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Alpha–Tocopheryl Phosphate: a biomolecule for the modification of chemically treated Ti6Al4V alloy surfaces for antibacterial and anti-inflammatory purposes

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Abstract

The role of vitamin E in human health has been widely studied since its discovery as the nature's most potent lipid-soluble antioxidant. Now, other properties have been discovered as its anti-inflammatory and antibacterial activities. In this thesis, a water-soluble form of Vitamin E, the α-tocopherol phosphate, associated with titanium surfaces will be discussed. In particular, the purpose of this this work is to develop an anti-inflammatory and antibacterial surface by coupling a chemically treated Ti6Al4V surface with the above biomolecule for orthopedic and dental prosthetic devices. The titanium alloy (Ti6Al4V) disks used were firstly treated using a patented thermo-chemical surface treatment consisting of an acid etching followed by a controlled oxidation. The surface obtained was characterized in previous works. The treated titanium disks were then immersed in a $CaCl_2$ solution to achieve a positively charged surface, thus creating a suitable surface where negatively charged α-tocopherol phosphate could create an electrostatic bond. Two different samples were created, one functionalized and one coated with α-tocopherol phosphate, and they were characterized by means of different physico/chemical techniques. The results showed the successful grafting of the molecule and the expected differences between the functionalized and the coated sample. The topography obtained through the thermo-chemical treatment was preserved (Kelvin probe analysis) on both samples. Release tests were performed to analyze if the molecule remains on the surface if immersed in solutions at different pH values and different results were obtained for the two samples analyzed. Lastly, biological characterization was carried out by cytocompatibility test using hMSC, which viability resulted constant throughout the duration of the test, and antibacterial activity was assessed by evaluating the ability of *S. epidermidis* to grow on the treated surface.

1 Introduction

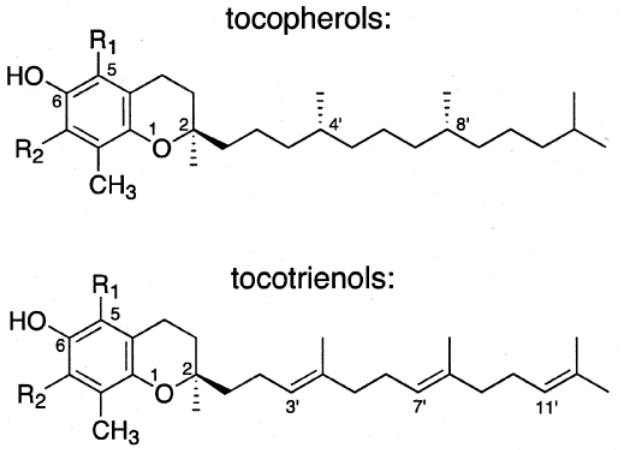
Prosthetic bacterial infections are a burden for the global health care industry as well as for individual patients.[1] Moreover, the antibacterial drug resistance is increasing, mostly due to their overuse or misuse, and new strategies to fight or prevent infections are in high need. Surface modification techniques and new molecules with antibacterial activity are being tested in order to create antibacterial surfaces for medical implants. So far, not many attempts have been made to couple natural biomolecules to inorganic biomaterials.[2] In the search of new antibacterial agents, many studies have been carried out and led to good *in vitro* results using vitamins. In particular, vitamin E in recent years has been studied not only for its antioxidant and anti-inflammatory properties but also as enhancer of antibacterial activity of different antibiotic compounds [3] and as direct antibacterial agent itself even if this capacity is still under investigation[4]. In this thesis, a water-soluble form of Vitamin E, the α-tocopherol phosphate, associated with titanium surfaces will be discussed. Therefore, the first part of this work will focus on the different roles in human health and the current applications of the above cited vitamin E and, in particular, the phosphate ester of its most active isoform, the α-tocopherol. The material chosen as substrate in this thesis will be discussed in the second chapter. Titanium and its alloys are the most used materials in the prosthetic implants field thanks to their fatigue resistance, stable chemical properties and good biocompatibility. Different surface modification techniques and agents used to make the titanium surface antibacterial present in literature have been collected and described. The second part of this work will be dedicated to the description of the process and the characterization of the developed anti-inflammatory and antibacterial surface. The samples obtained were characterized to evaluate the presence of the vitamin on the surface by means of different physico/chemical techniques such as contact angle, Z potential, X-ray photoelectron spectroscopy (XPS), UVspectroscopy, FTIR analysis and Kelvin probe force microscopy. In addition, biological characterization was carried out by cytocompatibility and antibacterial tests.

2 Vitamin E

Vitamins are organic substances essential in small quantities for the proper functioning of an organism's metabolism. They are generally classified as either fat-soluble or water-soluble; the fat-soluble (A, D, E, and K) are accumulated in fat tissues while the water-soluble (B1, B2, niacin, B6, folic acid, pantothenic acid, biotin, B12, ascorbic acid) can be stored only in small amounts. Vitamin E, that belongs to the first category, can only be obtained through diet but the body is able to store it so it has not to be consumed frequently. Vegetable and animal origin fats, in fact, are sources of vitamin E, especially plant derived oils that represent the major source of this vitamin in the human diet. The term vitamin E refers to two groups of related compounds, the tocopherols and tocotrienols, each with four forms (α-, β-, γ-, δ-). All the forms are not redundant as regards their biological functions. [5] Shortly after its discovery, Vitamin E antioxidant capacity was studied and in the 1980 its antioxidant action against lipid peroxidation was assessed[6]. The more studied isoform is the α-tocopherol as it is the most abundant in the human body and works as a peroxyl radical scavenger protecting polyunsaturated fatty acids in membranes and lipoproteins [6, 7].

2.1 Chemistry and Biochemistry

As said before, the term Vitamin E encases eight different but structurally related forms divided into two main groups: the tocopherols and the tocotrienols. They are all formed by a chromanol ring system with two to four methyl groups and a 16 carbon chain in position two. The side chain is saturated in tocopherols and unsaturated in tocotrienols (double bound in position 3',7' and 11') while the α-, β-, γ-, and δ-forms differ as regards the number and the position of methyl groups on the chromanol ring (the α-forms have three methyl groups, the β- and γ-forms have two and the δ-forms have one methyl group). The tocopherols present three chiral centers at carbon 2, 4' and 8' and all the natural isomers have the RRR-configuration. All the forms possess antioxidant activity,



tocopherol/ tocotrienol	R_1	R_2	relative antioxidant activity (%) in vitro
α-	CH_3	CH_3	100
β-	CH_3	H	71
γ-	H	CH_3	68
δ-	H	H	28

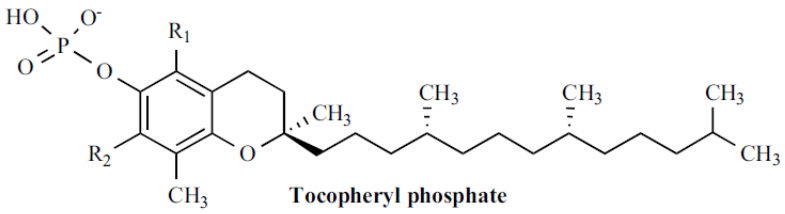
Figure 1: Vitamers

but the α-tocopherol is the most active from a chemical and biological point of view [8]. The tocopherols are exclusively biosynthesized by photosynthetic organisms and more precisely in the membrane of chloroplasts. In recent times, an enzyme, the tocopherol cyclase enzyme, has been identified as responsible for the tocopherol biosynthesis[5].

2.2 a-Tocopherol

Only α-tocopherol (α-T), of the 8 major analogues of vitamin E, has raised a particular interest for its strong antioxidant properties and because it is present

in the highest concentration in plasma [9]. In fact, a specific protein in the liver, the α-T transfer protein (α-TTP), has the greatest affinity for this form of the vitamin while the others are secreted via the bile or the urine [10]. The α-T form also accumulates at sites where free radical production is greatest (the membranes of the mitochondria and endoplasmic reticulum in the heart and lungs for example) [11]. All Vitamin E isomers are practically insoluble in aqueous solutions, but soluble in oils, acetone, ethanol, ether, and other organic solvents [8]. Over the past few decades, a water-soluble form of vitamin E, the alpha-tocopherol phosphate (α-TP), has gained interest especially after it was found in the plasma and the tissues of animals and humans in a similar amount as the α-T [12].



Tocopheryl phosphate

R_1	R_2	
CH_3	CH_3	α
CH_3	H	β
H	CH_3	γ
H	H	δ

Figure 2: α-tocopherol phosphate

The structures of the α-T and α-TP differs in the presence of a phosphate group instead of a hydroxyl group on the phenolic ring [13]. The stability and, at the same time, the chemical reactivity of the α-tocopherol are given by the location of the reactive -OH group that is in between two methyl groups on the phenolic ring: when there is a reaction with a lipid peroxide, the unpaired electron delocalizes over the completely substituited chromanol ring.The fact that the

chromanol -OH group in the α-TP is phosphorylated, there is no antioxidant activity *per se* but through indirect antioxidant effects, possibly by acting at membrane level preventing the propagation of free radicals or directly by interfering in their generation at an enzymatic level. In cells and tissues have been detected the enzymes α-T kinase and α-TP phosphatase or esterase that are capable of phosphorylating α-T to α-TP as well as the reverse reaction to maintain cellular homoeotasis or its inter-conversion may have some cellular signalling functions [12]. When the α-tocopherol is phosphorylated to the phosphate form, the new molecule results negatively charged ($PO_4$2-) which allows the binding to charged surfaces [14]. Studies suggested that α-TP may exhibit some regulatory activities at the cell level different from the α-T. In fact, it can act like an active lipid mediator modulating the signal transduction and gene expression. As concern the gene expression, it was demonstrated that α-TP more effectively reduced the THP-1 cell proliferation and modulated the expression of the CD36 scavenger receptor, suggesting its important role in the prevention of atherosclerotic and inflammatory events [12]. The potentiated effect on angiogenesis and vasculogenensis of the phosphorylated α-T could have important pathophysiological implications: in fact, angiogenesis has an important role in the development of a tumor and migration of the neoplastic cells making the α-TP a form of vitamin E without anti-cancer properties [15]. Recent studies have demonstrated the bacteriostatic and biofilm penetration properties of this form of vitamin against bacterial strains such as *Streptococcus oralis* in dental implants and antibacterial and antiadhesive properties in prosthetic implants for *Staphylococcus aureus* tested *in vivo* on animal models [16, 14]. Moreover, the same studies have noticed a better osteointegration of the implants in the presence of α-TP leading to the hypothesis that it could also stimulate bone precursor cells and osteoblast to enhance new bone deposition [16].

2.3 Role of Vitamin E

In 1922 Evans and Bishop, while they were studying the dietary factors essential for reproduction in rats, discovered vitamin E as a required substance to prevent fetal resorption [6]. In the past decades, many publications have been produced regarding the functions of this Vitamin, especially the α-tocopherol form, and the importance of it in the human diet. The efficacy of Vitamin E supplementation is still not proven and Vitamin E has not yet any clinical applications. The most known activity of Vitamin E is its antioxidant properties: it consist of the scavenging of reactive oxygen species (ROS) by donating the hydrogen from the hydroxyl group present on the phenolic ring. It neutralizes free radicals before they originate lipid peroxidation or DNA damage [5]. It also protects, by peroxyl radical-scavenging, the polyunsaturated fatty acids that are present in membrane, phospholipids and plasma lipoproteins [11]. Studies determined the effects on DNA damage of a mixture of tocotrienols and the α-tocopherol discovering that the mixture has a stronger antioxidant effect in healthy humans than α-tocopherol alone. The oxidative stress can be associated with different ailments including cardiovascular disease, cancer, neurodegenerative diseases, cataracts, macular degeneration and more [9]. More recently, a large number of studies that aimed to confirm the antioxidant capacity of vitamin E in vivo had failed showing rather no benefits of vitamin E supplementation. Moreover, high doses vitamin E significantly increased overall mortality [17]. Another role of Vitamin E in the human body is the inhibition of platelet aggregation: it has been observed that an increase in the concentration of α tocopherol in the endothelial cells increases the release of prostacyclin, an effective vasodilator with the ability to inhibit platelet aggregation. Other studies associate the role on tocopherols in platelet aggregation to its ability to inhibit the protein kinase C (PKC) and to increase the action of nitric oxide synthase [11]. The protein kinase C is a family of enzymes that promotes lipid hydrolysis. More in detail, the α-tocopherol acts by attenuating the generation of diacylglycerol, a lipid that increases the activity of PKC. Also in this case, as seen previously

discussing the antioxidant activity of Vitamin E, mixed tocopherols prove to be more effective then just α-tocopherol in the inhibition of platelet aggregation [11]. Vitamin E has also a regulatory role as concern the activation or the inhibition of VEGF: the fetal resorption in rats initially studied could be a consequence of VEGF inhibition that may cause an impaired formation of vascular net in the placenta, inadequate nutrient supply to the fetus and placental ischemia. This ability of Vitamin E can be associated with the positive results in the prevention of ischemia/reperfusion injury in the cardiovascular and nervous system, the stimulation of neurite outgrowth and wound healing and the prevention of neurodegeneration. Lastly, Vitamin E has showed to significantly diminish inflammation biomarkers both in vivo and in vitro proving its effective anti-inflammatory proprieties [17]. Nowadays antibiotics are the most common therapy for microbial infections, but their overuse is creating a multidrug resistant strains of microorganisms that is leading the research into finding alternative antibacterial substances. Vitamin E has demonstrated, through agar diffusion method, to be a valid antibacterial agent for clinical strains of bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa*). These bacteria are resistant to antibacterial agents such as erythromycin, cephalexin, clindamycin and tetracycline but showed positive results with Vitamin E. Different concentration works for different strains: for example, 200 IU/ml was effective with *S. aureus* and *S. epidermidis* while *E. coli* needed a concentration of 400 IU/ml to respond. In general, gram negative bacteria are more resistant than gram positive bacteria towards antibacterial substances including vitamin E. This is related to the morphology of the cell wall: gram negative bacteria present an outer membrane containing phospholipids and lipopolysaccharide that acts as a protective layer and it is also the only feature that distinguish them from gram positive bacteria [18].

2.4 Vitamin E and diseases

Due to its strong antioxidant properties, vitamin E was expected to be effective in many oxidative stress related diseases, but the results *in vivo* are quite disappointing. It was hypothesized and reported that vitamin E supplementation could improve cardiovascular functions that are caused by the oxidation of low-density lipoproteins and the consequent inflammation. Recent interventional clinical trials denied the previous results showing no cardiovascular benefit of vitamin E supplementation. Indeed, an increased risk of hemorrhagic stroke was observed. Some results have also supported the antiproliferative and apoptotic activities on human cells supporting the theory that vitamin E could possess anticancer activity. As seen previously, α-tocopherol inhibits the production al PKC which, for some researcher, is strictly linked cancer cell growth [11, 19]. The form of vitamin E that proven to be the most effective as concern the growth inhibitory effect on cancer cell, in particular the prostate cancer cells line, is the γ-tocopherol, while δ-tocopherol showed inhibitory effects on mammary cancer cell line in murine model. γ-tocopherol, in particular, has demonstrated a number of mechanisms to inhibit cancer cells growth: it traps free radicals like nitrogen species that are a cause of deoxyribonucleic acid strands mutations and malignant transformation in cells and it also downregulates cyclins that are control molecules that prevent cancer cells proliferation. Tocotrienols as well are able to induce apoptosis in cancerous and healthy cells through mitochondria-mediated pathways or the suppression of cyclin D, that is involved in the cell cycle, or thanks to the inhibition of vascularization. As well as for the cardiovascular effect, the anticancer activity shows to be promising in vitro while human trials have pointed out no significant reduction in the risk of developing cancer in individuals taking Vitamin E supplementation. The documented in vivo effects of vitamin E include a protective role against neurological complications in ataxia with vitamin E deficiency disease(AVED), the modulation of innate immune response to enhance resistance to bacteria pneumonia and as treatment of nonalcoholic steatohepatitis (NASH) in adults without diabetes. Dietary vita-

min E deficiency is not very common in humans and it is mostly a consequence of other syndromes such as abetalipoproteinemia, chronic cholestatic liver disease, chronic pancreatitis, cystic fibrosis, progressive systemic sclerosis, short-bowel syndrome or other lipid malabsorption syndromes [15].

2.5 Current Applications

While there is no scientific evidence to support the use of vitamin E in most of the conditions discussed (with the exception for AVED and Vitamin E deficiency), it is largely used in dermatology: it is available in cream form and as oil for topical use as it works as free radical scavenger protecting the skin from solar radiation [20]. Another important field where vitamin E is having success is as dietary supplement, even if, also in this case, there is not enough evidence of real benefits. Currently, vitamin E is starting to gain ground is the biomedical field where in the past 20 years many scientific studies have been made. Starting from prosthetic implants, this molecule can be blended in the implant's material or used as a coating. The most well known application in prosthetic implants is in stabilizing high molecular weight polyethylene (UHWMPE). UHMWPE is a polymer which biocompatibility, high wear resistance, good toughness, high impact strength, good resistance to corrosive chemicals and low cost makes it a great material for orthopaedic implants, particularly at interfaces subject to high stress, such as those in hip or knee replacements. This material is has been used clinically for over 60 years, at the beginning for acetabular components, then as bearing material in total hip replacement. The problem encountered at the time was that the contact with harder materials, metals or ceramics, led to the continuous reorientation of the chains of the polymer and the production of wear debris. The wear debris resulting from the friction cause an inflammatory response which may induce osteolysis, the resorption of periprosthetic bone, and the consequent loosening of the implant and weakening of the bone structure. To decrease the wear rate of UHWMPE, high crosslinking process has been developed which consists of radicalizing the side chains with different type of

radiation (gamma ray, electron beam) or chemicals (peroxide) decreasing in this way the mobility of the polymer chains but it also triggers the formation of free radicals. The cross-linked polymer was then thermally treated to improve the oxidation resistance. Until the 1990, prior to implantation, the UHWMPE components were sterilized by gamma irradiation in air that induced the formation of free radicals in the polymer that reacted with oxygen during storage or in vivo, inducing, again, the oxidation of UHMWPE. In order to prevent oxidative degradation, antioxidant vitamin E (α-tocopherol) was added in UHMWPE either by diffusion or by blending vitamin E with UHMWPE powder before consolidation and then crosslinked by irradiation. Recent literature evidences indicate that oxidative stress is present even when UHMWPE is modified with Vitamin E but cell damage is prevented by the antioxidant effect in vivo of the vitamin which works as a scavenger of oxidant molecules when they are present in high concentration. Vitamin E did not show any cytotoxic or genotoxic effects when blended with UHMWPE and gamma-sterilized in vitro. Another cause of implant loosening is biofilm-related infections caused by pathogens' adhesion (mainly by Staphylococcus species) on the surface of metal implants. Bacterial infections is a severe complication of a joint replacement surgery and not always is curable with antibiotics as once the biofilm has formed, it is very difficult to eradicate as the extracellular polymeric substances that forms the biofilm protects the bacteria embedded in it. If not rapidly cured, the infected implant has to be removed, the infection cured, and a new implant must be inserted. Bacterial adhesion occurs in the first hours after invasion of the host tissue and it is believed that most of the prosthesis-related infections are a result from direct contamination during the operative procedure [1]. Different antibacterial coatings have been tested during the last decades among which two forms of α-tocopherol: the acetate ester and the water-soluble phosphate ester. To prevent the bacterial adhesion and the biofilm formations, the α-tocopherol phosphate resulted particularly successful in vitro and on animal models, both on Gram-positive and Gram-negative. The bacterial strains tested in the studies were S. epidermidis, S. aureus, Pseudomonas aeruginosa which are the ones that easily

form biofilm on titanium. S. aureus and S. epidermidis, are considered the most frequent pathogens responsible of prosthetic joint infections [1]. The coatings, as for now, are created by simple adsorption of the vitamin E on the surface and the substrates used in these studies that are mostly commercially pure titanium, predominantly used in dental implants. The samples coated with α-tocopherol phosphate showed a notable anti-adhesive ability especially towards the strains of S. epidermidis and S. aureus [4]. To assess the biofilm formation, the traditional colorimetric essay cannot be used because the α-tocopherol phosphate coated on titanium interferes with the spectrophotometric reading as it binds crystal violet. For this reason, the amount of biofilm produced on coated and uncoated samples was evaluated through CFU method (conventional plate counting). Another interesting result was found evaluating the presence of the vitamin E in the culture medium by means of high-performance liquid chromatography (HPLC): very low amounts of the vitamin were found in the medium meaning that the absorption method effectively binds the molecule to the substrate [4]. The mechanisms of action and the spectrum of activity of α-tocopherol esters are still under investigation but so far, the results obtained make them valid candidates as coating to prevent implant-associated infections. Coatings using Vitamin E are also tested on other materials such as polystyrene and silicone to evaluate if the antibacterial activity of the vitamin could also work in the prevention of ventilator-associated pneumonia (VAP), lower respiratory tract infections and catheter-associated urinary tract infections (CAUTI) [21]. Wound healing is a complicated biological process in which four different phases can be distinguished: the homeostasis phase, the inflammation phase, the proliferation phase and the remodeling phase [22]. In patients affected by different conditions like diabetes, vascular diseases or obesity, this process may fail in one or more phases leading to chronic wounds in which the tissue cannot regain its structural and functional integrity. Diseases predisposing to poor wound healing are more and more common and represents a major cost in the healthcare system [23]. In this scenario, bioengineered scaffolds or hydrogels represent a great option promoting wound healing as they facilitate cell migra-

tion and exchange of nutrition and wastes creating a favorable environment for complete skin wound healing. The main requests for this type of wound dressings are biocompatibility, biodegradability and good mechanical properties. In this regard, it is very important to choose the right polymer. For tissue engineering, the most used are biodegradable polyesters like poly-e-caprolactone (PCL), poly(lactide) (PLA) and poly(lactide-co-glicolide) (PLGA) or polysaccharides, including cellulose, hyaluronic acid and cellulose which, beyond having a great biocompatibility and biodegradability thanks to their hydrolysable groups, have a structure that is very similar to the extracellular matrix (ECM) [7]. Incorporating Vitamin E in these materials to crate different wound dressings allows to combine the antioxidant properties of the vitamin with the benefits of a bioengineered system for wound healing. It has been studied a Vitamin E (α-tocopherol)-loaded chitosan/alginate porous hydrogel for the healing of skin injuries that has proven to be successful on a rat model ensuring the highest epidermal cells proliferation compared to other dressings and even the growth of new hair follicles. The rapid wound closure makes this dressing based on Chit/Alg/Vit E hydrogels promising for the successful wound treatment [24]. Vitamin E-functionalized scaffolds were also produced. The main advantage of using a scaffold is the greater control of the mechanical properties as well as the biodegradability time but the poor cell attachment makes surface functionalization of these structures the only way to create a cell-friendly environment. The cell-attachment is obtained by functionalizing the scaffold with extracellular matrix proteins such as collagen, fibronectin, fibrin, etc. As well as for the hydrogels, the purpose of loading these structures with bioactive macromolecules like vitamin E is to provide antioxidant, skin barrier stabilizing, photo-protective and anti-tumorigenic properties. In literature few attempts have been made in this direction using, for example, PLA-CL nanofiber scaffolds, obtained through electrospinning, for better mimic the ECM, where silk fibroin to allow cell adhesion and it has been reported that the nanofibrous scaffold functionalized with silk fibroin and vitamin E- loaded enhance cell proliferation in skin fibroblast. The only concern in using synthetic polymer wound dressing devices is the lack

of flexibility and the vitamin E release kinetics. Another solution, other than hydrogels and scaffolds, could be using a bidimensional polymeric film that are able to cover large surfaces, present a grate flexibility and can be easily loaded with macromolecules like vitamin E.

The topic of wound healing is strictly linked to tissue regeneration. In this context three-dimensional structures are preferred and needed to offer mechanical support to the tissue while allowing cell adhesion and proliferation. As well as for the wound dressings, the scaffold has to degrade in the physiological environment while the new tissue is building up. The polymers chosen in this case have to consider the stiffness of the tissue being regenerated: high mechanical properties are required only for hard tissues. As well as in any other case, Vitamin E is loaded in these structures mainly to carry out its role as antioxidant, but some studies also shown its ability to enhance protein adsorption and to reduce some cell attachment and spreading. Different studies, for example, have been made on vitamin E coated PLA: the material results to be anti-adhesive for bacteria and osteoblast, and hydrophilic.

The last field in which Vitamin E is used in bioengineering is drug delivery. Vitamin E is a lipophilic molecule, characteristic which makes it possible to cross the cell membrane. It is also insoluble in water and its digestion has to occur through emulsion into lipid droplets [7]. In this way, vitamin E is absorbed in the different tissues. This natural method of transport has inspired the drug delivery systems used for this molecule: polymers or biopolymers in form of hydrogels, micro- and nano- particles, liposomes and nano-emulsions are all means for encapsulating vitamin E and deliver it to the target. Among biopolymers, particularly successful is chitosan which water solubility increases the bioavailability of water-insoluble molecule like Vitamin E. In literature chitosan nanoparticles loaded with α-tocopherol can be found as well as chitosan micelle with both α-tocopherol and doxorubicin for site-specific anti-cancer effect through grafting to a ligand. Another noteworthy biopolymer used in drug delivery system for with α-tocopherol is hyaluronic acid, also an hydrophilic compound with the peculiar characteristic of forming a gel when immersed in

water. As for polymers, PCL was used to fabricate nanoparticles loaded with α-tocopherol through O/W emulsion and successive ultrasonification to allow the encapsulation. Vitamin E has also been used in combination with thermosensitive materials, useful for controlled release of drugs. We just discussed the ways to deliver the vitamin into the desired tissue but Vitamin E itself, after proper adjustment, can also work as the delivery system. The amphiphilic structure of tocopherols and tocotrienols is characterized by a small hydrophilic part that makes it challenging for the molecule to self-assemble into micelles. To overcome this limitation, the phenolic part of the vitamin is converted into esters using acetic or succinic acid to optimize the amphiphilic structure and make compounds like α-tocopheryl ether-linked acetic acid or α-tocopherol succinate that can be used for drug delivery and, in addition, show some anti-cancer activity. Moreover, the α-tocopherol succinate can be esterified with polyethylene glycol (PEG) to form a new compound , the D-a-tocopheryl polyethylene glycol succinate (TPGS) which is a popular non-ionic surfactant used especially as permeation enchancer, a prodrug carrier and to produce other copolymers with PLA, PLGA or PCL. Its main advantages are the high biocompatibility, the cellular uptake of the drug and works also as anti-tumor. The α-tocopherol succinate can be also esterified with a glucidic polymer, the inulin (INU) to form a compound called INVITE which can easily self-assemble into nanocarriers and present hydrolyzable groups. This compound is stable in water and is used ad drug carrier for different targets, especially for the urinary tact and intestinal site. Lastly, the the α-tocopherol succinate has been esterified with hyaluronic acid to crate delivery system targeting tumors as hyaluronic acid has the ability to bind CD44, a protein overexpressed by tumor cells. It has been tested with Doxorubicin showing its potential as a promising strategy for efficient tumor therapy. In contrast with what have been said, nanoparticles of pure α-tocopherol have been successfully produced in a water-surfactant-oil emulsion. The nanoparticles thus produced were able to encapsulate antioxidant molecules and anti-cancer agents.

3 Titanium functionalisation

The steady aging of the population is accompanied by the increasing demand for replacing bone tissue as bone and joints pathologies as osteoarthritis and osteoporosis are the most common in elders[25]. The most common surgeries, as for now, are hip replacement and knee arthroplasties, counting both replacement and revision surgeries. Unfortunately, revision surgeries have a quite low success rate and generate a great discomfort in the patient both for the pain and the psychological burden of undergoing a second surgery. Different reasons can lead to implant failure such as microbial infection, debris generation, wrongly chosen modulus, to low strength or release of metal ions and the consequent foreign body response [26]. Titanium, stainless steel, cobalt, magnesium and tantalum and their alloys have been used as metallic biomaterials for orthopedic devices but not all of them resulted successful as regards the load-capacity and the release of metallic ions that are toxic for the host tissues [25]. It has been demonstrated that Ti, Nb, Ta, Zr, Au, Mo and Sn are biocompatible meaning that the reactions that take place when they are placed in the host tissue are safe for the human body. While Al, V, Cr and Ni etc. are not biocompatible [26]. When choosing the right material for the specific implant, mechanical properties both of the material and the replaced bone have to be considered. Elastic modulus, for example, has to be comparable to the replaced bone to avoid the stress shielding effect the leads to bone resorption and consequent implant loosening. To date, there are no metallic biomaterials used for implant devices with a Young's modulus equal to the replaced cortical bone [27]. Other properties that have to be considered are fatigue and tensile strength and elongation. Moreover, if the material lacks the proper wear and corrosion resistance, wear debris could form and metal ions could be released in the host tissue causing allergic and toxic reactions. Not only the implant has not to cause any adverse reaction but it should also promote osseointegration [26, 25]. This bond between the implant and the adjacent bone can be obtained if the implant has the proper surface topography, the right chemical composition as well as an ac-

tive surface coating. All of consideration listed above can prevent the formation of fibrous tissue, the development of an inflammatory response and consequent loosening of the implant reducing the incidence of revision surgeries [25].

3.1 Titanium and its alloys

The most employed materials have been 316L stainless steel, Co-Cr basted alloys and titanium and its alloys. Among the three, stainless steel and Co-Cr are potentially hazardous as they tend to release Ni, Co, Cr as a result of corrosion and wear. Ni showed toxicity towards skin, Co resulted carcinogenic in animal studies while Cr damages through oxidation reaction different organs and tissues such as kidney, liver and blood. Moreover, both materials possess an incompatible elastic moduli whit the bones in the human body. Therefore, Ti results the bast choice and it is used in its commercially pure (CP) form and its alloys for biomedical implants since the early 1970s [28]. At room temperature, pure Ti has hexagonal close packed structure (hcp) that is called α-phase. At 881°C a phase transformation to body-centered cubic (bcc) structure, the β phase, take place.

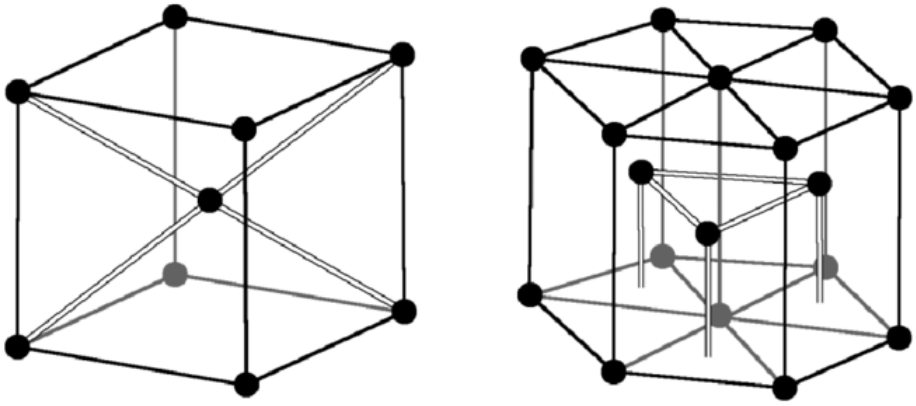


Figure 3: Crystal structure of Titanium. Left: β structure (bcc) Right: α structure (hcp) [29]

The β-transus temperature depends on the amount of impurities in the pure Ti which are mainly Nitrogen (N), Hydrogen (H), Oxygen (O) and Iron (Fe) and on the alloying elements.

The alloying elements for titanium can be neutral or stabilize the α-phase or the β-phase: the first ones rise the β-transus temperature while the other ones lower it. Some of the elements that belong to the first category are Al, C and O while Mo, Ta and Nb are classified as β-stabilizers.

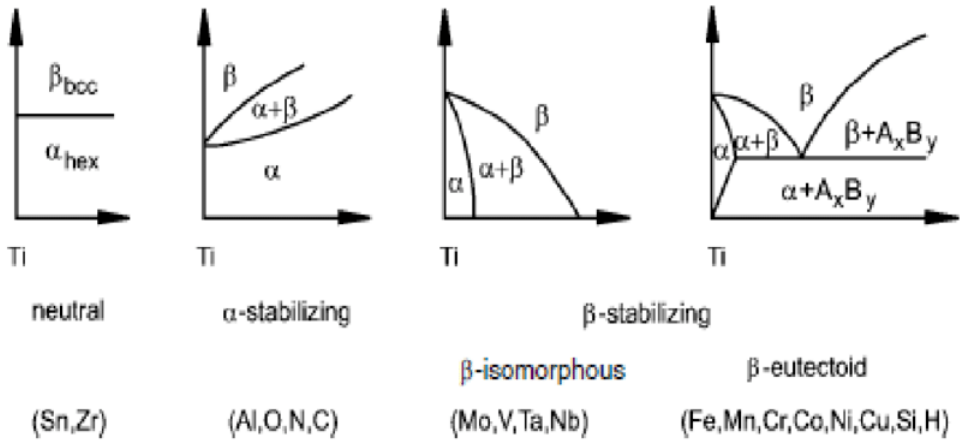


Figure 4: Effects of different alloying elements on Ti alloys. [30]

Commercially available titanium can be found in different forms: α, near-α-, (α+β)- and β-alloys. The CP-Ti belong to the α-forms. The α alloys are formed by a single solid solution of α-phase while near α Ti alloys usually contain, besides the α-phase, less than 10% of β-phase. Both of them present similar properties such as an excellent corrosion resistance, a good weldability and creep resistance but low mechanical strength at room temperature that cannot be improved by heat treatment due to the high stability of the hcp phase. The β-phase in the near α-alloys is added to improve the mechanical strength but still, both of these Ti alloys are inappropriate for load bearing applications but are widely used in the aerospatiale field.The β-alloys contain higher amounts of β stabilizer which allows the retention of the β-phase at room temperature and

make them heat treatable by ageing in order to improve their strength. The treatment promotes the partial transformation of the β phase into the α-phase and the precipitation of a very fine α-phase into the β-phase matrix. These alloys can be hardened to strength level over 1400MPa [28]. By increasing the β phase in the alloys, toughness, heat treatment capability and plasticity are improved while the elastic modulus decreases. The low elastic modulus owned by these alloys can reduce the stress shielding effect. Moreover, the non-toxic stabilizers of the β phase as, Mo, Ta or Zr, enhance the biocompatibility and the abce of the micro-galvanic effect present between the different phases and provides a higher corrosion resistance when in contact with human body fluids [26]. β titanium alloys have been developed in the 1980s when an alternative to the vanadium based $\alpha+\beta$ alloys was needed as concerns on the presence of vanadium in long term implants have been raised due to its toxicity both as element and as oxide and because it was reported that vanadium based alloys stimulates phagocytic cells and induced the release of proinflammatory and osteolytic mediators when in contact with human bone marrow [31]. Despite the disadvantages just discussed, Ti-6Al-4V is the most used titanium alloy in the biomedical field. It belongs to the group of $\alpha+\beta$ alloys which contain up to 16% of β-stabilizers [28]. Also these alloys are heat treatable to optimize their mechanical properties and in general they feature good fabricability and high strength at room temperature, a higher yield and tensile strength compared to α-type alloys and an excellent corrosion resistance and, when in contact with the human body environment, they rapidly form TiO_2/OH film that promotes fast osteointegration [26]. This protective passive layer is retained at pH values of the human body [32].

3.2 Ti6Al4V

Ti-6Al-4V represents more than the 50% of titanium alloys used today and owns its fame to the extensive use in biomedical field both for hip and knee arthroplasty which are the most common orthopedic surgeries nowadays [28].

The strength, optimal fracture toughness and corrosion resistance has resulted in the recruitment of this material in biomedical applications . Usually it is employed in two different metallurgical conditions: solution treated or annealed. The treatment used on Ti-6Al-4V for orthopeadic and traumatology implants is commonly the annealing [26]. Temperature has a great influence on the properties of Ti-6Al-4V: the thermal conductivity increases substantially when the temperature in increased while, when temperature is decreased, the hardness increases as well as the tensile strength. The Young modulus is also influenced by the temperature but only if it exceeds 500°C a decrease in the modulus has been reported. There is also a strong correlation between the microstructure and the mechanical properties: as for the yield stress, it increases when the volume fraction of α-phase increases and decreases with its thickness and Feret ratio. The volume fraction of both phases is not the only parameter to have influence on the mechanical properties but also the stereological parameters have to be considered. The most used alloying element is aluminium, an α- stabilizer that increases thermal stability of the α phase and reduces the density of the alloy. β stabilizers have, as well, influence on the mechanical properties: the β isomorphic elements (Mo, V, Ta) are able to increase plasticity and decrease the ultimate tensile strength (UTS) of the β phase while the β- eutectoid elements (Cr, Mn, Fe, Si, Co, Ni and Cu) have the opposite effect [33, 34]. It has been reported that titanium alloys are inclined to fail by galling and show high and often unstable friction coefficients. Some restrictions in the use of Ti-6Al-4V could be undertaken because of the presence of vanadium, which shows strong toxicity for cells. In fact, implants could generate titanium alloys particles and ions that, by deposition in the surrounding tissues, may generate an inflammatory response that most probably will led to failure in the osseointegration. These particles and ions could also generate a toxic reaction in other tissues and organs or allergic reactions [35]. To overcome the problem of toxic ions release, new alloys have been developed such as Ti-6Al-7Nb and Ti-5Al-2.5Fe. They both present higher fatigue strength and lower elastic modulus [28]. The first one is already used for fracture fixation plates, femoral hips stems, fasteners,

screws and wires as it posses a better wear resistance compared to Ti-6Al-4V. Ti-5Al-2.5Fe is metallurgically similar to Ti-6Al-4V and has been used for hip prostheses and hip prosthesis heads. The three (α+β)-type Ti alloys contain Al which is also a toxic element linked to different diseases including Alzheimer's disease, Parkinson's dementia and osteomalacia [36]. As concern the production of biomedical implants, due to their complex shapes and different sizes, conventional techniques have the disadvantage of being time consuming and expensive from a material and energy point of view. In the last few decades, additive manufacturing techniques have been making their appearance in all different industry productions including the biomedical field. Selective laser melting (SLM) is one of the techniques applied to manufacturing Ti-based materials.It consist of selectively melt titanium alloy powder using a computer controlled laser, layer by layer, reducing the production time, optimizing material consumption, without geometric constriction and no need of further post-processing. The fast heating and cooling rate of this technique produces a distinctive microstructure characterized by a dominant fine acicular martensitic phase, called the α' phase, with some prior β grains. This microstructure presents better mechanical properties compared to Ti–6Al–4V conventionally produced. Another additive manufacturing technique for metals used for Ti-6Al-4V is electron beam melting in which the heat source is a high energy electron beam. Also in this case the laser follows a computer aided designed (CAD) model allowing the prototyping of complex shapes in a short time. This process has to be performed in a vacuum environment and thanks to the preheating of the powder, the parts produced present a higher density. The basket-weave microstructure shows a columnar prior β grains delineated by α grain boundary.Ti-6Al-4V EBM produced exhibits a better corrosion resistance than the SLM and conventionally-produced TI-6Al-4V, probably due to the refined lamellar α/β phases produced.

Ti alloys, regardless the production technique, present a higher elastic moduli than those of human bones resulting in the previously discussed stress shielding effect. In order to lower the moduli, Porous Ti alloy structures have been developed which, besides having a similar moduli to the bones, the macro and micro

pores allow bone cell ingrowth and vascularization. In this respect, pore size and shape, their distribution and orientation are fundamental for a successful osteointegration and can be controlled by different fabrication methods. It has to be considered that the porous structure suffers from a decline in mechanical properties. A balance between the porosity and mechanical performance has to be taken into consideration. Popular methods for the production of Ti alloys are: SLM and EBM, spark plasma sintering, space holder method and loose sintering powder. Ti porous alloys have great potential application in medicine but the research is still at early stages [26].

3.3 Surface modification on Ti6Al4V

Ti-6Al-4V or more generally CP-Ti and Ti alloys have an excellent biocompatibility but they are bio-inert. It means that no fibrous capsule forms around the implant but neither a strong chemical bond with the bone. Moreover, the poor tribological properties, such as wear resistance, low hardness and tendency to galling, represent a limitation in their use in the orthopedic applications [26, 37]. With surface modification techniques the already good corrosion wear can be improved as well. Surface modification of Ti alloys aims to combine different technologies to achieve bioactivity as well as wear and corrosion resistance [26]. As already discussed, the affinity of titanium to oxygen, leads to the formation on the surface of a thin oxide layer, mainly of TiO_2 and some Ti_2O_3 and TiO, at room temperature. This oxide layer is too thin (few nanometers) to prevent *in vivo* the release of toxic ions and wear debris. It has been observed that above 200°C, the oxide layer becomes thicker and in able to increase wear and corrosion resistance [38]. Oxidation treatments are the most used surface modification techniques for titanium alloys and they include anodic oxidation, thermal oxidation, plasma immersion ion implantation, plasma electrolytic oxidation and plasma oxidation [37]. The goal of this methods is to create a stable oxide layer, sufficiently thick with a good protection of the metal surface at room temperature. The anodic oxidation is a traditional technique in which the

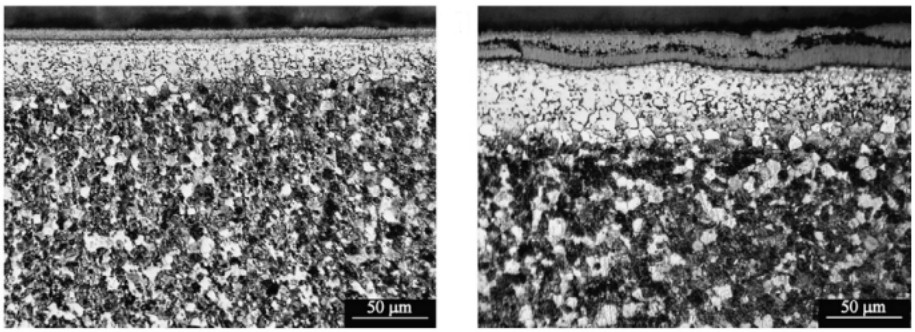


Figure 5: LOM micrographs of Ti-6Al-4V oxidised through thermal treatments.

oxide layer that forms on the surface of titanium is directly related to the anodizing voltage. This method of forming a thick layer of oxide does not prevent abrasive wear. Applying thermal oxidation is a simple and cost-effective method to create a thick and hard oxide layer. The two factors that influence the wear resistance of the oxide layer obtained by this method are temperature and time. It is in fact considered a time-consuming process and some suggested that the high temperatures applied for a long time could cause debonding stratification. Tribological properties of thermal oxidized titanium have been studied and an improvement in friction and adhesive wear has been detected. The tough, adherent rutile oxide layer is able to effectively eliminate the adhesive action and enhance boundary lubrification. As concern the lubrification, the boundary lubricating effect in strongly associated with wettability that, for its part, is related to the ionic character of the surface. TiO_2 rutile oxide that forms during the thermal treatment has a higher wettability due to its higher ionic character compared to bare titanium and this characteristics explains the enhanced boundary lubrification [39]. The oxide layer can be obtained also through chemical treatments with hydrogen peroxide often accompanied by a heat treatment. The oxide formed depends on the concentration of the hydrogen peroxide: Ti_2O_3 forms for low concentrations of the solution while TiO_2 anastase forms for higher concentrations. Among the anastase and rutile forms of TiO_2, the first one is more biocompatible while the second has higher hardness and is thermodynam-

ically stable. The heat treatment is responsible for the formation of rutile phase that increases the hardness of the oxide layer. The combined thermochemical treatment provides an excellent wear and corrosion resistance [40]. As concern the osseointegration, the topography and the roughness of the surface have to be modified to create a better mechanical and biological anchoring between the implant and the surrounding bone and tissues. Among the different treatments, the etching is the most widely used. Several studies demonstrated that the acid attack corrodes the surface of the titanium and creates a rough surface with micropores [41]. In this way, also the wettability of the surface in modified. Acids used for this type of surface treatment are HCl, H_3PO_4, HF, HNO_3 or H_2SO_4 being the last one the most effective one in terms of roughness of the surface obtained. This treatment has the advantage of creating homogeneous roughening no matter the shape and size of the substrate. Studies demonstrated that the modified surface promoted the attachment, the spread and the proliferation of osteoblast-like cells [42]. In recent years, an interesting combination of acid etching and thermochemical oxidation was developed to create a multiscale topography and a high hydroxylation on titanium surfaces for dental implant applications. The topography of the surface resulted to be composed of a micro-porous layer whit an overlapped nano-texture and was obtained by etching in HF and thermal oxidation in hydrogen peroxide. The hydroxyl groups present on the surface are important for apatite precipitation and can be used for biomolecules grafting. The obtained modified surfaces have showed bioactive behavior as well as improved wettability, protein adsorption and resistance to implantation friction [43, 44]. Surface modification of titanium alloys are also employed to improve biofunction of the material when implanted in the body. Bioceramic bioactive coatings have to be prepared on the surface with a good bond strength and no residual stress in order to avoid the peeling off from the implant. The coatings have to increase the osteointegration and the most common ones include hydroxyapatite and/or fluoroapatite, β-tricalcium phosphate, β-wollastonite and bioactive glasses. The methods employed to fabricate these coating are: plasma spraying, sputtering, electrochemical deposition, dip

coating, sol-gel, sintering and many more. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (HA) is present in the human body and constitute the inorganic phase of hard tissueswhich makes it a highly biocompatible material. It also promotes the adhesion of bone, propriety called osteoconductivity. The hydroxyapatite coating have to be coupled with a transition layer of ZrO_2 or TiO_2 between the implant and the coating to increase bond strength and the stability of the coating. In general HA coatings undergo cell-mediated resorption in most of the clinical cases but it is usually followed by the growth of new bone [45]. Fluoride is not a naturally occurring element in hard tissue but can be incorporated in them through therapeutic drugs like sodium fluoride or ingested in food. The exposure to fluoride leads to the formation of fluorapatite obtained from the substitution of the OH- in the hydroxyapatite with the F-. $Ca_5(PO_4)_3F$ is more stable in the human body and has a great bond strength with Ti-6Al-4V as well as a bone regeneration abilities [46]. These coatings, besides being bioactive, inhibit the release of metal ions from the implant to the surrounding tissues. Glasses, glass-ceramics and ceramics including CaO-SiO_2 were reported to have good biocombatibility and to improve bioactivity when used as coatings of Ti and Ti alloys. Both wollastonite ($CaSiO_3$) and diopside showed in vivo formation an apatite layer but clinical applications are still poor due to the rather poor mechanical properties. Kokubo demonstrated that major contribution to the bioactivity of these bioceramics is the presence of CaO-SiO_2 components, in fact calcium ions dissolved from the material increase the ion activity product of the apatite in body fluid in contact with the implant and the hydrated silica promotes apatite nucleation. This process take place in a short time proving the excellent bone conductivity of these glasses and glass-ceramics. To enhance the bonding to titanium substrates, similarly to the HA coatings, and control the dissolution, wollastonite/ZrO_2 and wollastonite/TiO_2 composite coatings have been developed [47]. Besides inorganic coatings, biochemical modification of the surfaces of biomaterials are performed in order to obtain bioactive and antibacterial properties. Biopolymers as well as biomolecules are immobilized on the surface to induce a specific response from the cells and the surrounding

tissues: ECM proteins or peptide sequences can be immobilized on the surface to promote bone cell adhesion; to trigger new bone formation, growth factors can be deposited; novel approaches used enzyme-modified titanium surfaces to enhance the process of bone mineralization [48]. Different techniques including photochemistry, self-assembled monolayers, protein resistance and immobilization are used for this purpose. These technologies are based on either physical adsorption such as van der Waals or electrostatic interactions, the use of barrier system or chemical bonding. Adsorption is the most simple among these methods as it consist of immersing the titanium implant into a solution containing biomolecules but the linkage is very sensitive to the process parameters including pH, temperature and solvent, and the loading is low and the desorption uncontrolled. A barrier system could be a coating that retains the biomolecule but there is no chemical interaction between the two. Often time used coatings to incorporate biomolecules are poly(D,L-lactide), ethylene vinyl acetate and collagen. The retain of the biomolecule on the surface is greater and the release is more controlled. Lastly, covalent binding is more complicated than the methods just discussed but it provides the highest surface loading and great biomolecule retention. It is used mainly for peptides, enzymes and adhesive proteins; the titanium surface has to be derivatized into reactive groups, amino groups or aldehyde groups, in order to react with the biomolecules. Among the covalent immobilization methods, the silane chemistry is the most common. Different biomolecules require different method of immobilization: for example, growth factors have to be immobilized for a short time before being released in the tissue while adhesion molecule and enzymes need a long term immobilization. In fact, GFs have to reach the desired cell to induce a biological response, which in this case is increasing ostoblast activity to accelerate bone regeneration [48]. Growth factors such as platelet-derived growth factor (PDGF), bone morphogenetic proteins (BMPs), insulin-like growth factor (IGF) or TGF-β facilitate the osseointegration of different kinds of implants and tend to be immobilized on the surface through barrier entrapment, especially in collagen which allows them to remain biologically active during preparation and implantation proce-

dures [49]. Fibronectin, vitronectin, type I collagen and other ECM proteins present specific amino acid sequences that bind the cell membrane integrins promoting cell adhesion. The amino acid sequence is the well known RGD that is also the most commonly used peptide sequence in titanium surface modification. In most cases, only short peptide fragments of ECM proteins are immobilized on the surface to target more specific cellular interactions and eliminate the possible undesired responses that an intact protein could generate. Peptides sequences are not meant to be released, therefore they are covalently attached to the surface via hydroxyl-, amino- or carboxyl- groups.[48] Organic-inorganic composite coatings are being developed to better mimic the double nature of the bone that is composed of an organic collagenous matrix and a inorganic CaP phase. The inorganic CaP coatings are often created using technologies that would damage biomolecules such as cytokines, growth factors or antibiotics, therefore the inorganic layer is formed before the attempt to adsorb the biological agents. To avoid the burst release either a coating incorporating the biomolecules is created or the CaP layer is produced under more physiological condition in order to allow the incorporation of the biomolecules [48]. For example, protein co-precipitated with calcium and phosphate ions on titanium implants creates a biomimetic layer with a high incorporation of the protein which remained stable in the coating [50, 51].

3.4 Antibacterial functionalization strategies

Ideally, orthopedic implant coating should be multifunctional, both facilitating the osteointegration and having antibacterial properties. In fact, implant-related infections are among the leading causes of implant failure: when the biofilm is formed after the adhesion of bacteria, destruction of the adjacent tissue leads to implant loosening or even detachment and dislocation. There are not treatments that can effectively destroy the biofilm or prevent the infection to recur, consequently antimicrobial properties of the implant surface are a major factor in the long term success of the implanted materials. In general, the

material must have or anti-adhesive or anti-infective properties, meaning that bacteria cannot adhere to the surface or that the implant should be able to kill them [52]. Chemical composition, charge, wettability and roughness are crucial surface features with regard to interaction with bacteria. Surfaces with low roughness as well as nano-textured surfaces do not increase bacterial adhesion compared to roughness Ra value higher than 0.2 μu [53]. The influence of wettability of the implant surface on microbial surfaces differs between bacterial species: S. epidermidis shows high adhesion on hydrophobic surfaces while S. sanguinis does not show any difference in adhesion between hydrophobic and hydeophilic surfaces [54]. The two properties can be achieved, even at the same time, by surface treatments that can be divide into two main categories: surface physical, chemical or the combination of the two modifications or through coatings. Different antibacterial molecules, antibiotics, antimicrobial peptides and inorganic metal element like silver or copper were used on titanium implants simply adsorbed or chemically linked. Simple adsorption, as already stated, may not be successful for long-term implantation, while covalent binding implies surface modification through chemical reactions that could be harsh towards the biomolecules [52].

Grafting

Covalently grafting molecules is the preferred strategy; it requires an anchor molecule and the most common are organofunctional alkoxysilane molecules, catechol and phosphates and phosphonates.*Silanization* Silanization of metallic surfaces has been widely used for the functionalization with bioactive molecules. The oganofunctional alkoxysilane molecules react with the exposed hydroxy groups on the surface of the titanium allowing the covalent binding of petides, proteins and polymers when in presence of a crosslinking agent. In literature, Melimine, an antimicrobial peptide, was grafted on silanized titanium surfaces that exposed amino groups. It antimicrobial activity was testet both *in vitro* an *in vivo* showing anti-adhesive properties on *P.aeruginosa* and *S. aureus*. Both vancomycin (VAN) and caspofungin (CAS), an antibiotic to treat bacterial infection and antifungal agents, were successfully grafted on titanium via silane

anchor.

Cathecol anchor. Different approaches can be used to graft onto the titanium surface a polymer with a catechol group that could be used as an anchor for chemical bonding. Direct polymerization on the surface in the presence of an initiator containing a catechol group, by functionalizing a polymer using a molecule with a catechol group and anchoring the complex onto the titanium surface or firstly anchoring rhe catechol onto the surface and then grafting the polymer. Dopamine could be used to anchor catechol groups on titanium surfaces just by simple immersion in aqueous dopamine solution; thus carboxymethyl chitosan, which has antibacterial properties, has been grafted to the surface. An effective grafting was also performed with hyaluronic acid, which inhibits the adhesion of bacteria and can be modified with dopamine to create hyaluronic acid-catechol [55].

Phosphates and Phosphonates. Other linkers for covalent grafting are phosphates and phosphonates that have the advantage of being more stable than silanes in aqueous environments at physiological pH when bounded to metals. The phosphate group creates covalent bounds with the TiO_2 layer. The chemisorption of molecules containing phosphate groups is obtained at really low pH and high temperature which increase the deprotonation of P-OH groups and the formation of Ti-O-P bonds [56]. Molecules like Myo-inositol hexaphosphate have been grafted without using a crosslinker and they showed anti-adhesive properties against oral bacterial strains (*S. Sanguinis*)

Coatings

Coatings as well can be physical, chemical or a combination of the two. Firstly, bacteriostatic materials, the ones that repel bacteria without killing them, can be obtained through hydrogel coatings based on PEG, negatively charged polymers or ultra-hydrophobic modification of the surfaces. Coatings can be also bactericidal by perturbing bacterial membrane, blocking DNA replication or interrupting protein synthesis. The most used bactericidal agent are the antibiotics but it is not the only strategy: antimicrobial peptides loaded in a calcium phosphate coating or polymer coatings with amphiphilic character have the

same effect [52].

Inorganic Antimicrobial Agents. In recent years, due to the drawbacks related to the use of antibiotics, alternative antibacterial agents have been investigated and special attention has grown around inorganic antibacterial agents as metal ions and nanoparticles. Silver (Ag), zinc (Zn), copper (Cu), iodine (I) or selenium (Se) ions, among others, can be integrated directly on titanium or by doping a TiO_2 or HA coating. Silver is the most well known for its antibacterial properties among the metals cited and its mechanism of action consist of binding to the thiol group of proteins altering their function and structure in the bacterial cell wall, leading to its disruption and the consequent death of the microorganism. Moreover, Ag ions are able to bind several enzymes responsible for cellular respiration and metabolism and also interfere with DNA. Also the other metal ions have similar mechanism and their activity is not limited to bacteria but they also work against fungi and yeast [53]. Nanoparticles interact with the bacterial wall by electrostatic interactions and disrupt its integrity [57] but could also penetrate healthy cells hence their concentration, dimension and shape have to be strictly controlled. The process of ion implantation in which ions of a desired material are accelerated in an electrical field and implanted into a solid material is used also for CP titanium and titanium alloys for all the metallic ions [58]. Ions content on the surface depends on the ion dose; for antibacterial properties and biocompatibility for osteoblast cell, doses have been establishedù [59]. Wear and corrosion resistance are improved as well. Alternatives to the traditional ion implantation have been developed: plasma immersion ion implantation(PIII) that induces silver nanoparticles precipitation or ion beam assisted deposition (IBAD) that deposits an oxide titanium layer doped with silver on titanium implants. Ion beam assisted deposition have been also used to form Ag-naoparticles-doped hydroxyapatite as a coating on titanium. Plasma electrolytic oxidation (PEO) which is a method to produce oxide-ceramic coatings on metals can be used to introduce silvers nanoparticles onto the oxide layer by just the addition of Ag nanoparticles in the electrolytic solution [60, 61]. Nanoparticles, besides the bactericidal activity, increased the

surface roughness and wettability. In the electrolytic solution Ca and P, among Ag-nanoparticles, can be added to obtain a layer with interconnected micro- and nano-porosity on a plasma treated microporous Ti6Al4V surface [59] A combination PEO and electrophoretic deposition, called plasma electrolytic process, has been used to create composite coatings made of titanium oxide and silver enriched HA [53]. The sol-gel technique is another technology to form a coating in gel form in which polymers or nanoparticles can be trapped. The synthesis consists of forming mineral phases obtained by the polymerization of a molecular precursor [52]. In this process, silver can be introduced both as nanoparticles or in ionic form ($AgNO_3$) and converted into NP in the calcination process. This technique was also used to coat microporous titanium structures with hydroxyapatite formed in the presence of silver nitrate [53]. Other noteworthy methods for integrating in the surface metallic antimicrobial agents are sputtering and plasma spray. Plasma spray technology consists of depositing the coating material in powder form at high temperature. The powder could contain silver nanoparticles as well as Zn-enriched calcium silicate. In sputtering, the material chosen as coating, the target or cathode, is bombarded in a vacuum chamber by ions generated in a glow discharge plasma which causes the sputtering of target atoms that condense in a thin layer on the surface that has to be coated [62]. Using a Ag-TiO_2 composite target, a TiO_2 coating containing silver nanoparticles has been deposited. ZrO_2 and ZrO_2 coatings doped with silver or copper have been deposited as well using the slightly modified technique of magnetron sputtering that controls the sputtered atoms by a magnetic field resulting in a higher deposition rete [62, 53]. Both copper and silver doped surfaces exhibited antibacterial properties against *S. aureus* and *A. actinomycetemcomitans* [63].

4 Materials and Methods

4.1 Samples Preparation

Ti6Al4V disks, measuring 10 mm in diameter and 2 mm in thickness, were employed as substrates. They were obtained from cylindrical bars by automatic cutting. Firstly, the samples were polished by abrasive paper (120, 320 than 400 grit) on an automatic mechanical polishing machine. The samples were polished on both sides in order to avoid the native oxide to interfere with the process and the side that was not going to be treated, was marked. All the samples were washed 5 minutes in acetone and twice for 10 minutes in double distilled water in an ultrasonic bath. To avoid surface contamination, no organic solvents have been used. The thermo-chemical treatment consisted of a first acid etching in diluted hydrofluoric acid (HF) for 2 minutes to remove the native oxide layer and create a macrorough surface then a controlled oxidation was performed by immersing the samples in a hydrogen peroxide (H_2O_2) solution for 2 hours in a shaking water bath at high temperature. The samples thus obtained showed surface microroughness and a high number of hydroxyl groups and will be named CT (chemical treated) Ti6Al4V.The treatment performed until this point was firstly describe by Ferraris et al [43].

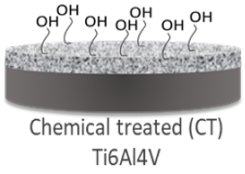


Figure 6: Chemical Treated (CT) Ti6Al4V

4.2 Grafting of natural molecules: functionalization or coating

The treated samples were then UV irradiated to improve the reactivity of the OH groups and to reduce the carbon contamination before the functionalization or coating. Immediately after the UV irradiation, the samples were immersed into a $CaCl_2$ solution (0,292 g in 1 L of double distilled water) for 24 hours minimizing the contact of the sample with the air during the transfer. An electrostatic interaction arises between the alloy surface in the Ti-O^- form and the Ca^{2+} ions charging positively the surface.

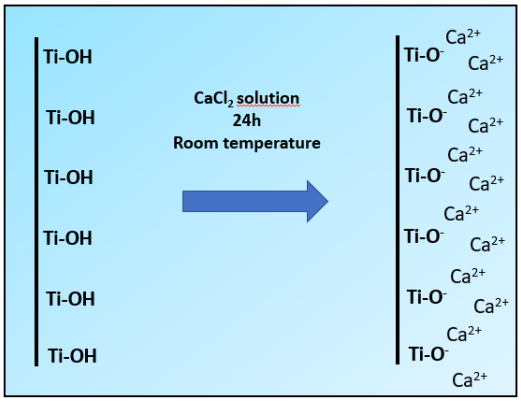


Figure 7: Scheme of possible interaction of Ca^{2+} ions with the titanium alloy surface.

A solution of α-Tocopherol phosphate disodium salt ((±)-α-Tocopherol phosphate disodium salt, T2020, Sigma-Aldrich, St. Louis, MO, USA) in TRIS-HCl with a concentration of 5 mg/mL was prepared by stirring the solution on a magnetic stirrer for 10 minutes or until the complete dissolution of the salt. The TRIS-HCl is a buffer solution used as a pH standard for the physiologically important pH range of 7.3 to 7.5. After 24 hours, the samples were dried under a laminar flow hood and then immersed into the solution of α-TP, 1 mL for sample, for 3 hours at 37°C.

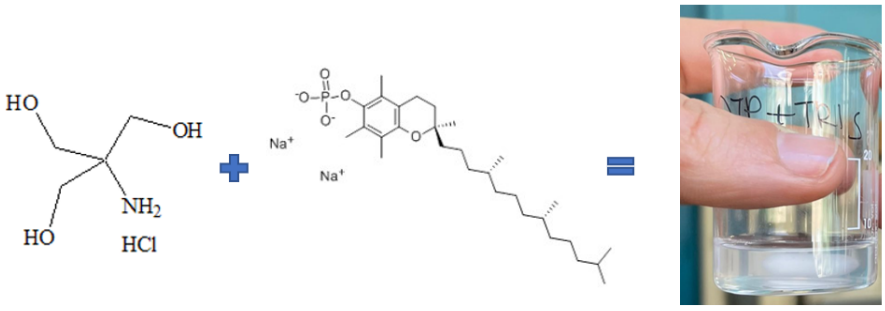


Figure 8: α-tocopherol phosphate salt in TRIS-HCl solution.

Following two different protocols, the surface was coated or functionalized. By coating it is intended a continuous layer while by functionalization the grafting of singular molecules to the surface. The functionalized surface was obtained by rinsing the samples 3 times in double distilled water immediately after the 3 hours spent in the solution with the molecule and letting it dry under the laminar flow hood. The coating, instead, was obtained by firstly letting dry the samples taken out of the solution and then rinsing them 3 times in double distilled water and letting them dry again under the laminar flow hood.

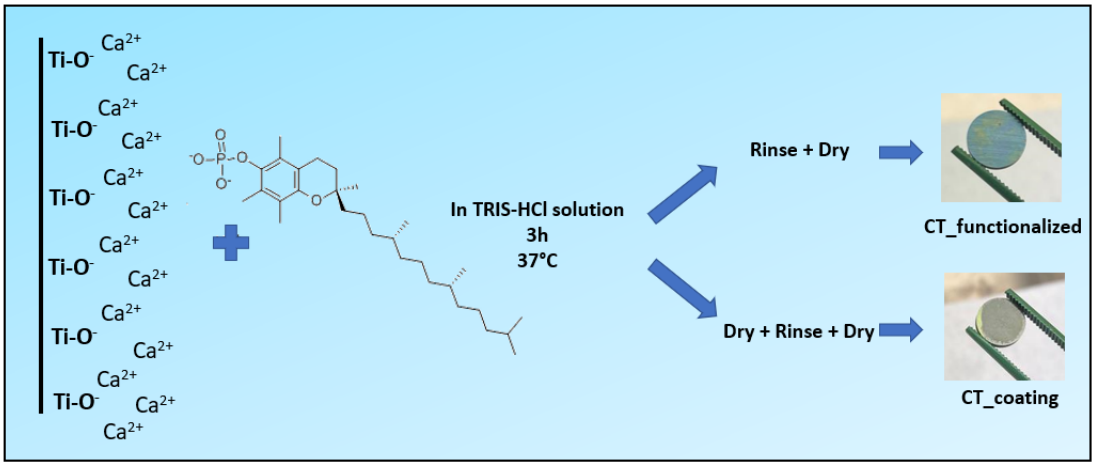


Figure 9: CT Ti6Al4V alloy coated and functionalized.

4.3 Contact angle

The wettability of the surface was evaluated by means of measurements of the contact angle obtained through sessile drop method: a drop of water was deposited by a syringe on the treated surface of the CT sample, the coated sample and the functionalized one and by acquiring and elaborating, by Image J software, the images captured by the camera placed in front of the sample, the value of the contact angle was obtained. The measures were performed two times on every sample. Mean and standard deviation were calculated and represented.

4.4 Z potential electrokinetic measurements

Surface charge in function of pH of CT, functionalized and coated samples was analyzed by means of electrokinetic measurements (SurPASS, Anton Paar). The Z potential was determined in function of pH in a 0.001M KCl electrolyte solution (titration curve) and the pH value was varied by adding 0.05 M HCl or 0.05 M NaOH using the instrument titration unit. The Z potential at constant pH values of 3.1, 4.5 and 7.4 was also acquired by manual titration with the 0.05 M HCL and 0.05 M NaOH solutions. For the acidic and the basic titrations as well as for the different measurements at constant pH values, separate couples of samples were used in order to avoid artifacts.

4.5 Kelvin probe

Potential difference between the functionalized/coated and the CT Ti6Al4V was assessed by means of Kelvin Probe which is a non-destructive method that measures the contact potential difference (CPD) between the sample and a vibrating reference electrode [64]. The samples had to be prepared in a slightly different way for this analysis. After the 24 hours immersion in the $CaCl_2$ solution, half of the sample was covered with Kapton ®, a polyimide film employed as it does not leaves residues after its removal. Then the samples were coated or functionalized according to the described procedure and, only after the complete drying, the Kapton®was removed. The sample thus obtained were coated or

functionalized only in half with a rather narrow edge. The scanning width in fact, is around 100 μm meaning that for a successful measure of the potential difference, the edge should not be wider than a few dozen of μm. Moreover, topography could also be investigated by means of Kelvin Probe analysis.

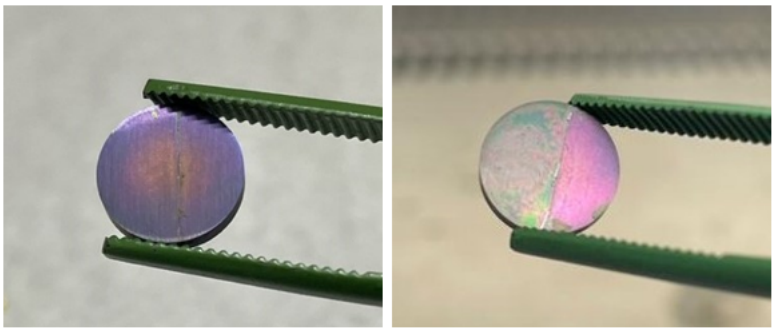


Figure 10: KP samples: CT-functionalized on the left and CT-coated on the right.

Kelvin probe analysis was also performed to assess the uniformity of the functionalized/coated half and its topography.

4.6 UV–Vis Diffuse Reflectance Spectroscopy

The physicochemical properties were assessed by Ultraviolet-Visible diffusive Spectroscopy (UV-2600i, UV-2700i – Shimadzu), a technique in which the sample surface is impacted by a collimated light beam directed with a certain angle and, if the surface is not smooth, multiple reflections, scattering and refraction form the so-called diffusive reflectance which contains the information about the examined surface [65]. This simple, cost-effective and non-disruptive analysis was performed on the functionalized, the coated and the CT ti6Al4V samples.

4.7 Fourier-Transform Infrared spectroscopy (FTIR) analysis

To confirm the presence of the α-tocopherol phosphate, chemical groups on the different samples were analyzed by means of Fourier-Transform Infrared spectroscopy (FTIR, FTIR Hyperion 2000 - Tensor 27, Bruker Optics, Ettlingen, Germany) in reflectance mode and confronted with the spectrum of the pure α-tocopherol phosphate. Spectra were acquired between 400 and 4000 cm^{-1}.

4.8 X-ray Photoelectron Spectroscopy (XPS) analysis

To further confirm the presence of α-tocopherol phosphate, X-ray photoelectron Spectroscopy (Al source, Surface Science Instruments, M-Probe) analysis was performed on both coated and functionalized samples. This type of analysis register the energy in form of photons that atoms returns after being previously excited by X-rays; each element has a characteristic X-ray spectrum which can be used to identify the element.

4.9 Release tests

For *in vivo* applications, the possibile release of the molecule had to be verified. For this scope, coated and funtionalized samples were immersed in PBS to study the possible release at physiological pH, and in a solution of PBS and H_2O_2 at pH 4.5 that simulates inflammatory environments. Four coated and four functionalized samples were immersed individually in 5 ml of PBS and PBS+H_2O_2. The solutions were then analyzed by means of UV-Vis spectroscopy after 1 day, 1 week and 2 weeks. After the end, another contact angle measure was performed on all the samples as well as FTIR analysis.

4.10 Biological analysis

To investigate the actual possibility of clinical application, biological tests in forms of cytocompatibility evaluation, fluorescence staining and antibacterial

activity (metabolic and SEM), were performed on mirror polished Ti6Al4V, on CT-Ti6Al4V and on the functionalized and the coated samples. The cells used for the cytocompatibility and the fluorescence evaluation tests were hMSC human mesenchymal stem cells while *Staphylococcus epidermidis* was the bacterial strain used for the antibacterial activity tests. Biological caracterization was conducted at Università del Piemonte Orientale in collaboration with Dr. Andrea Cochis and Professor Lia Rimondini.

5 Results and Discussion

5.1 Wettability

The chemical treatment performed on the Ti6Al4V alloy significantly increased the wettability of the polished samples because of the presence of the -OH groups exposed on the surface and the nanotopography of the oxide layer [43]. While studying the method of functionalization, it was noted that by measuring the contact angle before and after the rinsing, the values changed notably. It was

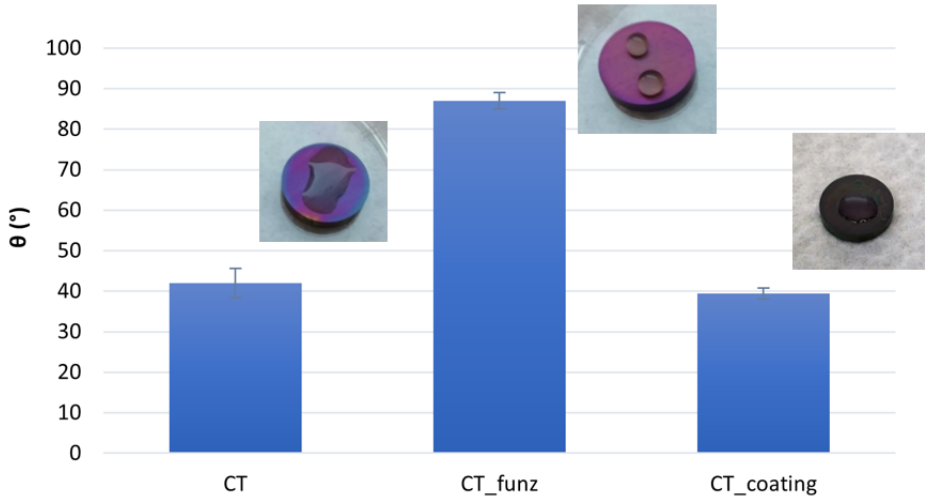


Figure 11: Contact angle measurement of CT Ti6Al4V, CT coated and CT-functionalized samples.

then decided to pursue the two different strategies of coating and functionalization previously described. As shown in the Figure 11, the CT functionalized sample has a higher contact angle than the coated one. It can be explained by assuming that in the functionalized sample, the α-tocopherol phosphate molecules are attached to the surface through the phosphate group with the Ca^{2+} ions that were prewiously absorbed, exposing in this way the long hydrophobic alkyl chain. The coated samples, instead, present a thicker layer in which the α-

tocopherol phosphate molecules are incorporated without a specific orientation exposing randomly also the hydrophilic phosphate group making the wettability of the surface similar to the CT Ti6Al4V sample. In every case, the surfaces can be considered hydrophilic with the functionalized one being on the edge of hydrophobicity. Wettability has a great importance when considering the biological response: it has been demonstrated that osteoblasts are reluctant to adhere on surfaces which have a contact angle for water higher than 60° (or a free surface energy lower than 40mN/m) [66].

5.2 Z potential

The titration curves of the CT and the coated and functionalized samples are reported in Figure 12. The isoelectric points (IEP) of the three samples are not determined because out of measurement range but can be obtained through interpolation. In literature it is reported that bare titanium surfaces present

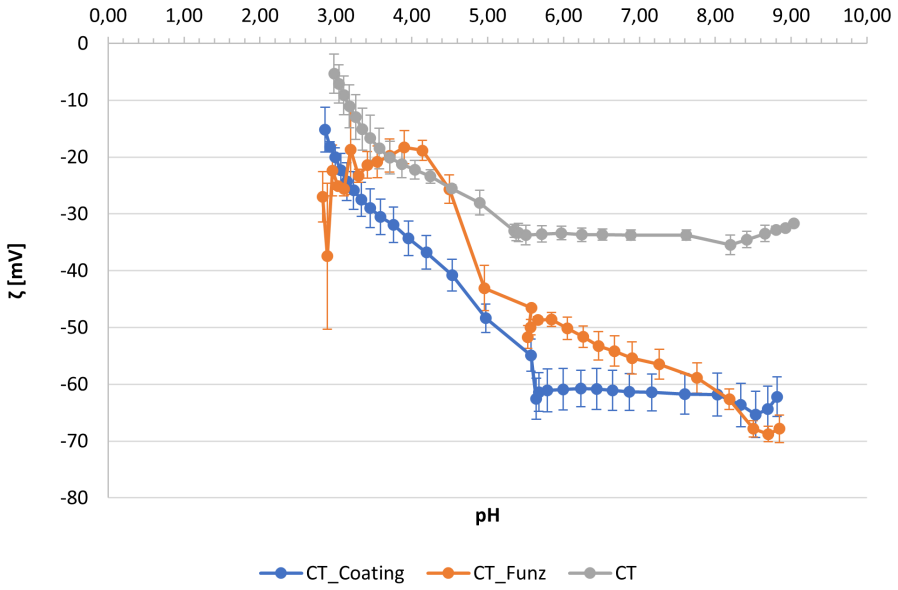


Figure 12: Zeta potential vs. pH of CT Ti6Al4V, CT functionalized and CT coated samples.

the IEP at pH 4.7, value consistent with the IEP of a surface without functional groups with acid/basic behavior, therefore a shift to acidic values for all of the three samples can be justified by the enrichment in hydroxyl groups with acidic behavior of the surfaces.

The CT and the CT coated samples show a similar trend. They both present a well-defined plateau for pH values higher than 5, indicating the presence of functional groups with a specific acid strength, in contact with the solution, that completely dissociate above a specific pH. Moreover, the similar slope, fairly low, between pH 3 and 5, can be linked to the high wettability of the two samples. In fact, water is highly absorbed on hydrophilic surfaces and when the pH of the solution in contact with the samples changes, the solution's OH groups are not easily able to substitute the water molecules present on the surface. Therefore, the Z potential of the two samples changes but slowly. The lower Z potential values for the coated sample confirm the presence of negatively charged phosphate groups on the surface. The CT functionalized sample presents a different trend: there is no plateau at basic pH while it seems to be one at very low pH values (3 and 4), and the slope is present but between pH 4 and 5 and is much steeper. The steep slope and the abrupt change at pH 4.5 can be justified by the hydrophobicity of the surface and by the detachment of the biomolecule respectively. The absence of the plateau in the basic region can be explained by the lack of functional groups with acidic behavior like the phosphate groups on the CT coated sample or the OH groups on the CT. The plateau present at low pH is due to the presence on the surface of functional groups with basic behavior but it is not clear which ones: it can be hypnotized that TRIS created a partial bond with the surface but further supporting data are needed. The Z potential electrokinetic measurement was also performed at constant pH to analyze surface changes in time (98 minutes) in different environments. At a particularly acid pH, 3.1 and 4.5, which can reflect the acidosis that stresses the cells during inflammation processes, the Z potential of the functionalized and coated CT samples is rather constant, taking also in consideration that a 10 mV variation can be considered negligible.

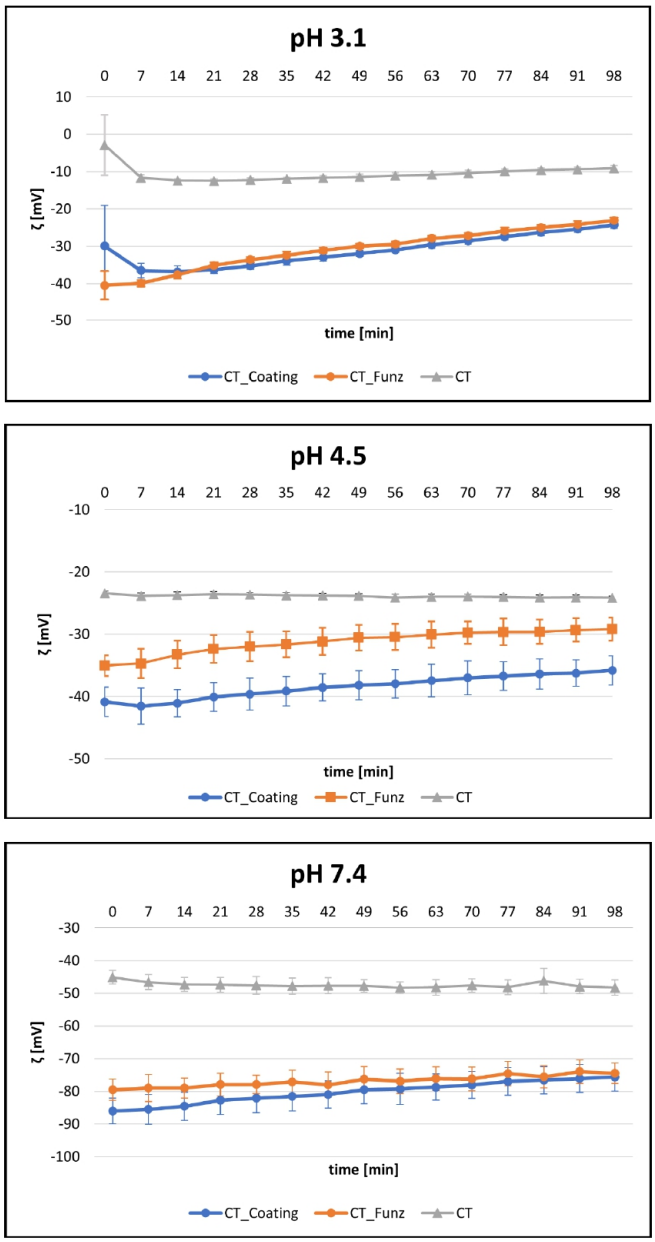


Figure: Zeta potential at constant pH vs. time of CT Ti6Al4V, CT functionalized and CT coated samples.

There is also a correspondence in the values of the Z potential between the measurements made at variable pH and these ones. The Z potential measured at physiological pH of 7.4, is constant too but, unlike the other two, deviates significantly from the values obtained at variable pH (-55/-60 mV vs. -80/-85 mV). It is necessary to report that the pH 7.4 is very unstable and the measurement was performed trying to stabilize the pH using a nitrogen pressure reducer. In conclusion, it can be affirmed that all procedures lead to a stable grafting of the biomolecule both at physiological and inflammatory pH.

5.3 Kelvin probe

The Kelvin Probe analysis permitted to assess the presence of the molecule on the surface of the samples by measuring the difference in the electrical potential between the just chemically treated surface and the functionalized and coated ones. The use of Kapton®to cover half of the sample during the functionalization or coating process resulted successful as no residues were found during the analysis. The absolute values of the potential should not be considered as this is a differential measurement and they depend on the measuring condition rather than the actual potential of the surface. As Figure 13 shows, the potential difference between the coated and the CT is around 60 mV, with the coating having a lower potential. This result could be confirming the presence of a continuous layer of α-tocopherol phosphate grafted to the surface which is also visible without the engaging of an optical device (Figure10). Despite the high potential difference and the visible coating, on the AFM image of the topography, no difference between the two halves is detected meaning that the micro- and nano-structure of the CT surface is not covered. The contact potential difference between the functionalized half and the CT half is around 40 mV, 20 mV less than the coated/CT, which can be considered a meaningful result in terms of the different concentration of α-tocopherol phosphate molecules on the surface. Also, just by sight, the difference between the two halves is hardly visible compared to the difference visible on the other sample.

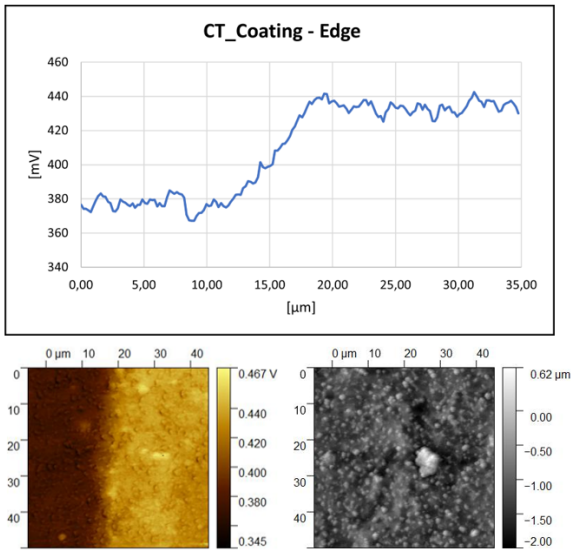


Figure 13: Kelvin Probe of CT coating vs CT Ti6Al4V measured at the edge.

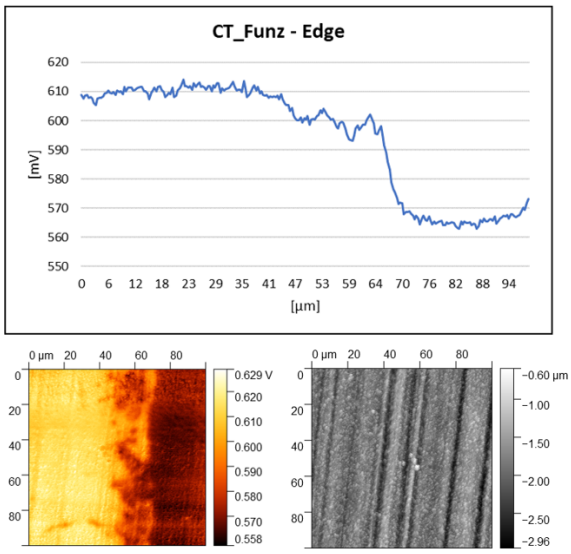


Figure 14: Kelvin Probe of CT functionalized vs CT Ti6Al4V measured at the edge.

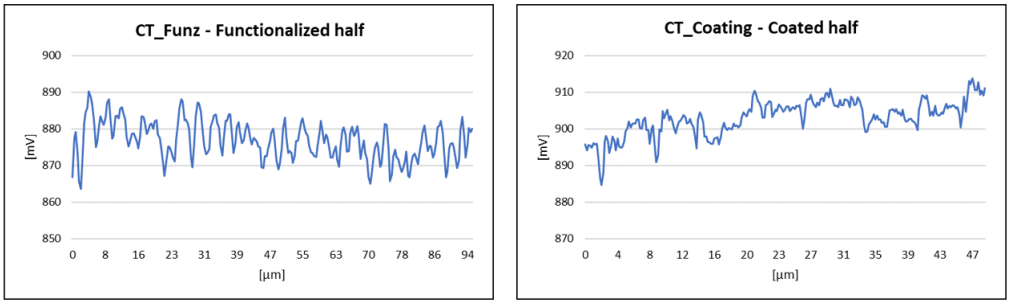


Figure 15: Kelvin Probe analysis of the functionalized and coated half.

Figure 15 represents the Kelvin probe analysis made on the coated and functionalized halves to analyze the uniform distribution of the molecules on the surface. The absolute values are not to be considered as meaningful. As shown, both the samples have a rather constant potential over the entire length analyzed. In summary, the measurements confirm that both procedures lead to an effective modification of the surface, show that the molecule is homogeneously distributed and that the surface topography, previously optimized to be osteoinductive, is not masked.

5.4 FTIR

FTIR analyses was performed on the pure α-tocopherol phosphate dissolved in double distilled water and on CT samples after functionalization and coating. The spectra obtained are represented in Figure 16 in the frequency range between 400 and 4000 cm^{-1}.

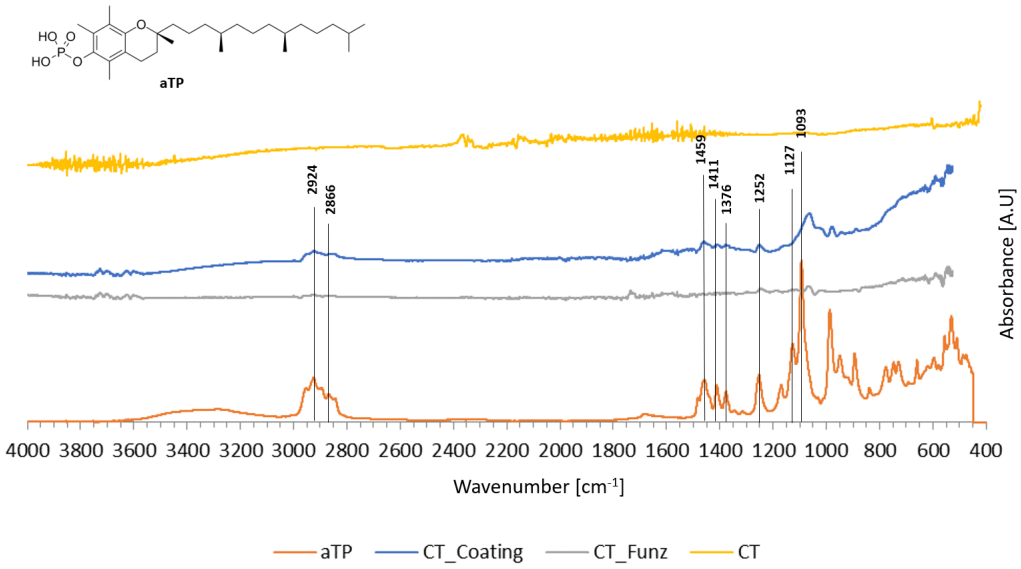


Figure 16: FTIR analysis of CT functionalized and CT coated samples and pure α-tocopherol phosphate.

The spectrum of the pure vitamin presents different and well defined peaks useful for identifying the molecular species grafted on the surface of the functionalized sample or that cover the surface of the coated sample. By just observing the spectra, it is evident that the CT coated has higher and more defined peaks in correspondence of the peaks of the α-tocopherol phosphate which can be due to the higher presence of the molecule on the coated sample. The band between 3726-3700 cm^{-1} of the free OH stretching is detected rightfully only on the samples and could be related to the OH groups of the titanium oxide layer or groups absorbed during the different immersions in different aqueous solu-

tions. The first common peaks encountered are at 2924 and 2866 cm^{-1} and are attributed to the stretching vibration of asymmetric and symmetric -CH_3- and -CH_2- [67]. Absorbance at 1459 cm^{-1} is associated to aromatic –C=C stretching vibrations present in the aromatic ring of α-tocopherol phosphate. Peak at 1411 cm^{-1} represent the bending vibrations of CH [68] while the one at 1376 cm^{-1} the aliphatic C-CH_3 [69], present at the extremity of the α-tocopherol phosphate molecule. This groups of three peaks is clearly visible on the CT coated sample but not determined on the functionalized one.

The peak at 1252 cm^{-1} is attributed to the significant stretching vibration of the C-C bond. This peak is clearly visible in both of the samples confirming the presence of the long lateral chain of the vitamin. In the spectrum of the molecule, two peaks are particularly evident, one at 1127 cm^{-1} and the other at 1093 cm^{-1}, the first attributed to the presence of acid phosphate-containing species [70] while the other to the stretching vibrations of P-O^- [71]. This exact two peaks are not present in the analyzed samples but it is possible that they merged into one slightly larger one shifted to the right (1054 cm^{-1})clearly visible for the CT coated sample, probably due to a complex formation with the Ca^{2+} ions.

Wavenumber [cm^{-1}]	Group vibration
2924 and 2866	symmetric and asymmetric stretching of -CH_2 and -CH_3
1459	-C=C of the phenyl skeleton
1411	-CH
1376	symmetric bending of -CH_3
1252	C-C stretching
1127	acid phosphate species
1093	-PO^-

Table 1: FTIR peaks of interest.

5.5 XPS

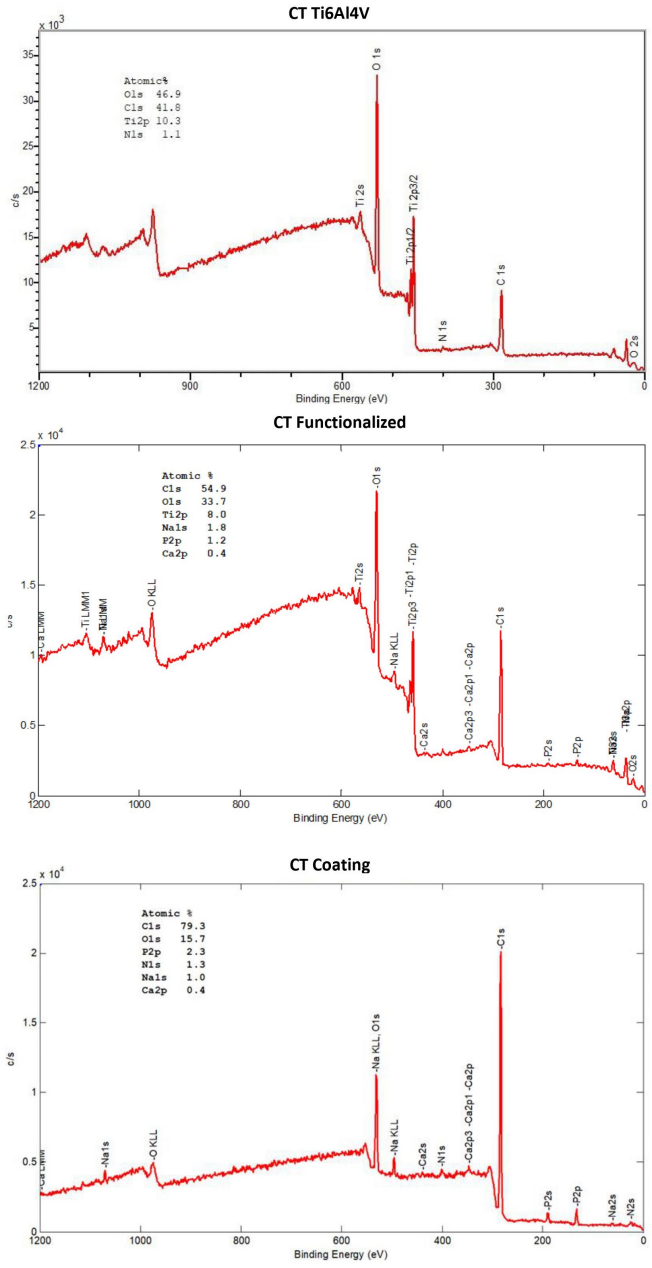


Figure 17: XPS spectra of CT, CT Functionalized and CT coated samples.

	Elements [at. %]					
	O	C	Ti	P	Ca	Other
CT	46,9	41,8	10,3	-	-	1,1
CT Funz	33,7	54,9	8	1,2	0,4	1,8
CT Coat	15,7	79,3	-	2,3	0,4	2,3

Table 2: Atomic percentages of the elements detected on the samples by XPS survey analyses.

XPS analysis was performed on the coated and functionalized samples in order to investigate the elements present on the surfaces and the two survey spectra were compared with the CT one. It has been chosen the XPS analysis instead of the SEM/EDS analysis because of the penetration depth, in the order of nanometers for the XPS and micrometers for the SEM/EDS, was considered more adequate for the situation. On the CT sample, a large amount of oxygen was detected confirming the presence of hydroxyl groups on the surface. Carbon is a common surface contaminant in titanium and titanium compounds and for this reason it will always be present in the spectra. The Ti signal has a similar amplitude for the CT and CT functionalized sample while as regards the CT coated sample, the Ti alloy substrate is not detectable, confirming the presence of a continuous layer that masks the signals of the metal surface. For the same reason, higher percentages of oxygen are detected on the bare CT and on the functionalized sample. Moreover, the percentage of carbon is considerably higher in the CT coated sample confronted with the CT and CT functionalized one, another evidence of the increased presence of the -tocopherol phosphate molecule on this sample. The phosphate is present in small amounts (1.2 vs 2.3 atomic %) on both samples but it was not detected on the CT, indicating the successful grafting of the molecule to the surface. Calcium, also in small amounts, can be detected on both surfaces resulting from the 24h immersion in $CaCl_2$. For a better understanding of the chemical groups, a high-resolution analysis was performed on the oxygen and carbon regions. In the O1s region

on the CT sample, the contribution of the OH groups present on the surface is clearly visible, as well as a great contribution in the range of 530 eV attributed to the Ti-O bond. The hydroxyl groups on the functionalized samples are slightly less but there is still an important peak around 530 eV confirming that the functionalized sample exposes the oxide layer on the metal surface. The spectrum of the CT coated sample is significantly different from the other two: is slightly shifted to the left not presenting the signal attributed to the Ti-O bond, evidence that confirms the presence of a coating, but instead shows the signal of the phosphate group at around 531 eV. An interestingly high signal for the OH group is present on the coated sample, most likely attributable to the OH groups of the TRIS that was used as a solvent in the solution of aTP. All three samples in the oxygen region show the CO peak at around 531.3 eV.

Looking at the spectra of the carbon region the main signal is at 284.8 which is attributed to C-C and C-H bonds that are always present on the titanium surface due to the presence of hydrocarbon contaminants but are also present in the α-tocopherol phosphate molecule and, as expected, the peak is higher on the CT coated sample. The other two peaks present on the carbon spectra are associated with C-O (286.3 eV) and C-O-C (286-287 eV) [72], which could also be associated to surface contamination but, similarly to C-C and C-O bonds, are present in the vitamin molecule as well. The spectrum of the functionalized sample presents a contribution on the right due to the detection of the C-Ca signal around 283 eV: as said, the calcium is absorbed to the surface while the carbon with which it interacts is most likely the one from the contaminants as the grafted molecule interacts whit the surface through the phosphate group. It cannot be ruled out that this bond is also present on the coated sample, but it is masked by the thickness of the coating.

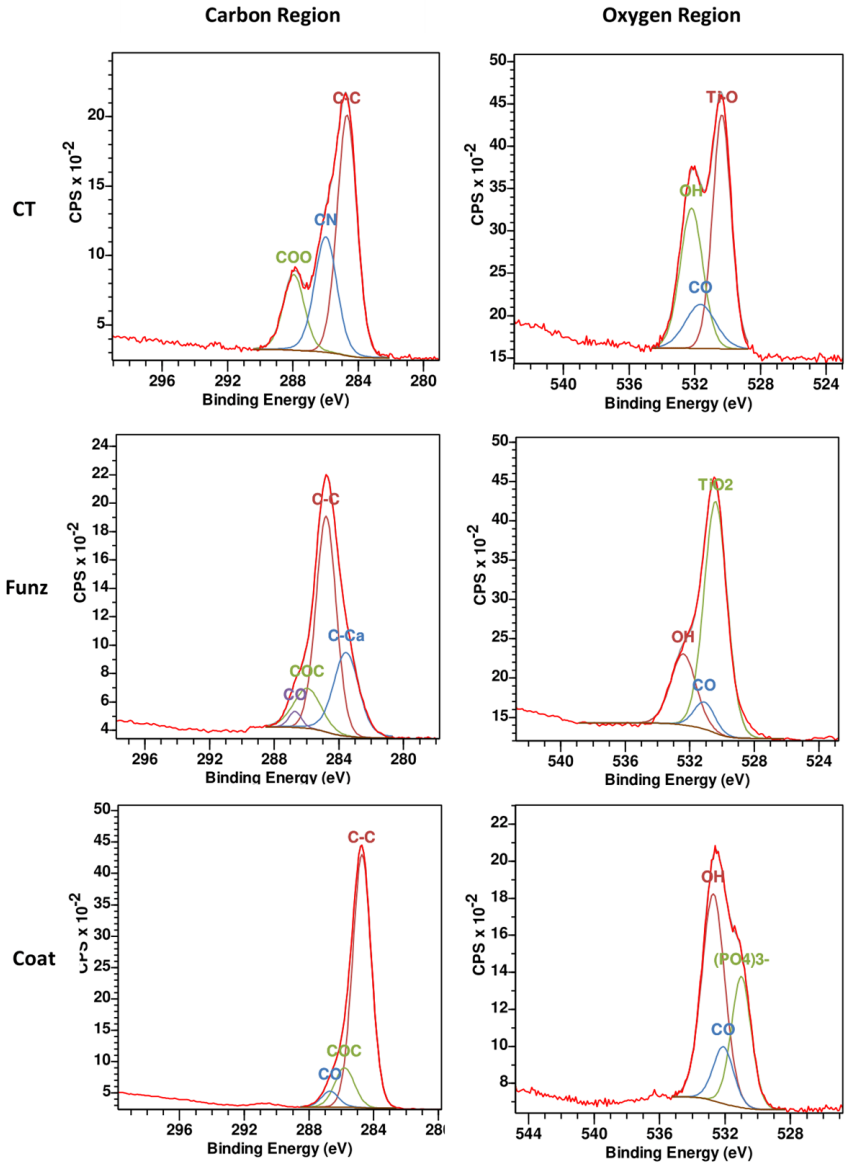


Figure 18: High resolution XPS spectra of C and O regions of CT, CT functionalized and CT coated samples.

5.6 Reflectance

The UV-Vis spectroscopy was used to detect the presence of the molecules grafted and of the coating on the CT functionalized and CT coated samples. The CT Ti6Al4V sample has been taken as reference for this analysis. Moreover, the absorbance spectrum of α-tocopherol phosphate was considered for the evaluation.

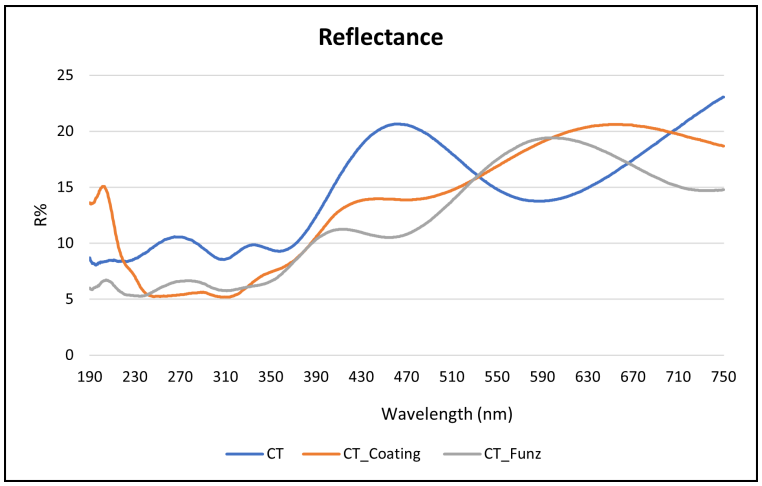


Figure 19: Diffuse reflectance spectra of CT Ti6Al4V, CT functionalized and CT coated samples.

The spectrum of the CT sample presents clear interference ripples caused by the titanium oxide layer: between the surface of the sample and the bulk metal, different reflections occur creating the typical spectral effect. The spectrum of the CT functionalized sample shows a quite pronounced ripple effect as well while for the coated sample the ripple seems less marked suggesting that the oxide layer was masked at least partially. All the three curves present an upward trend probably caused by the titanium dioxide as well which absorbs UV light of the wavelength less than 400 nm [73]. The presence of the molecule was assessed by the flattening of the functionalized and coated samples in the region

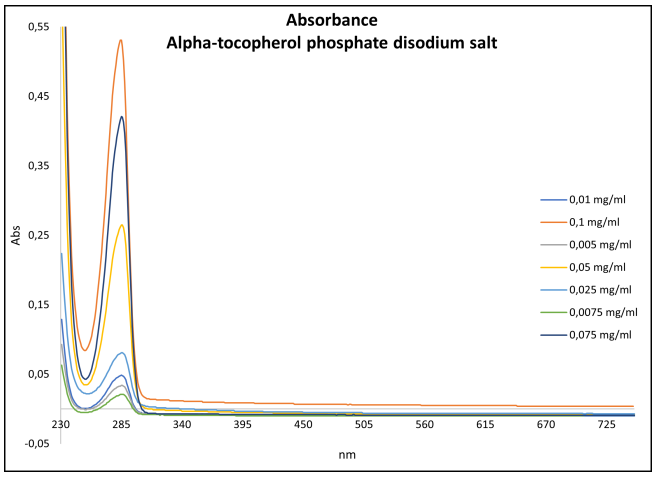


Figure 20: Absorption Curve of α-Tocopherol phosphate disodium salt solutions at different concentrations

between 250-300 nm at which the absorbance peak of α-tocopherol phosphate occurs. Confirming the presence of a coating, the flattening of the curve in the above cited region is more pronounced for the CT coated sample.

5.7 Release tests

Due to the low sensitivity of the UV-Vis spectroscopy, the results obtained by this analysis were not informative so will not be showed. However, after the unsatisfactory results, it was supposed that, if the release took place, it was so minimal to remain undetected. Therefore, further analysis were performed on the samples after the two week.

Only by observing the surface of the samples it was possible to detect the detachment of the coating on the CT coated sample (Figure 21), after the immersion in both PBS and PBS+H_2O_2, while on the surface of the functionalized sample no major differences were noticed. The contact angle was measured again and big variations could be observed for the CT coated sample. In fact, the wettability decreased to the levels of the functionalized sample probably meaning that the coating desorbed and only the vitamin's molecules grafted to the sur-

Figure 21: Detached coating on the CT coated samples after a two weeks immersion in PBS (left) and PBS+H_2O_2 (right).

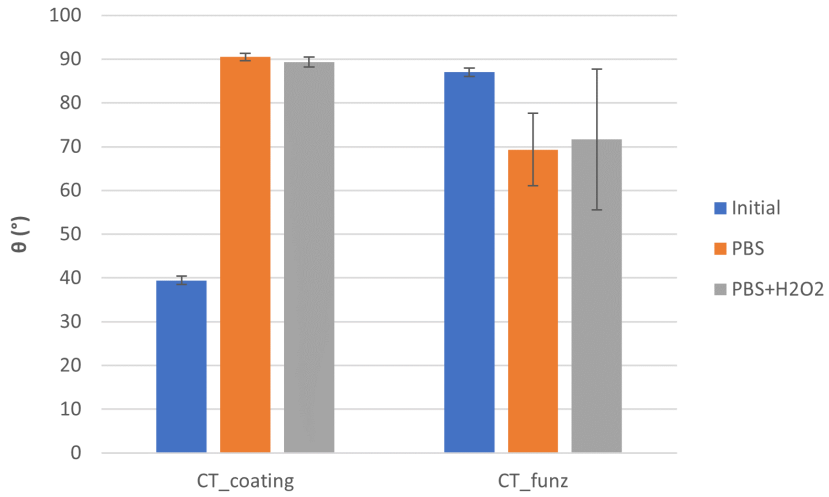


Figure 22: Comparison between the contact angle values obtained for the CT functionalized and CT coated sample before and after the immersion in PBS and PBS+H_2O_2.

face remained in place. This supposition was confirmed by FTIR analysis: it was performed on the coated sample both on the remaining coating and where the coating detached (CT coating-detached).

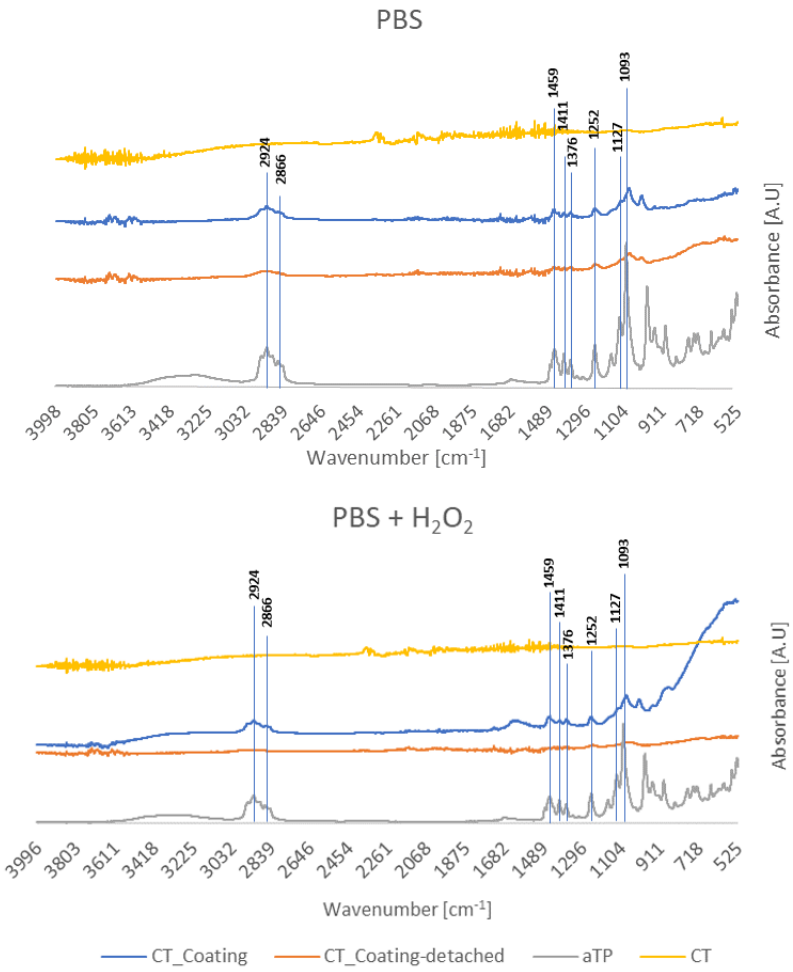


Figure 23: FTIR of the CT coated sample after immersion in PBS and PBS+H_2O_2.

The surface of the coated sample immersed in the PBS solution showed rather no differences on the coated part compared to the one acquired before the two week immersion, while the surface where the coating detached resembled more the spectrum of the functionalized sample analyzed before the immersion. More evident differences were observed for the samples immersed in the acidic

solution: the peaks of the intact coating were a little attenuated while on the spectrum of the detached coating even the peaks found on the functionalized sample have not been obtained meaning that probably most of the α-tocopherol phosphate molecules have been released.

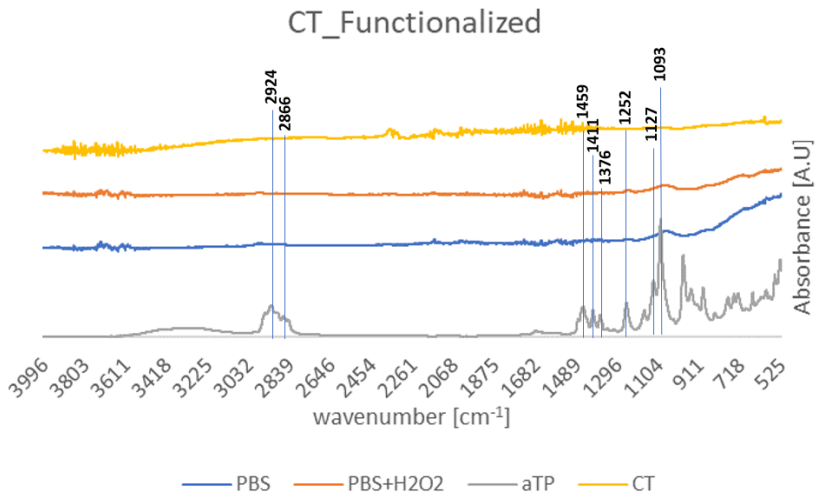


Figure 24: FTIR of the CT functionalized sample after immersion in PBS and PBS+H_2O_2.

No significant differences were observed on the spectra of the functionalized sample after the two week immersion in PBS and PBS+ H_2O_2 implying that the functionalization method creates a more stable bond between the surface and the molecule.

5.8 Biological Analysis

5.8.1 Antibacterial Activity

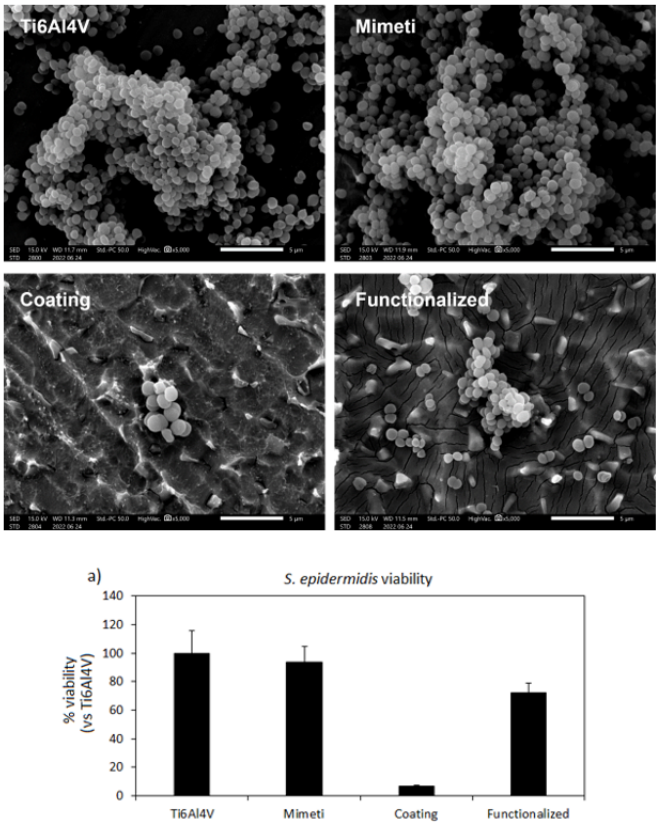


Figure 25: Bacterial viability

To study the antibacterial activity of the prepared samples in terms of inhibition of bacteria ability to growth or reproduce , viability tests were performed on the CT functionalized and CT coated samples and CT Ti6Al4V and mirror polished TI6Al4V (up to 4000 grit) samples were used as control series. *S. epidermidis*, a gram positive bacterium, was used for the testing as it represents one of the most common strains responsible for nosocomial infections due to its ability to adhere and form biofilm on medical devices. A drop of the medium containing bacteria was deposited on the surface of the samples and the results

were acquired after 24 hours of incubation through SEM. As shown in Figure 25, the number of bacteria on the surface of the coated sample is far less than the bacteria on the control samples and fairly less then the bacteria found on the functionalized sample. The images demonstrate the strong antibacterial activity of α-tocopherol phosphate and it is possible to suppose that the better results obtained on the CT coated sample are due to the more uniform distribution and the higher concentration of the vitamin on its surface. In fact, considering the viability of the bacteria on the mirror polished sample as 100%, the CT surface, considering the standard deviation, exhibits the same behaviour while the functionalized sample shows a decrease in viability up to 75%. The coated sample exhibits a ten times lower viability than the mirror polished and CT Ti6Al4V.

5.8.2 Cytocompatibility Evaluation

The same experimental set up seen with bacterial activity test was performed with cells: a drop of medium containing human mesenchymal stem cells (hMSC) was deposited on the surface of mirror polished and CT Ti6A4V and on the functionalized and coated samples that were then stored in an incubator.
To verify the viability, cells were stained with the fluorescent live/dead assay and data were collected after 24, 48 and 72 hours (Figure 26). The results showed an increase of the viability for the mirror polished and CT samples while the cells on the functionalized and the coated samples exhibited a rather constant viability. This result is important to assess that α-tocopherol phosphate does not have a cytotoxic effect on the cells analyzed.

An important difference between the coated and the functionalized sample, however, can be observed by analyzing the images obtained by fluorescence staining of cytoskeleton and nuclei (Figure 27) the shape of the cells seeded on the coated sample is elongated and flat, typical characteristics of a cell when it adheres to a surface. Lamellipodia growth through the processes of actin fila-

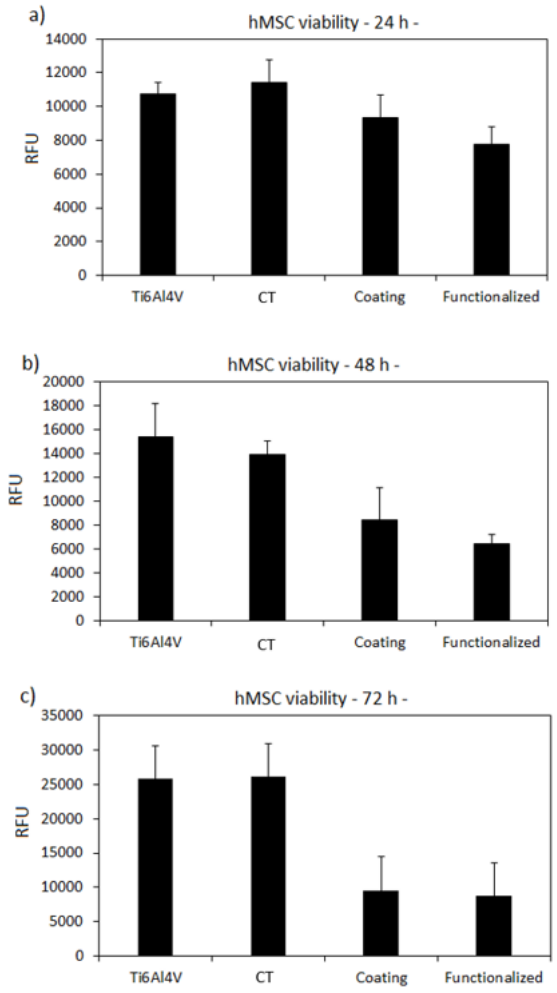


Figure 26: Cell Viability

ment polymerization and branching is also clearly visible on the coated surface. On the other hand, the cells found on the functionalized surface clearly did not adhere: they are small and rounded and not interconnected. This means that cells prefer the hydrophilic surface of the coated sample rather than the more hydrophobic of the functionalized one. As matter of fact, it has been demonstrated that on surfaces which wettability is higher than 70°, as for the functionalized sample, the adhesion and the proliferation of hMSC are discour-

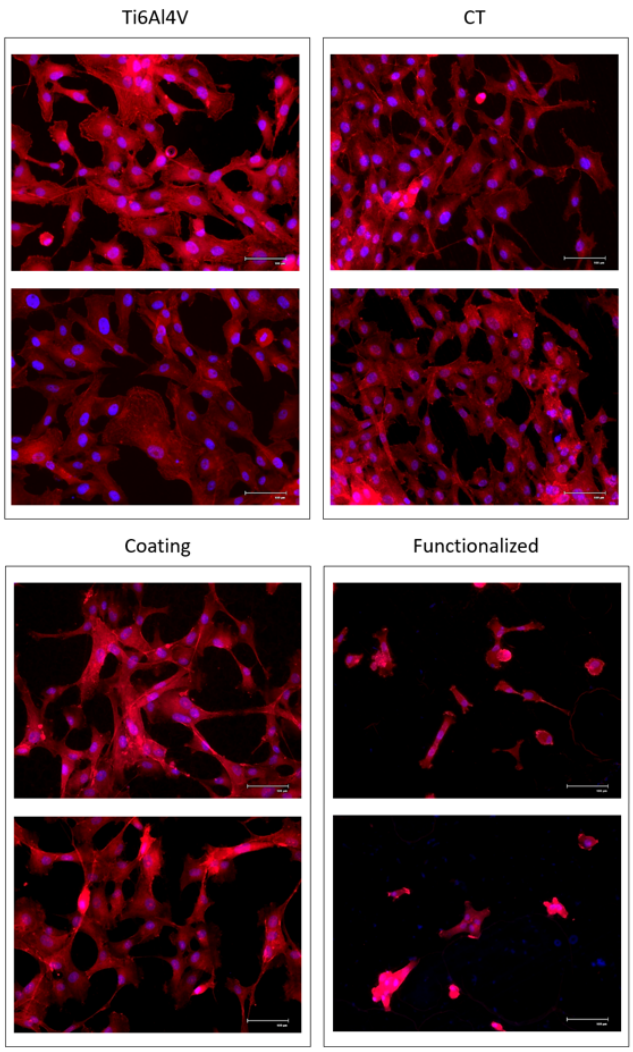


Figure 27: Alpha-tocopherol doped materials – fluorescence staining (cytoskeleton and nuclei) of hMSC-human mesenchymal stem cells.

aged [74]. Another important obsrevation is that the difference between the two methods used to modify the surface of the CT Ti6Al4V is also perceived at the biological level.

6 Conclusions

The thesis presented a novel application of α-tocopherol phosphate, a water soluble form of vitamin E with anti-inflammatory and antibacterial properties. The aim was to graft the molecule on the surface of chemically treated Ti6Al4V alloy in order to obtain surfaces for prosthetic devices that could actively reduce the inflammation and minimize the risk of bacterial infections. On the surface of the CT Ti6Al4V the molecule was applied both in the form of grafting and as a coating. The analyzes on the two samples produced were pursued in parallel in order to study their similarities and differences and to understand which of the two could be more successful in a future medical application. The studies carried out demonstrated the presence of the molecule on the surface both in the form of grafting and coating and the biological tests confirmed the strong antibacterial properties of the alpha tocopherol phosphate and its cytocompatibility. For future applications, a more in-depth study on the stability of the coating and on the release kinetics should performed.

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