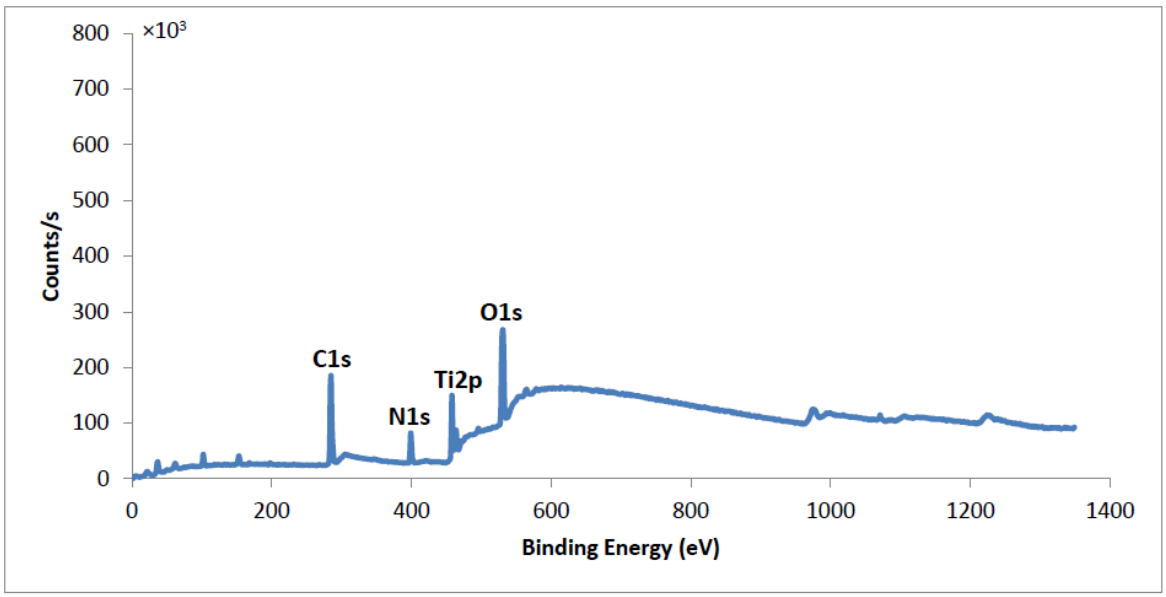

Figure 4.10 XPS Survey Spectrum of Trp-SAM Modified Ti.

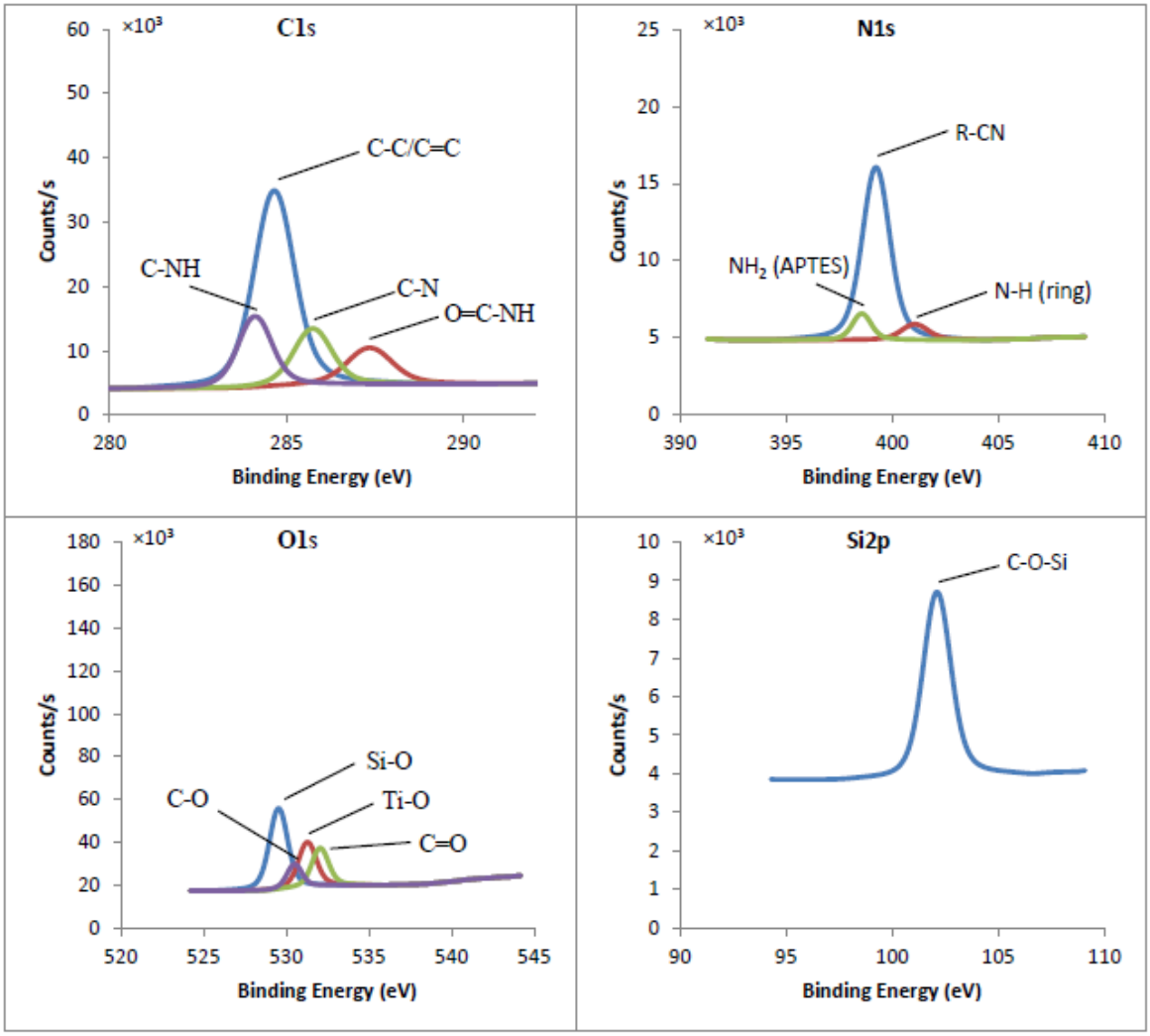

Figure 4.11 High-resolution C1s, N1s, O1s, and Si2p Peaks of Trp-SAM Modified Ti.

4.3 Cell Culture Studies

4.3.1 MTT Assay Results of Bare and Modified Ti Surfaces

MTT assay was used to monitor the health of the cells seeded on Ti surfaces and to measure cell viability. MTT assay was performed on 1^{st} and 4^{th} day of incubation on 24-well plates. Cells were seeded on modified Ti surfaces, and on bare Ti surface. The medium containing no cells was assessed as control. Results are presented in Figure 4.12; higher absorbance represents higher viable cell concentration.

Significant difference was found between 100% His and bare Ti on the 1^{st} day. 100% His, 80% His+20% Leu, 20% His+80% Leu, 100% Leu were found significantly different from bare Ti on the 4^{th} day.

By the end of the first day, viable cell concentrations on all six surfaces were comparable to each other. On the 4^{th} day, influence of surface properties on cell viability became recognizable; cell viability was highest on bare Ti surface, followed by 50% His+50% Leu modified surfaces.

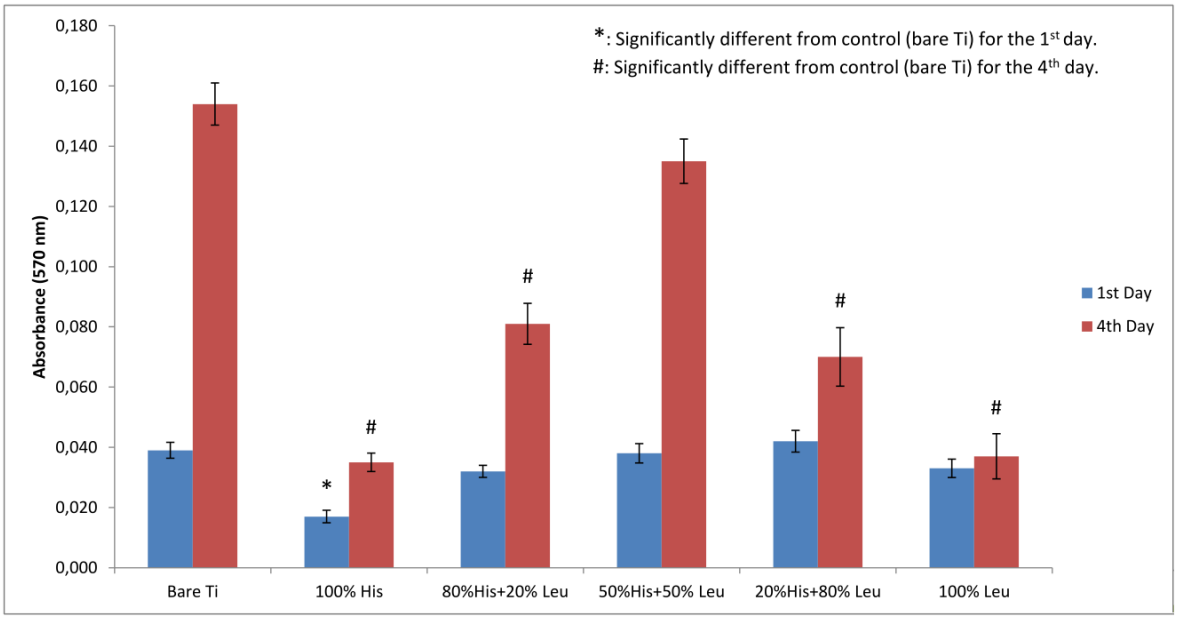

Figure 4.12 MTT assay for assessing cellular viability of osteoblasts on all experimental groups after the 1st and 4th day of application.

5. DISCUSSION

When any part of bone tissue is deteriorated because of congenital reasons, accidents or diseases; and human body is not able to preserve the integrity of the body and to regenerate correctly; different therapeutic methods are used. These methods mostly involve bone grafting not only by biological material but also by synthetic materials [35, 38]. Biomaterials have been used for repairing damaged tissue or organ within the body for decades. Polymeric, ceramic and metallic implants have been used as bone substitutes [38]. Among metallic biomaterials titanium has an important place because of its mechanical properties, its bioinertness and its ability to integrate with bone [55, 56].

Body response is an important factor in rating success of a biomaterial and it has been shown that microenvironment around a biomaterial has a role in regulating tissue homeostasis. Therefore, interaction between implanted material and surrounding cells is of paramount importance [9, 38]. Surface modification of implanted material has been used in order to enhance biocompatibility [4, 58]. Modification by self-assembled monolayers (SAMs) is one of the techniques utilized for surface modification. They provide arrangement of desirable properties on the surfaces [5]. In this study, 3 different amino acids were coupled with APTES ((3-aminopropyl)triethoxysilane) and one-layer thick SAMs were created on titanium surfaces. Exposed ends of 3 amino acids present different scales of hydrophilicity. Human osteoblast cells were seeded on modified titanium surfaces and cell behavior was examined in the scope of this study.

5.1 Characterization of Amino Acid Conjugated Self Assembled Molecules

NMR was used to characterize amino acid conjugated SAMs. Analysis was performed by use of ^{1}H-NMR and physical and chemical features of the molecules were

investigated. In the first place, Cbz-AA molecule was obtained. Afterwards, Cbz-AA was activated with benzotriazole by using carboxyl ends of amino acids and Cbz-AA-Bt molecule synthesized.

In ^{1}H-NMR spectra of Cbz-Amino acids, in addition to the peaks belonging to amino acids, 5H multiplet peak seen around 7.0-7.3 ppm belongs to the benzene ring in Cbz group, and the 2H singlet peak seen around 5.0 ppm belongs to the protons of CH_2 within the Cbz group. Also, ^{1}H doublet peak that is seen around 6.0 ppm refers to the NH group. This is another indicator that the amino acid has bonded with its amine end [79].

As a result of Cbz-protected amino acids' reaction in the presence of benzotriazoliletionilchloride, -OH group of carboxylic acid function is replaced with benzotriazol (Bt) and bonds to benzotriazolcarbonyl group. This way, carboxyl end of amino acid is functionalized. Whether the benzotriazol has bonded is checked via ^{1}H-NMR spectrums. -OH peak seen around 10 ppm in ^{1}H-NMR spectra of Cbz-amino an acid compound is not observed in the spectra of Cbz-AA-Bt compounds. Instead, two triplets seen around 7.5 ppm and two doublet peaks seen around 8.3-8.5 ppm are characteristic peaks of 1-substitute benzotriazole [79].

As a result of a reaction between Cbz-AA-Bt compounds and amines carrying equal amounts of silicon, phosphor and thiol functions Cbz-amido amino acids were acquired. While characteristic peaks belonging to benzotriazole were not seen in ^{1}H-NMR spectra of the acquired products, signals belonging to functional groups on the amine were seen.

At the final stage of the reaction protective group Cbz is removed from the amino acid's amine end by catalytic hydrogenation method with palladium as the catalyzer, to obtain the target molecules, which are amido amino acids (Amino acid-Si). In ^{1}H-NMR spectrums of the products, the 5H multiplet peak at 7.0-7.3 ppm and the 2H singlet peak at 5.0 ppm, which are characteristic of the Cbz group, were not seen. This confirms that the Cbz group was removed.

5.2 Surface Characterization

Surface characterization of modified Ti surfaces were performed by 3 techniques which are surface thickness measurements with ellipsometry, hydrophilicity determination by water contact angle measurement and elemental composition measurement by XPS.

Thickness of His-SAM was measured 1.8 ± 0.3 nm in ellipsometry analysis which is higher than the theoretical value of 1.63. Difference between theoretical and experimental values may derive from the interaction angle of His-SAM with the surface. Theoretical thickness value is obtained when it is assumed that His-SAM molecule creates and angle of 90° with the surface. In some regions of modified surfaces, it is possible that His-SAM molecules oriented in a way more than one layer. According to water contact angle results, Ti surfaces modified with His-SAM exhibit hydrophilic characteristics (32.28°C ± 2.34°C). It is because full coverage of Ti surface of with His-SAM and nature of exposed groups such as imidazole and carboxylic acid.

According to ellipsometry analysis, thickness of Leu-SAM is 1.8 ± 0.2 nm. This value is higher than the theoretical value and it shows that Leu-SAM oriented on the surface with a different angle. Contact angle of Leu-SAM modified surface measured 68.05 ± 2.84. This might be resulted from interaction of side chain methyl groups with each other instead of interacting with the surface. Hence, carboxyl groups of Leu-SAM were directed to the surface and hydrophobicity decreased. Another explanation for low hydrophobicity may be that the surfaces are not fully covered with Leu-SAM.

Thickness of Trp-SAM was measured 2.2 ± 0.4 nm in ellipsometry analysis, which is higher than the theoretical value of 1.7. It is possible that Trp-SAM molecules oriented in a way more than one layer. According Tryptophan is an aromatic and amphipathic amino acid. Modified Ti surface shows partial hydrophobic properties (65.97 ± 3.47); this is because Ti surfaces were not covered fully with Trp-SAM and nature of exposed carboxylic acid groups. Trp-SAM oriented on the surface with a certain angle and polar groups of the amino acid (carboxylic acid and pyrrole ring in

the indole group) repel and mask each other. In this situation, hydrophobic groups in APTES molecule may be exposed and increase hydrophobicity. Indole groups may be repelled because of the steric effect, Trp-SAM interacts with the surface at a steeper angle. Covered area with Trp-SAM increase and indole groups are pushed out toward the outer region, exposing the hydrophilic group.

XPS was performed in order to get molecule structure of modified surfaces. High resolution C1s, O1s, Ti2p and peaks were investigated for bare Ti. For modified surfaces, N1s and Si2p spectra were recorded in addition to C1s and O1s peaks.

According to XPS analysis of His-SAM modified Ti surfaces specific bonds in C1s, O1s, N1s, Si2p and Ti2p peaks were defined. In C1s high resolution spectrum C-C/C=C, C-Si, C-N, C=N and C=O bonds at 284.38, 284.68, 285.18, 286.08 and 287.58 eV, respectively, proved success of the modification. C1s spectrum of Leu-SAM modified Ti surfaces reveals C-C bond at 284.18 eV, C-Si bond at 285.98 eV, C=O bond at 287.48 eV and C-N bond at 285.08 eV. C1s spectrum of Trp-SAM modified Ti surfaces reveals C-Si bond at 284.08 eV, C-C/C=C bond at 284.68eV, C-N bond at 285.68 eV and C=O bond at 287.48 eV. These specific bonds indicate surfaces were covered with amino acid conjugated SAMs.

Acquired XPS data for His-SAM, Leu-SAM and Trp-SAM was analyzed. Specific bonds in O1s spectra were defined and compared with XPS data of bare Ti. Appearance of Si-O bond at 531.98 eV, 531.88 and 529.58 for His-SAM, Leu-SAM and Trp-SAM, respectively, confirm the successful modification of the surfaces because Si-O bond appears in the presence of APTES.

In N1s core level analysis of His-SAM modified surfaces, binding energies at 398.48, 399.38 and 399.68 eV correspond with C=N, N-H and C-N peaks, respectively. According to N1s core level analysis of Leu-SAM modified surfaces, binding energies at 398.18, and 399.18 eV are correlated with N-H, and C-N peaks, respectively. When Trp-SAM modified surfaces were investigated deconvolution of N1s peaks show bonds at 398.58 and 399.28 which correspond to N-H and C-N bonds, respectively. Presence

of amino acid conjugated SAMs on Ti surfaces is demonstrated by N-H and C-N bonds.

In Si2p core level analysis, O-Si-C bond found at 102.28, 101.58 and 102.08 eV on His-SAM, Leu-SAM and Trp-SAM modified surfaces, respectively. Existence of O-Si-C bond on modified surfaces shows that there are APTES molecules on the surfaces.

According to contact angle results His-SAM was chosen as the most hydrophilic group and Leu-SAM as the most hydrophobic molecules. Because main aim of this study is to investigate surface wettability on cellular behavior, mixtures of His-SAM and Leu-SAM was prepared to modify the surfaces. Hence, 3 groups were obtained in addition to 100% His-SAM and 100% Leu-SAM, which are 80% His-SAM+20% Leu-SAM, 50% His-SAM+50% Leu-SAM and 20% His-SAM+80% Leu-SAM (v/v). Contact angle results of these mixture groups are given in Table 4.2. It can be detected that most hydrophilic group is 80% His-SAM+20% Leu-SAM and most hydrophobic group is 20% His-SAM+80% Leu-SAM, as expected.

5.3 Cell Culture Studies

In this study, MTT assay was utilized to evaluate osteoblast cell growth and proliferation on different Ti surfaces. Experiments were performed in 24-well plates and measurements were taken on days 1 and 4. Cells were seeded on six different surfaces.

1st day MTT results show that there were no dramatic differences in terms of cell viability between groups except for the 100% His group. On day 4, increase in cell population was observed for all groups. Bare Ti group had the highest cell number on day 4, followed by 50% His+50% Leu group.

It is known that moderately hydrophilic surfaces are osteogenic and promote osteoblast adhesion on Ti surfaces [66]. According to MTT results (Figure 4.12), viable

cell concentration is at its minimum on the most hydrophilic and the most hydrophobic surfaces on day 4, which are 100% His group and 100% Leu group, respectively. Nevertheless, when compared to the results of the 1st day, higher cell proliferation is observed on the 100% His surface. That is, the increase in viable cell number from 1st day to 4th day is greater on the 100% His surface. This observation suggests that hydrophilic surfaces are more favorable for osteoblast cell proliferation than hydrophobic surfaces, although hydrophilic surfaces allow less cells to adhere on the first day. This can be attributed to the shell effect created by water molecules. Comparing the 80% His+20% Leu and 20% His+80% Leu samples, it is observed they have similar viable cell concentrations on the 4th day. Same relation between hydrophilicity and early cell adherence is noticed. More cells attached on the more hydrophobic surface (20% His+80% Leu) by the 1st day of incubation; however, cell proliferation was superior on the more hydrophilic surface at the end of 4th day (80% His+20% Leu).

Highest viable cell concentration was obtained on bare Ti and 50% His+50% Leu modified surfaces. Both surfaces show the greatest proliferation between 1st day and 4th day. As shown in Table 4.1 and Table 4.2, hydrophilicity of bare Ti and 50% His+50% Leu modified surfaces is similar and cell proliferation can be correlated with that. MTT results support the idea that moderately hydrophilic surfaces optimally promote cell proliferation [66]. Hydrophilicity is not the sole arbiter of biocompatibility, specific interactions between osteoblast cells and functional groups of amino acids could emulate osteoblast viability.

Lee et al demonstrated that more osteoblast cells could attach on hydrophilic titanium surfaces. Cell attachment on surfaces is determined by chemical activities occurring within the cell-material interface and mostly associated to surface energy [72]. According to an animal study conducted by Erikkson et al, bone fixation has better results when rats' tibiae were implanted by titanium having hydrophilic surface [80]. Other parameters affecting bone formation and osteogenesis can be listed but not limited to surface topography, roughness and surface chemistry. A study demonstrated that surface roughness and chemistry of titanium guide adhesion and proliferation of osteoblasts by alternating length of integrins. Integrins are transmembrane proteins

involved in cell adhesion. They do not only attach cell cytoskeleton to the ECM but also sense the physical environment biochemically [73]. Schwartz et al. emphasized that surface modification of titanium with RGD peptide tethering enhanced adhesion and spreading of osteoblast cells [81].

Although viable cell concentration data gives a perspective on health of the cells, other properties of osteoblast cells should be considered such as differentiation and adhesion. MTT assay a proper method to evaluate cell viability and proliferation. Different techniques can be used to see if an osteoblast is differentiated into an osteocyte or the extent of attachment of the osteoblast cells. It might be the case that the characteristics of the cells cultured on 50% His+50% Leu modified surface are more likely to adhere on an implant surface and differentiate into osteocyte.

5.4 Future Studies

This study supported the idea that Ti surfaces having different hydrophilicity characteristics affect cell behavior. Additional studies in the future may provide a better insight on cell proliferation and cell differentiation. For this purpose, studies with osteoblast cells can be conducted on modified Ti surfaces; and techniques, such as scanning electron microscope imaging, alkaline phosphatase activity measuring and/or alizarin red staining, can be used to show differentiation of cells into osteocytes.

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