# Surface modification of Ti-6Al-4V alloy for biomineralization and specific biological response: Part II, Alkaline phosphatase grafting

# Surface modification of Ti-6Al-4V alloy for biomineralization and specific biological response: Part II, Alkaline phosphatase grafting

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**Abstract**

Titanium and its alloys are the most widespread materials for the realization of orthopaedic and dental implants due to their good mechanical properties and biocompatibility. Surface functionalization of biomaterials aimed to improve and quicken implant integration and tissue regeneration is an active research field. The opportunity to confer biological activity (ability to directly stimulate cells with proper biological signals) to the Ti6Al4V alloy, previously modified to be bioactive from the inorganic point of view (apatite precipitation), was explored in this research work. The alkaline phosphatase (ALP) enzyme was grafted to metal surface via tresyl chloride activation, maintaining its activity. A synergistic effect between biological functionalization and inorganic bioactivity was observed.

**Keywords:** surface functionalization, enzyme grafting, ALP, titanium alloys, osteointegration

## Introduction

Prosthetic devices are widely employed in orthopaedic surgery for different kinds of disease, from articular reconstruction after trauma or degeneration caused by arthrosis, to bone substitution after surgical treatment of cancer. A good integration between the artificial bone substitute and the natural tissue is required in all these cases, in order to obtain a satisfactory physiological recovery.

Numerous studies have been made and clinical applications have been developed, in the last decade, in order to improve tissue integration of orthopaedic implants. Examples of these are the morphological modification of surfaces in order to make them rougher or porous and improve mechanical adhesion of tissues [1] and bioactive coatings or chemical treatments in order to induce inorganic bioactivity in terms of *in vitro* and *in vivo* hydroxyapatite precipitation (biomineralization) [2, 3].

Nowadays the main goal of research is to accelerate integration kinetics in order to obtain effective bone regeneration in a short time, reducing the costs of hospital treatment and patient

discomfort. Current research in this field aims at developing new surface treatments on prosthetic materials that conjugate good mechanical properties, inorganic bioactivity and also the ability to directly stimulate cells with proper signals in order to induce rapid bone regeneration (biological bioactivity). A number of different solutions have been proposed to date in order to induce biological bioactivity [2, 3] such as covalent grafting of proteins via silanization or via surface activation.

With this aim in mind this paper proposes a new patented [4] process. The first phase of the treatment is a thermo-chemical one that induces inorganic bioactive behaviour (precipitation of hydroxyapatite) on the metallic surface [5, 6, 7] and subsequently a functionalization with biomolecules, in order to obtain a bioactive surface, also from the biological point of view. This primary phase is also essential in order to obtain an active surface for the direct linking of biomolecules onto the metal, so polymeric coatings and spacers, (potentially toxic, such as glutaraldehyde) can be avoided.

Alkaline phosphatase (ALP) has been chosen for surface grafting in this research work. It is a well known enzyme because it is widely employed in *in vitro* testing as a marker of osteoblast differentiation and also in the clinical diagnosis of some bone and liver pathologies, so reliable techniques for its detection are available. Several research articles also suggest that the local application of ALP promotes bone mineralization and regeneration [8, 9, 10]. Therefore alkaline phosphatase can be a good model molecule and concomitantly it presents an interesting practical application in orthopaedic and dental fields.

## Materials and methods

*Surface activation*

Organic chloride was employed to directly link ALP onto a titanium surface. 2,2,2-Trifluoroethanesulfonyl chloride (99% Aldrich) was chosen and it is indicated as tresyl-chloride or TC from now on. The molecule was used without further purification. Thermo-chemically treated

samples were prepared as reported in [7]. They were activated by soaking in pure TC for 48 hours at 37°C, as described by Hayakawa et al [11]. Untreated (polished) samples were also treated for comparison purposes. At the end of the incubation period samples were gently washed with anhydrous acetone in order to remove unbounded TC and left to dry at room temperature.

*Biomolecule grafting*

5mg/ml ALP solution was prepared by dissolving lyophilized alkaline phosphatase (Phosphatase Alkaline from bovine intestinal mucosa lyophilized powder, ≥10DEA units/mg solid, Sigma Aldrich) in PBS (Phosphate buffered saline, pH 7.2 25°C, Sigma Aldrich) in a beaker with a magnetic stirrer, as reported in [12]. By reading the nominal enzymatic activity of the specific lot (reported in the product certificate of analysis), the nominal enzymatic activity of the prepared solution can be estimated as 50 U/ml. It was observed that the real activity of different ALP solutions prepared with the same nominal enzymatic activity is not completely reproducible. This can be explained by taking into consideration the intrinsic variability of protein solutions derived from animal tissues. Recently other authors [12] have also noticed a significant variability in enzymatic activity data for ALP functionalized silica coatings. These considerations can account for the statistical variability in enzymatic activity of functionalized samples.

TC activated samples were soaked in ALP solution for 24 hours at different temperatures (4°C in a refrigerator, 25°C in a thermostatic bath and 37°C in an incubator), in order to evaluate whether enzyme bonding to material surface is sensitive to its activity at different temperatures [13]. At the end of the soaking period the samples were gently washed in TRIS solution and left to dry under a laminar flow cabinet. Polished and thermo-chemically treated samples were also soaked in ALP for control purposes. Different washing procedures were performed and enzymatic activity of washed samples were measured in order to study bonding stability. Washing procedures included one or two gentle washings in TRIS solution, ultrasonic washing in TRIS solution and washing in urea. All

the procedures were performed after the gentle washing, described above, which aimed at stopping the grafting reaction.

*Biomolecule detection on material surface*

The presence of ALP on the titanium surface after grafting was investigated by means of XPS analysis (Al source, Surface Science Instruments, M-Probe). Both survey spectra and detailed analysis of carbon region were performed. In this way it was possible to determine the detailed chemical composition of the outermost surface layer and also to identify the elements chemical bindings in order to detect characteristic functional groups of the biomolecule.

Enzyme presence on the surface is not sufficient to induce a biological response to functionalized biomaterials, but the biomolecule should also maintain its activity after grafting.

Enzymatic activity tests were performed to verify this hypothesis. Functionalized samples were put into a reactive mixture containing equal volumes of $MgCl_2$ (2mM), p-nitrophenilphosphate (2 mM) and 2-amino-2-methyl-1-propanol (AMP- 2 mM) (all reagents were purchased from Sigma Aldrich Fluka). The pH value of the reactive mixture is 10.5. p-nitrophenylphosphate is a good substrate for the enzyme alkaline phosphatase, although it is not the physiological one. The reaction between ALP p-nitrophenylphosphate produces paranitrophenol which presents a yellow colouring in an alkaline environment. It can be quantified by means of UV-visible spectroscopy. The reaction was stopped after 2 mins by adding NaOH (1N), and the solutions, after sample removal, were analyzed for absorbance at 405 nm (GENIUS Spectra FLUOR plus TECAN) at room temperature. ALP solutions were also tested after storage at different temperatures, for comparison; enzymatic activity tests on solutions were performed that were analogous to samples ones, in short reactive mixture was added to an aliquot of ALP solution in a multi-well plate, after 2 mins NaOH was introduced to stop reaction and finally absorbance was measured.

Finally, several ALP dilutions (from 5 mg/ml to 0.01 mg/ml) were prepared and tested in order to draw a calibration curve.

For each multi-well plate, used for testing samples and solutions, three measurements have been carried out on the reactive mixture alone, as reference. This reference value was subtracted to each measure, in order to obtain the normalized enzymatic activity of the tested sample.

All the enzymatic activity tests were performed in triplicate. The data were analyzed by means of one-way ANOVA, with a $p<0.05$ significance level and represented as mean ± standard deviation.

*In-vitro Bioactivity evaluation*

ALP grafted samples were soaked in simulated body fluid (SBF), prepared according to Kokubo protocol [14], at 37°C for 15 days and then observed at SEM/EDS (SEM – FEI, QUANTA INSPECT 200, EDS - EDAX PV 9900) in order to investigate hydroxyapatite precipitation and to evaluate an eventual synergistic effect between biological functionalization and inorganic bioactivity.

**Results and discussion**

*Surface activation*

The direct linking of biomolecules that promote a specific cell response and osteointegration, such as ALP, can not be achieved on untreated titanium and titanium alloys, because they are covered by a stable and un-reactive oxide layer. Pre-treatment is needed in order to introduce reactive chemical groups on the surface. To this purpose thermo-chemical treatment of hydroxylation of the surface was considered in this work [7]. The reactive -OH groups can be used to directly anchor the biomolecules to the surface or to introduce a good leaving group for further activation of the surface beforehand. A leaving group is a molecular fragment that easily departs with a pair of electrons in heterolytic bond cleavage. Soaking of samples in tresyl chloride was employed in this work with this aim. The reaction between the hydroxylated titanium surface and TC molecule is proposed in

figure 1a. The presence of TC on the surface of activated samples (thermo-chemically treated samples soaked in TC) was tested by means of XPS. Survey analysis (figure 1b) reveals fluorine presence (at about 3% in atomic percentage) and sulphur presence (at about 1.7% in atomic percentage), as an indication of the presence of TC molecule on Ti6Al4V surface. The detailed analysis of the carbon region detected a specific signal for C-F bond, as shown in figure 1c. It can be therefore confirmed that TC is linked to the titanium surface. The other signals are related to the inevitable carbon contaminations that are always present on titanium surfaces [15, 16].

*ALP grafting*

The final step of a functionalization process is the grafting of the biomolecule (ALP) to the surface. The presence of the biomolecule on the surface after the grafting process was firstly confirmed by XPS. XPS survey analysis on thermo-chemically treated and activated samples showed enrichment in carbon and nitrogen after ALP anchoring, together with a significant reduction of the fluorine signal (1,4 %at) and the complete disappearance of the sulphur one, (figure 2a). The detailed analysis of the carbon region (figure 2b) indicated specific signals for C-O and C-N bonds (characteristic of the enzyme) and a peak that is typical for aromatic rings flattened onto the surface (shake up features [17]). These are attributable to phenylalanine (an amino acid present in ALP molecule) [18]. This is relevant because both signals are present on all analyzed samples and also on ALP grafted bioactive glasses [19], so it confirms the link of the biomolecule to the metal surfaces and a non random orientation of the enzyme can be supposed.

*ALP activity*

Biological molecules, like ALP, are sensitive to pH and temperature. ALP can tolerate temperatures up to 40°C and pH values below 5 without denaturing [20, 21]. Different temperatures were investigated, for the storage of the solutions and the grafting process, in order to reach the best compromise between the effectiveness of the bonding reaction and the preservation of the

biomolecules. First of all an analysis of the activity was carried out of prepared ALP solutions, that had been stored for 20h at 4°C, 25°C and 37°C, respectively. Enzymatic activity of ALP solutions after 20h storage at the different temperatures (4°C, 25°C and 37°C) is reported in figure 3.

The reaction of the enzyme with the substrate and the measurement of the absorbance of the solutions are always performed at room temperature. It can be observed that the activity of the enzyme in solution is higher if it is stored at 37°C rather than at 4°C ($p<0.05$). On the basis of these data, the grafting of treated and activated samples was carried out by soaking samples in ALP solution at different temperatures (4-25-37°C). Enzymatic activity tests were also performed on ALP grafted samples. The enzymatic activity of Ti6Al4V samples, functionalized with ALP at different temperatures, is reported in Figure 4. It can be noted that samples grafted at 37°C show a higher activity of ALP. So it can be concluded that low temperatures (such as 4°C) could reduce enzyme activity and also its reactivity towards metal surfaces. For this reason ALP grafting was performed at 37°C from this point onwards.

Figure 5 compares the enzymatic activity of samples grafted with ALP at different stages of the treatment. The effects of hydroxylation and surface activation with TC were investigated. It is clear that the biomolecule can not be grafted onto the polished and untreated surface of Titanium alloy (Ti+ALP sample – first column). The activation of the polished surface with TC allows the grafting of a low amount of the biomolecule onto the untreated titanium alloy (Ti+TC+ALP sample - second column). This is in agreement with the low amount of -OH groups detected on untreated (polished) samples by XPS [7]. The higher density of -OH groups on the thermo-chemically treated surfaces and their higher surface area, due to the nanoporous surface layer, are in agreement with a better response to grafting (Ti+HF+$H_2O_2$+ALP sample – third column) compared to the polished one (first column), ($p<0.05$). Furthermore, activation with TC makes the grafting onto thermo-chemically treated samples (fourth columns) even more effective by introducing a good leaving group onto the surface. The increase in ALP activity of functionalized samples induced by TC activation of thermo-chemically treated samples is statistically significant ($p<0.05$). Considering the calibration

curve performed on diluted ALP solutions, the enzymatic activity of a functionalized sample (at about 1cm$^2$ area) is in the order of magnitude of a 0.6 mg/ml ALP solution. It can be concluded that the complete process (thermo-chemical treatment and surface activation by TC before functionalisation) allows the best functionalization result.

The enzymatic activity was compared on samples functionalized with ALP after complete chemical treatment ($HF+H_2O_2$) and after a chemical and thermal treatment at 300°C ($HF+H_2O_2$+TT300) or 400°C ($HF+H_2O_2$+TT400). All samples were grafted after TC surface activation. The difference in the amount of ALP grafted onto $HF+H_2O_2$ samples and $HF+H_2O_2$+thermal treatment ones is not statistically significant ($p>0.05$) (data not shown) [7]. It can be concluded that the thermal treatment does not reduce the reactivity of the surface, as already discussed in part I of the present paper [7].

Considering that the functionalised surfaces are intended for implantation, it is important to investigate the stability of the grafting and the release of the grafted biomolecules. To this aim figure 6a reports the enzymatic activity of grafted samples after different washings. It can be noted that several gentle TRIS washings can remove a significant amount of enzyme from the surface ($p<0.05$). This aliquot decreases with the number of repetitions (second and third columns of figure 6a, $p<0.05$). The mechanical action of ultrasounds (fourth column in figure 6a) causes a higher removal of the grafted enzyme if compared to two sequential gentle TRIS washings ($p<0.05$), but part of the grafted enzyme is still anchored to the surface after it. The complete detachment of the enzyme can be obtained only by a further washing in urea (last column in figure 6a). Urea is able to dissolve the entire grafted enzyme also without the mechanical action of ultrasounds (data not shown). Urea was used to verify bonding stability because it is a well-known chaotropic agent that is able to detach proteins, and not to simulate physiological conditions.

Thanks to the micro- and nano-porous topography of the modified titanium, described in the first part of the work [7], it can be supposed that a certain amount of the biomolecule can be adsorbed on the surface. On the other hand, the chemical activation with tresyl chloride supports covalent grafting of ALP on the modified metal. So both absorption and covalent bonding of the enzyme will

occur during the functionalization procedure. On the basis of this, it can be hypothesized that after washing the adsorbed portion of enzyme can be released in solution and only stronger washings are able also to detach the covalently grafted molecule.

If we consider the washing solutions (figure 6b), it can be observed that ALP maintains its activity in solution and that the amount detected in solution is consistent with that removed from the solid sample. In particular, it can be observed that the second gentle TRIS washing is able to remove a significantly lower amount of ALP from the surface than the first one ($p<0.05$) and that ultrasonic TRIS washing can detach a significantly higher amount of enzyme if compared with gentle washing ($p<0.05$). It can also be noted that urea does not inhibit the activity of the enzyme in the washing solution. This data confirms the strong nature of the link between the enzyme and the surface and also reveals that the grafted biomolecule is partially available for interaction with body fluids while maintaining its activity.

*In vitro bioactivity*

The final goal of this work is to obtain a bioactive surface able to induce both in-vivo apatite precipitation (inorganic bioactivity and biomineralization) and a specific cell response due to functionalization. The ability of the surface to induce apatite precipitation must therefore be verified also after functionalization with ALP. ALP functionalized samples were observed by SEM after 15 days of soaking in SBF. Figure 7 shows typical precipitates on the surface, with the characteristic morphology of hydroxyapatite, and EDS analysis confirmed a Ca and P rich composition. EDS analysis performed on the surface thet was free from precipitates revealed a widespread enrichment in Ca and P. The quantification of Ca and P on the whole area of the samples after 15 days in SBF is reported in table 1; values for thermo-chemically treated samples and thermo-chemically treated and ALP grafted ones are compared. Particle dimension is about 10 μm, so it is double if compared to the precipitates on thermo-chemically treated samples without ALP grafting [7, 22]. The total amount of particles is also higher. Bigger and more numerous hydroxyapatite particles are an

indication of more pronounced bioactivity of the surface and of faster precipitation and growth kinetics. The material ability to induce hydroxyapatite precipitation in vitro has been correlated to the in vivo ability to bond to bone [14], so it can be supposed that a surface that is able to induce a more effective and rapid hydroxyapatite precipitation in vitro should induce a faster and higher bone formation in vivo. The higher ability of promoting hydroxyapatite precipitation of the ALP functionalized samples can be explained by taking into account the chemical properties of this enzyme. Alkaline phosphatase is a hydrolase enzyme which catalyzes the hydrolysis of phosphate monoesters. It is characterized by an active site including 2 $Zn^{++}$ ions that presents high affinity for phosphate binding [23]. It can be therefore hypothesized that in SBF, which is a solution rich in phosphate ions, but free of organic phosphates, ALP molecules grafted onto a material surface will attract and bind these ions, acting as a nucleation centre for hydroxyapatite. Analogous behaviour was observed on ALP grafted bioactive glasses [19, 22]

**Conclusions**

The use of an innovative and patented process is described in this research work. the first phase involves the thermo-chemical surface modification of Ti6Al4V alloy, in order to make it bioactive from the inorganic point of view (ability to induce hydroxyapatite precipitation), enabling it to graft alkaline phosphatase (ALP), while also inducing biological bioactivity. In this way it was possible to obtain biomimetic surfaces, where inorganic bioactivity of the modified titanium alloy could be coupled to the biological one (ability to stimulate cells), due to the grafted molecule. It was verified that ALP was successfully grafted in its active state to the thermo-chemically treated titanium alloy. It was also observed that grafting is possible both after a two step chemical treatment ($HF+H_2O_2$) and after a further thermal treatment, carried out in order to mechanically stabilize the modified layer. It was possible to perform ALP grafting at different temperatures (4°C, 25°C and 37°C), but the best result was for 37°C grafting. It was observed that the functionalization with ALP is more

effective if the process of inorganic modification is followed by a surface activation with a good leaving group such as tresyl chloride (TC).

Finally it emerged that there is a synergistic effect between biological functionalization and inorganic bioactivity, in fact ALP grafted samples present a more marked ability to induce apatite precipitation.

The effects of sterilization and storage on functionalized biomaterials are under investigation and will be discussed in detail in a future paper. The investigation of cellular response of modified and functionalized Ti6Al4V is also in progress.

**Acknowledgements**

Regione Piemonte is acknowledged for its funding to the BIOSURF project.

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**Figure legends**

**Fig 1** Reaction scheme of tresyl chloride grafting to titanium surface (a), XPS survey spectrum (b) and XPS detailed analysis of carbon region (c) for thermo-chemically treated samples activated by TC.

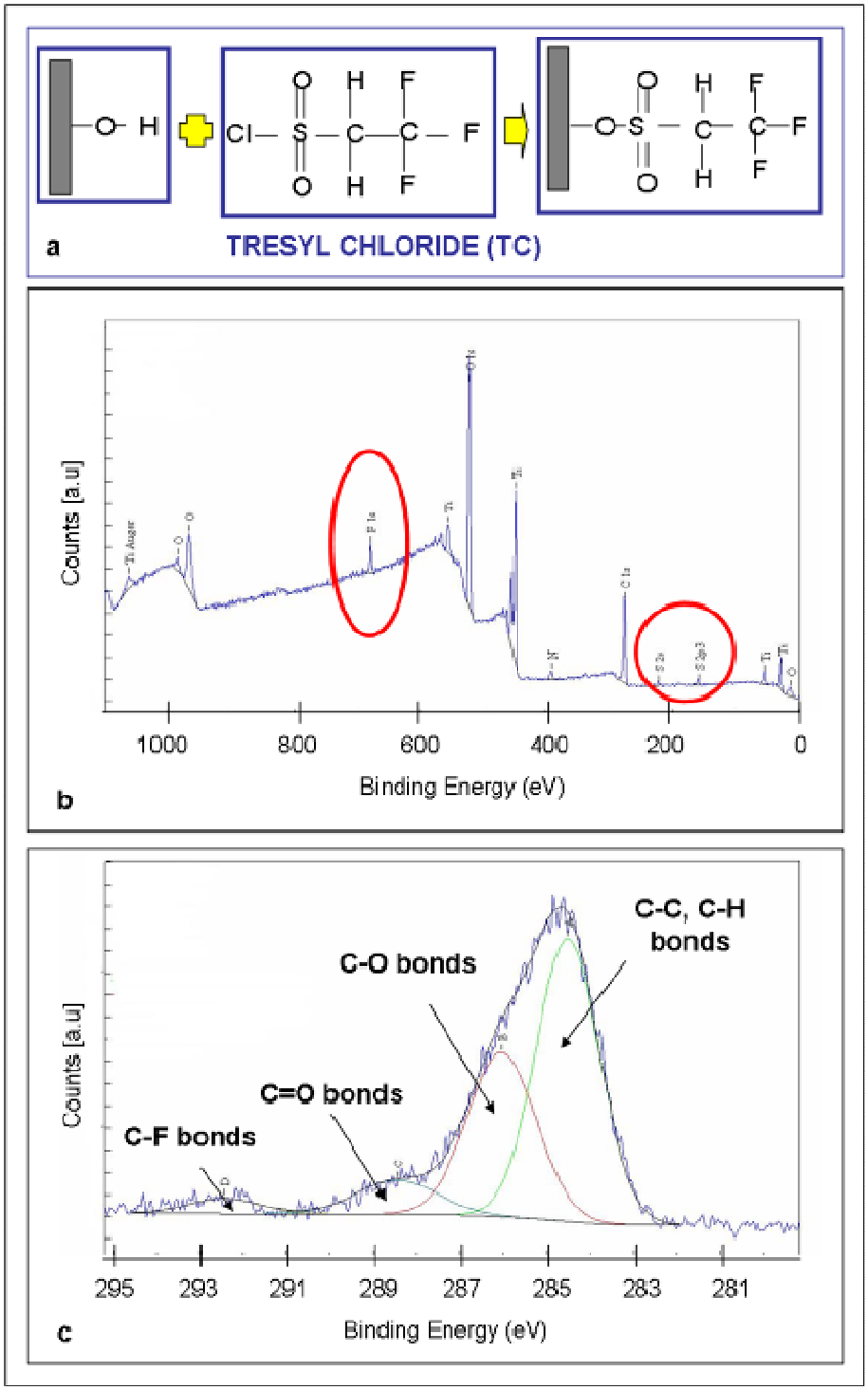


**Fig 2:** XPS survey spectrum (a) and detailed study of carbon region (b) for a thermo-chemically treated and functionalised sample

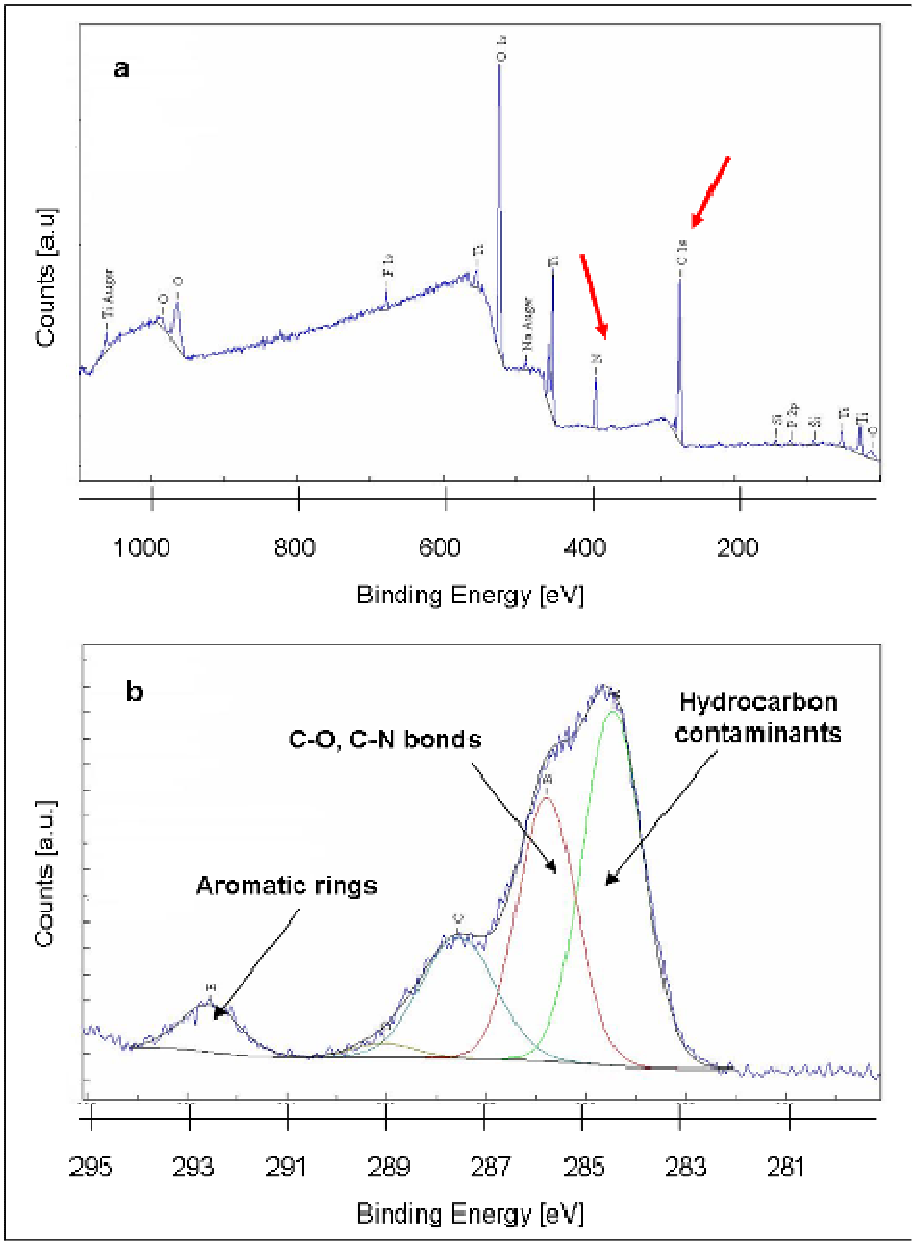


**Fig 3**: Enzymatic activity of ALP solutions after 20 hours storage at different temperatures (4°C, 25°C and 37°C)

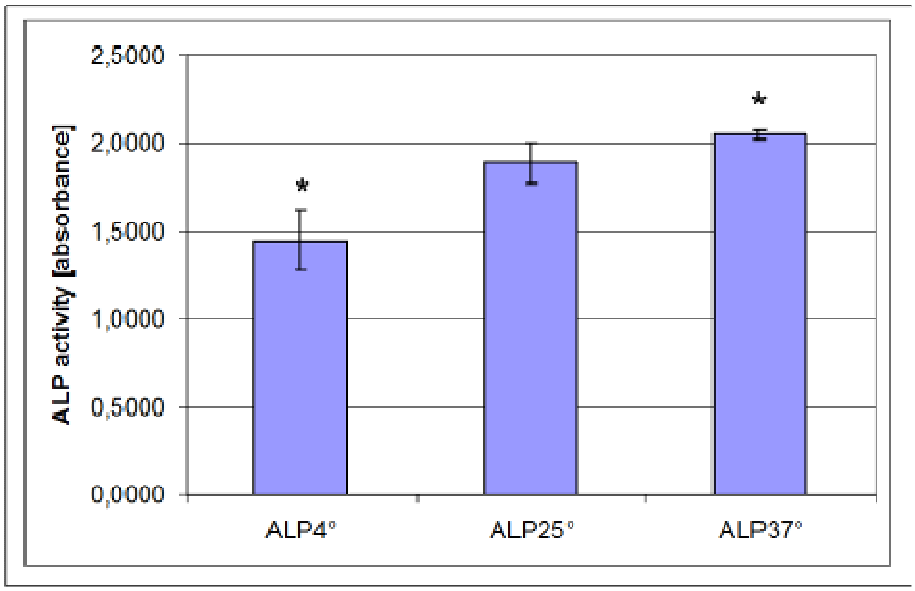



**Fig 4:** Enzymatic activity of Ti6Al4V samples after thermo-chemical treatment, TC activation and ALP grafting at different temperatures (4°C, 25°C and 37°C)

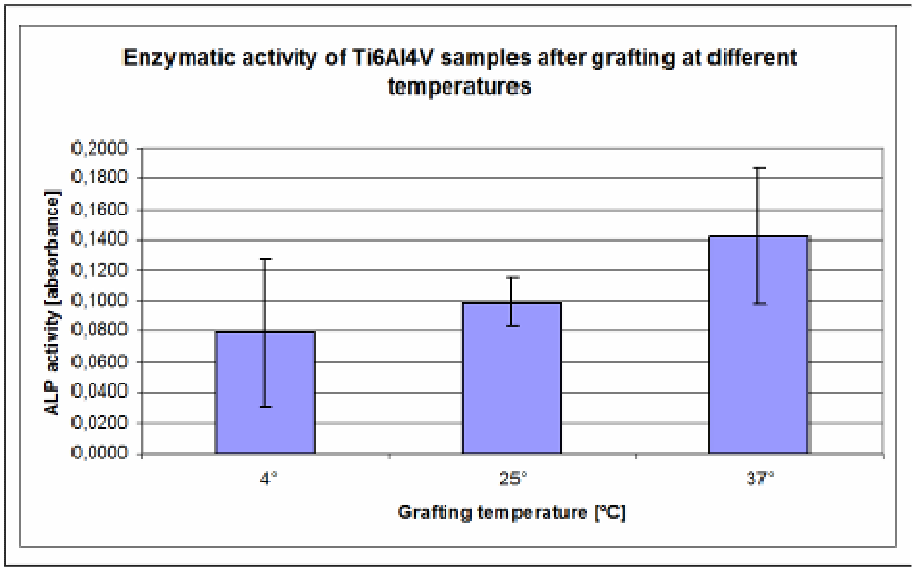


**Fig 5:** ALP activity of polished and grafted Ti6Al4V samples (first column), polished, activated with TC and grafted samples (second column), polished, thermo-chemically treated and grafted samples (third column), polished, thermo-chemically treated, activated with TC and grafted samples (fourth column).

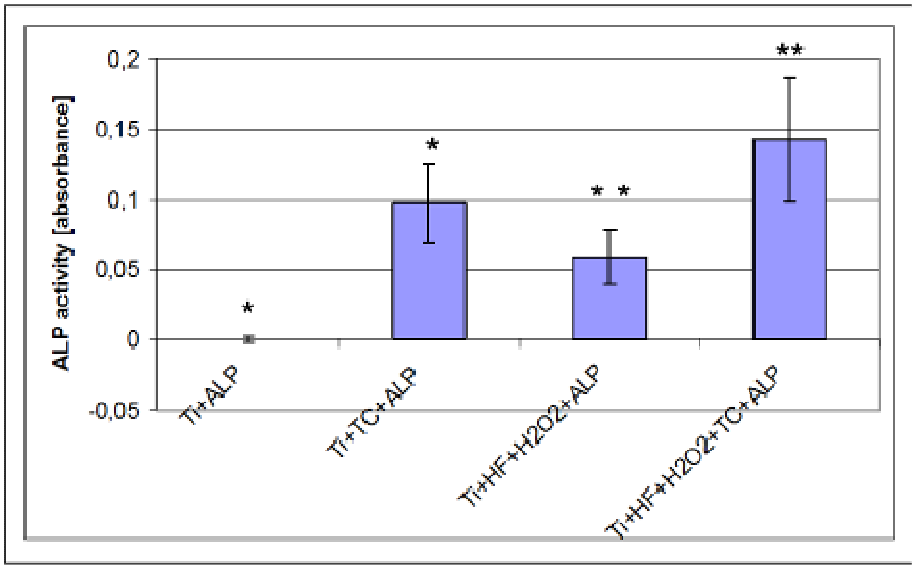


**Fig 6:** Enzymatic activity of Ti6Al4V before and after different washings. a) Enzymatic activity of Ti6Al4V samples: first column (ctrl) – no washing (except the one to stop reaction), second column (TRIS 1) - 1 gentle washing in TRIS solution, third column (TRIS 2) - 2 gentle washings in TRIS solution, forth column (TRIS US1) - 1 ultrasonic washing in TRIS solution, fifth column (TRIS US+Urea US) - 1 ultrasonic washing in TRIS solution + 1 ultrasonic washing in Urea 8M solution. Figure b) enzymatic activity in washing solutions: first column (TRIS 1) – TRIS of the first gentle washing, second column (TRIS 2) TRIS of the second gentle washing, third column (TRIS-US1) – TRIS of the TRIS ultrasonic washing, forth column (Urea-US2) – Urea of the ultrasonic washing after the TRIS one.

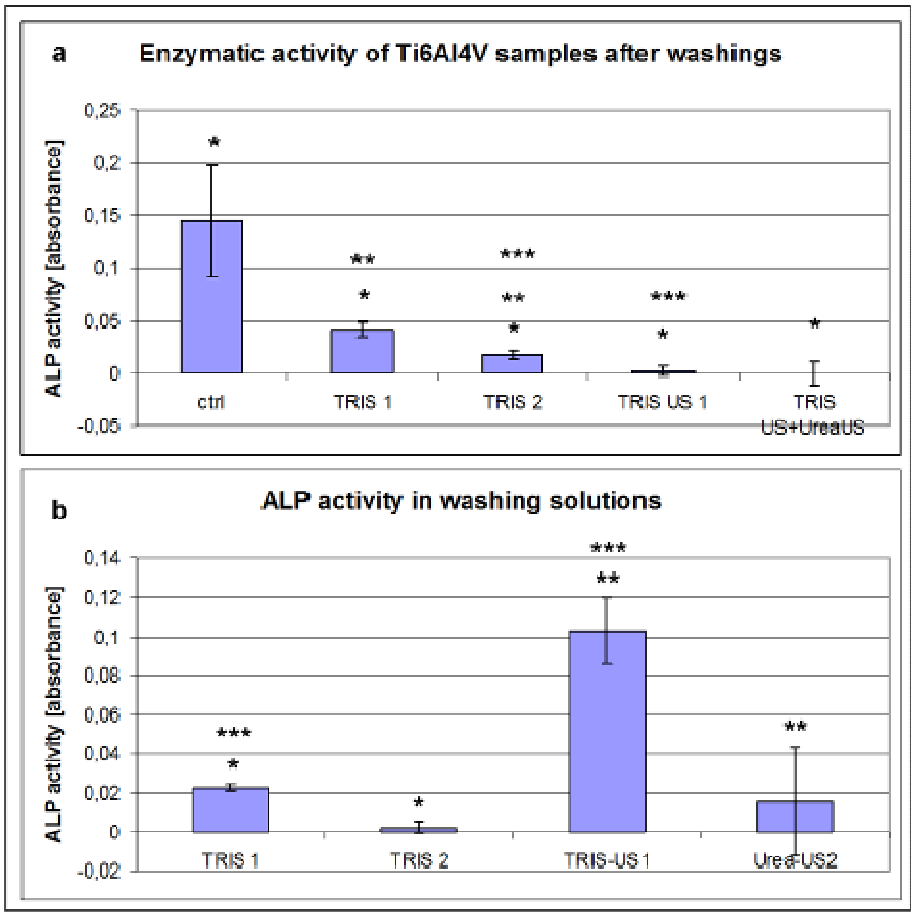
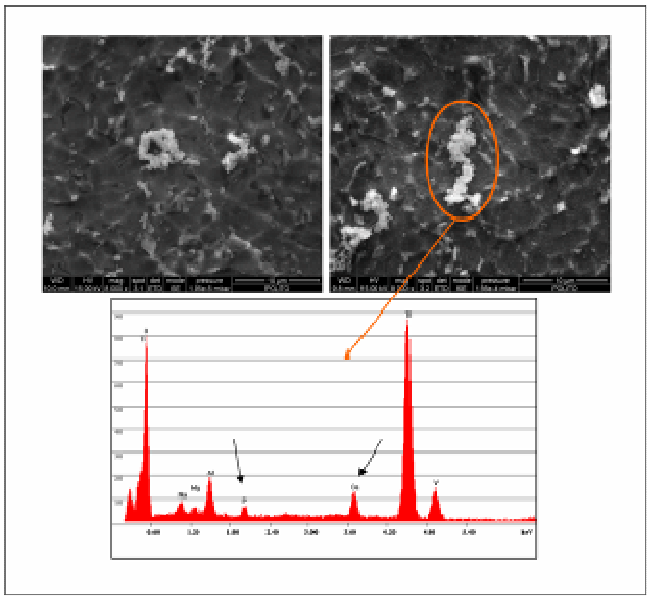


**Fig 7:** Hydroxyapatite precipitation onto biologically functionalized samples after 15 days in SBF



## Tables

**Table 1:** Ca+P atomic percentages on different samples after 15 days in SBF

**Table 2: Ca+P atomic percentages on different samples after 15 days in SBF**

| Sample | Ca+P atomic% [mean±st.dev] |
|---|---|
| $HF+H_2O_2$ | 1.83±0.01 |
| $HF+H_2O_2$+TT300 | 1.63±0.06 |
| $HF+H_2O_2$+TT400 | 1.46±0.31 |
| $HF+H_2O_2$+TC+ALP | 2.11±0.35 |