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Bioactive and antibacterial titanium surfaces through a modified oxide layer and embedded silver nanoparticles: effect of reducing/stabilizing agents on precipitation and properties of the nanoparticles

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Abstract

The final goal of this research is to get a titanium surface simultaneously able to induce bone tissue integration in case of critical bone quality and/or quantity (elderly population) and to prevent infections at the implant site and around it. A metallic antibacterial agent is used against bacteria colonization and biofilm formation, in agreement with the well-established strategy to reduce the use of antibiotics. A surface treatment able to induce the formation of a chemically modified and nanotextured surface oxide layer (with several functionalities suitable for fast and effective osseointegration), as well as the nucleation of silver nanoparticles on the surface was developed. This paper describes the selection and dose of the required additives, such as reducing agents and nanoparticles stabilizers, in order to get a good control of the series of chemical reactions concurrently occurring during the surface chemical treatment. Surface topography (oxide nanotexture and nanoparticle precipitation) was investigated by means of Field Emission Scanning Electron Microscopy (FESEM) equipped with Energy Dispersive Spectroscopy (EDS) for semi-quantitative evaluation of chemical composition. Silver release was measured in ultrapure water through a photometric method and surface ability to induce *in vitro* apatite precipitation was investigated by soaking in Simulated Body fluid (SBF) followed by FESEM-EDS observations. Finally the antibacterial activity of modified titanium surfaces was investigated against *Staphylococcus aureus* by means of both inhibition halo and adhesion assays.

Keywords: titanium, silver nanoparticles, reducing/stabilizing agents, antibacterial

1. Introduction

Advanced bone implants (orthopaedic, trauma, spine and dental implants) have to address two main medical needs: tissue integration (osseointegration) in case of critical bone quality/quantity, without a plethora of inflammatory reaction, and prevention of infections at the implant site and around it.

These issues are interconnected with each other and must be simultaneously addressed by the surface of an implant. It is well known that when an artificial material is implanted in the human body a series of events occurs at its surface, starting from hydration, protein adsorption and as last cellular colonization. If the bacteria are present at the implant site, in addition to the cells, a sort of “race for the surface” will start [1, 2]. Therefore, the bacteria will be obstructed if their cellular adhesion and proliferation on surfaces are reduced. The infection is mainly due to the bacterial colonization of the implanted materials through adhesion, accumulation and persistence of the bacteria onto the material surface and to the consequent formation of a biofilm. This three-dimensional structure protects the bacteria from the systemic therapies and patient own defense, so it is particularly difficult to be treated and it often leads to the necessity of removing the implant. The specific clinical target is elderly population, considering the widespread occurrence of criticism concerning bone quality and/or quantity.

An increasing problem in healthcare is the formation and spread of so-called multi-drug resistant bacterial strains that are resistant to many antibiotics and therefore difficult to eliminate once they are established. Infections may result in prolonged hospital stays, need for special antibiotics and the need to care for infected patients separated from non-infected (so-called barrier nursing). The progress of this research beyond the state of the art consists in realizing a metal implant surface able to stimulate tissue integration,

hindering bacterial adhesion, and directly fighting bacterial colonization, without using antibiotics. No antibiotic was used in the approach of this research, in agreement with the well-established European strategy to reduce the antibiotic therapies considering that some antibiotics induce hyper-sensitivity and cause allergic response, most antibiotics cause toxic side effects, as well as mutant bacteria are resistant to the antibiotics [3, 4, 5]. Silver nanoparticles were selected considering that silver is well known and used from ancient times for its broad spectrum antibacterial activity (based on a multiple mechanism of action) and low resistance development [6, 7, 8]. Silver nanoparticles present a large surface area to volume ratio with consequent higher ion release combined with the possibility to directly interact with bacterial cells offering the opportunity of an improved antibacterial effectiveness [9, 10]

The here adopted strategy is based on a surface chemical treatment able to simultaneously induce a surface texture and chemistry suitable for faster and more physiological bone integration, with respect to the standard implants, as well as the presence of an inorganic antibacterial agent on the surface (silver nanoparticles) [11]. Bone integration is obtained through bioactive behaviour (apatite precipitation) of the treated titanium surface, which in addition to this shows several multifunctionalities: high osteoblast differentiation, high degree of hydroxylation, high wettability, high protein adsorption, high implantation friction resistance [12, 13, 14, 15]. All these properties are related to the presence of a chemically modified and nanotextured surface oxide layer produced during a chemical treatment of surface controlled oxidation. An additional antibacterial action is introduced on the surface through the nucleation of silver nanoparticles formed during the same chemical treatment starting from silver ions.

The key factor for a successful result of the surface chemical treatment is therefore the simultaneous occurrence of a controlled oxidation reaction of the titanium surface, reduction of silver ions, nucleation of silver nanoparticles and growth control of them. The control of this set of concurrent chemical reactions requires the use of some additives. The specific aim of this research work is the selection and dose of the required additives, such as reducing agents and nanoparticles stabilizers to obtain surface nanotexture, hydroxyl exposition, precipitation of well dispersed silver nanoparticles avoiding both the formation of large aggregates and the surface contamination with additives, and reducing the bacterial adhesion.

2. Materials and methods

2.1 Samples preparation

Plane disks (2 mm thickness and 10 mm diameter) were obtained from cylindrical bars of commercially pure titanium (Ti-cp; ASTM B348, Gr2, Titanium Consulting and Trading) and Ti6Al4V alloy (ASTM B348, Gr5, Titanium Consulting and Trading) by means of an automatic cutting machine (Struers Accutom 5) equipped with an alumina blade (356 CA). The samples were then polished with SiC abrasive papers (up to 4000 grit), washed in acetone (one time for 5 min) and in water (two times for 10 min) in an ultrasonic bath.

The sample surface was modified, according to a patented chemical treatment (CT), to obtain a nanotextured surface oxide layer rich in hydroxyl groups, bioactive (able to induce hydroxyapatite precipitation upon contact with physiological fluids) and antibacterial (due to the presence of silver nanoparticles on it) [16, 17, 12, 13,14, 15, 11]. The treatment foresees a first surface etching in hydrofluoric acid to remove the native oxide layer and a subsequent controlled oxidation in hydrogen peroxide enriched with silver nitrate (AgNO₃, Silver Nitrate PA-ACS-ISO 131459,1611, Panreac) to obtain the nanotextured titanium oxide embedded with silver nanoparticles.

The lowest effective concentration of silver nitrate (0.001M AgNO₃) investigated in a previous work [11] was selected for the present research to obtain an antibacterial behavior avoiding the risk of cytotoxic side effects.

The addition of various additives (reducing/stabilizing agents) was considered in the present research, to control the silver nanoparticles size, distribution and ion release with the final aim to control surface antibacterial activity and biocompatibility.

The considered reducing/stabilizing agents with the employed concentrations and literature reference are reported in Table 1.

Table 1: The additives employed for reduction and stabilization of silver nanoparticles on titanium surfaces, during a controlled surface oxidation treatment, and reference papers used for the selection of the concentrations.

Additive	Abbreviation	Product	Activity	Concentration in Hydrogen peroxide	Reference
Glucose	GLU	Glucose syrup food grade	Reducing agent	0.23 g/l	[18]
Poly Vinyl Alcohol	PVA	Poly(vinyl alcohol) average Mw 13,000-23,000, 98% hydrolyzed, 348406 Sigma Aldrich	Stabilizing agent	0.01 g/l	[19]
Gallic Acid	GA	Gallic acid 97.5-102.5% (titration), G7384 Sigma Aldrich	Reducing and stabilizing agent	0.1 g/l	[20]
Starch	ST	Starch puriss. p.a., from potato, reag. ISO, reag. Ph. Eur., soluble, 33615 Sigma Aldrich	Reducing and stabilizing agent	0.003 M	[21]
Tannic Acid	TA	TA, Tannic acid ACS reagent, 403040 Sigma Aldrich	Reducing and stabilizing agent	0.001 M	[22]

2.2 Surface characterizations

Field Emission Scanning Electron Microscopy (FESEM - SUPRATM 40, Zeiss) equipped with Energy Dispersive Spectroscopy (EDS) was employed to investigate surface morphology and silver nanoparticles presence, size and distribution upon the use of different additives.

Fourier Transformed Infrared Spectroscopy (FT-IR, IR Hyperion 2000, Tensor 27 - Bruker S.p.A) was employed for the analysis of the surface hydroxyl groups.

The surface chemical composition and chemical state of the elements were investigated by means of X-Ray Photoelectron Spectroscopy (XPS, PHI 5000 VERSA PROBE, PHYSICAL ELECTRONICS) in survey and high resolution mode.

2.3 Silver release

The amount of silver released from the surface of the modified samples was determined in ultrapure water by means of a photometer for silver analysis (HI 93737, Hanna Instruments). Each sample was soaked in 25 ml of ultrapure water at 37 °C. After 3 hours, 1, 2, 7, 14 and 28 days the solution was changed with fresh one and analyzed for silver content.

2.4 *In vitro* bioactivity

In order to evaluate surface ability to induce *in vitro* precipitation of hydroxyapatite upon soaking in simulated body fluid (SBF), the samples were immersed in SBF for 15 days at 37 °C. SBF was prepared according to the protocol proposed by Kokubo [23] and changed every two days in order to mimic *the in vivo* physiological turnover of the body fluids. The pH was measured at each solution refresh.

2.6 Zeta potential measurements

Isoelectric point and zeta potential in function of pH were evaluated by means of the streaming potential technique through an electrokinetic analyzer (SurPASS, Anton Paar). The measurements were performed in 0.001M KCl as electrolyte and pH was varied by the addition of 0.05M HCl or 0.05M NaOH through the instrument automatic titration unit. The acid and alkaline sides of the curve were obtained in two different steps, usually firstly testing the acid one.

2.7 Tape adhesion tests

The adhesion of the modified surface oxide layer was evaluated by means of cross-cut tape test, performed according to ASTM D3359 [24]. Ti6Al4V CT and Ti6Al4V CT – GA+PVA+Ag were considered for this test, the first one as reference and the second one because it resulted the most promising from the previous characterizations. A grid of parallel cuts was obtained on each sample by a cutter, the surface was cleaned with a brush, the tape was positioned and removed. The surface was then visually inspected and compared with the reference schemes reported in the standard, to estimate the coating damage degree.

2.8 *In vitro* antibacterial tests

The antibacterial behavior of the treated Ti6Al4V samples was investigated by means of the inhibition zone evaluation test, in accordance to NCCLS standards [25], and by bacterial adhesion assay by applying methods used in previous papers of ours [26, 27, 28].

Biofilm-producing *Staphylococcus aureus* strain (ATCC 29213) was employed for both the tests because it is one of the main pathogens involved in both orthopedic and dental infections [29, 30].

For the inhibition halo test, a 0.5 McFarland bacterial suspension (containing approximately $1-2 \times 10^8$ CFU/ml) was spread using a sterile swab on Mueller Hinton agar (Becton Dickinson and Company, BD, New Jersey, USA) plates on which the modified Ti6Al4V sterile samples were placed (with the treated surface in contact with the plate) and incubated for 24 h at 37 °C. The antibacterial behavior was estimated by observing the inhibition halo (a zone around the samples where bacterial proliferation was stopped).

For the adhesion assays, each Ti6Al4V sample was immersed in 5 ml of bacterial suspension in Mueller Hinton broth, containing about 10^7 bacteria/ml, and incubated at 37 °C for 24h under shaking. At the end of the incubation, the samples were extracted from the bacterial broth, washed in sterile water, transferred into sterile plastic bags containing 1.5 ml of physiological solution (NaCl 0.9%; Bieffe Medital S.p.A., Grosotto, Italy) and sonicated (40 kHz) for 7 min at 22°C to detach adhered bacteria. This is an ideal protocol

for dislodging biofilm producing bacteria without affecting bacterial viability [31]. Controls represented by bacteria incubated in broth, in the absence of the samples, were also prepared. Both the incubation broths and sonication solutions were serially diluted and spread on Nutrient agar plates (BD), to obtain, after incubation for 24h at 37 °C, the number of Colony Forming Units (CFU) referred to the non-adhered and adhered bacteria, respectively [26, 27, 28].

The adhesion experiments were assayed in triplicate and repeated a minimum of three times.

2.9 Statistical analysis

The adhesion assay results (CFU/mL) were analyzed by descriptive statistics (mean \pm SD). The results were tested by unpaired t-tests to highlight significant differences ($p < 0.05$; 95% confidence interval) between the Ti6Al4V samples, using Graphpad Prism, Version 6 for Windows (Graphpad Software, San Diego, CA, USA).

3. Results and discussion

The main goal of the present research is the development of a chemical surface process able to get titanium surfaces with the following required features:

- nanotextured oxide layer with functionalities suitable for osseointegration as previously demonstrated by the authors [12, 13, 14, 15]. In this respect, surface morphology, bioactivity and surface zeta potential were evaluated;
- high density of hydroxyl groups able to induce surface bioactivity, high wettability and possibility of further surface modifications [12, 13, 14, 15, 16, 32];
- silver nanoparticles embedded in the surface oxide layer and silver ion release able to induce antibacterial behavior;
- absence of silver micro-aggregates, which can be responsible of burst/uncontrolled release and cytotoxic concerns, as previously observed by the authors in a preliminary work [11];
- absence of contamination of the surface by the additives at the end of the process;
- unchanged adhesion of the surface oxide layer, in order to guarantee that it will be not removed during implantation [15].

For this purpose, starting from an already developed surface chemical treatment (controlled oxidation in hydrogen peroxide enriched with silver ions), different additives have been tested for the control of the reduction reaction of silver ions and growth of silver nanoparticles, as reported in Table 1.

As first, a criterion of biocompatibility was used in the selection of the additives to be tested. The addition of glucose (GLU) was considered because of its reducing activity [18], while poly vinyl alcohol (PVA) is a nanoparticles stabilizer [19]; gallic acid (GA), starch (ST) and tannic acid (TA) were used because they are reported in literature both as reducing agents and nanoparticles stabilizers [20, 21, 22].

The results of FESEM observations (Figures 1 and 2) and FTIR measurements have been collected and compared in Table 2 to verify the above required features and used for a screening of the best chemical mix for the intended purpose.

3.1 FESEM observations

Figures 1 and 2 report an overview of the surface topography of Ti-cp and Ti6Al4V treated with the various additives described in the materials and methods section. Low magnification images (5000x, first column of both the figures) were acquired to investigate the eventual presence of undesired silver micro-precipitates. In addition, high magnification images (150000x, second column of both Figures 1 and 2) were acquired to verify the development of a surface nanotextured oxide layer and the deposition of silver nanoparticles embedded within it.

As shown in the first column of Figure 1, micro-precipitates (5-20 μm) are visible on all the treated surfaces of commercially pure titanium. EDS analyses (not reported) confirmed that they are mainly constituted of silver. Observations of multiple areas on the sample surface suggest a moderate reduction of the number of these precipitates on the CT – GA+PVA+Ag and CT – TA+Ag samples; however none of the considered additives was able to avoid the precipitation of silver micro-aggregates on Ti-cp.

In regards to the treated surfaces of the Ti6Al4V alloy (Figure 2), micro-precipitates are visible only on the samples treated with the addition of starch and in limited amount and dimensions on the samples treated with glucose. Conversely, TA, GA and PVA (used alone or in combination) are effective to avoid precipitation of micro sized silver aggregates.

The nanotextured oxide layer was successfully developed on all the surfaces: this result confirms that nor silver nor additives negatively affect its growth (Figures 1 and 2, second column). Bright nanoparticles (few nanometers) embedded within the nanotextured oxide layer can be observed on all the surfaces (Figures 1 and 2, second column). EDS analyses (not reported), despite of some difficulties due to the small dimension and the technique penetration depth, confirm that they are made of silver. The highest amount and most homogenous distribution of nanoparticles has been obtained on the Ti6Al4V CT – GA+PVA+Ag samples.

In addition to the confirmation of the surface nanotextured topography and silver precipitation, some other observations should be of interest in the whole samples characterization. In particular, some evident organic deposits (attributable to additive residues on the surface) have been observed on all (both Ti-cp and Ti6Al4V) the surfaces treated with ST and this contamination should be taken into consideration. Moreover, some cracks have been evidenced on the surface of CT – TA+Ag samples. This phenomenon may be due to an initial surface corrosion induced by the drop in pH (from 3 to 2.0) caused by the addition of TA with respect to the other additives here tested.

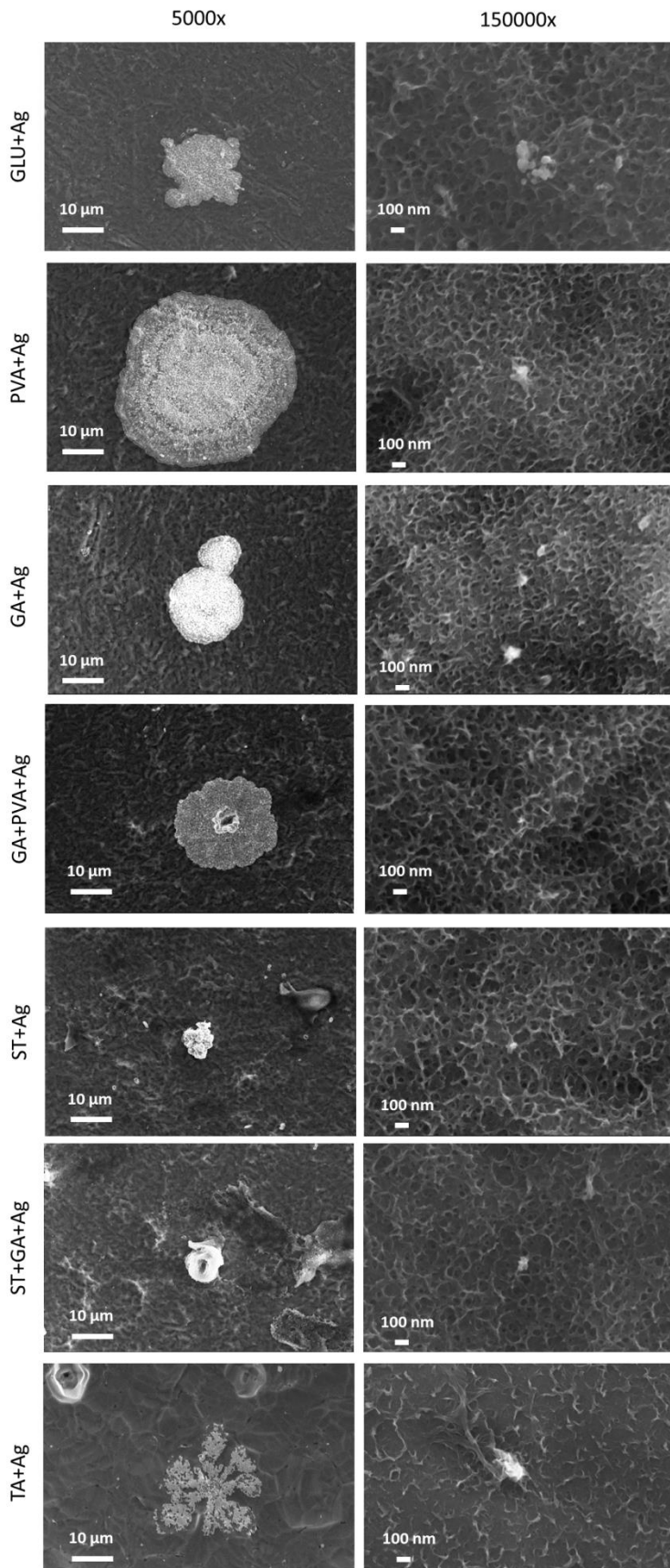


Figure 1: FESEM images of Ti-cp chemically treated with a controlled oxidation surface treatment in hydrogen peroxide with the addition of silver ions (Ag) and various additives: glucose (GLU), poly vinyl alcohol (PVA), gallic acid (GA), starch (ST) and tannic acid (TA).

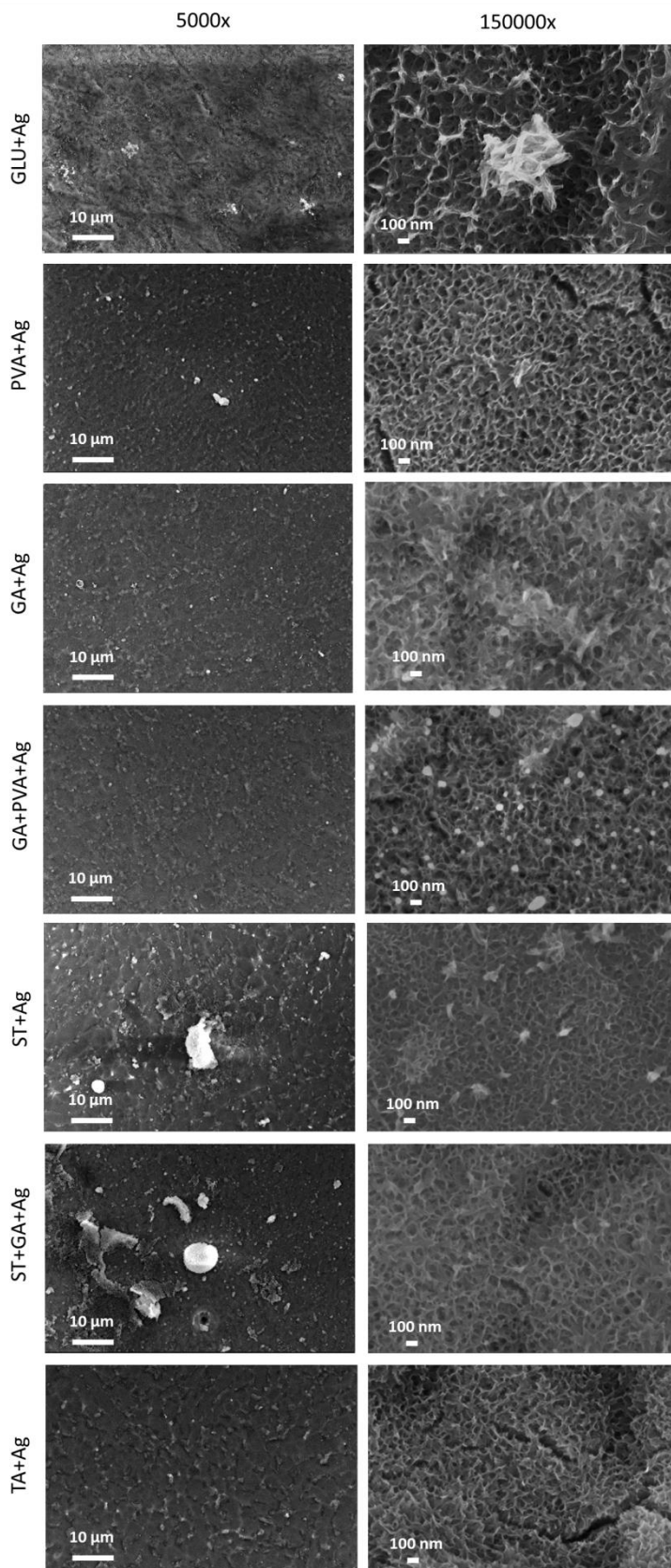


Figure 2: FESEM images of the Ti6Al4V alloy chemically treated with a controlled oxidation surface treatment in hydrogen peroxide with the addition of silver ions (Ag) and various additives: glucose (GLU), poly vinyl alcohol (PVA), gallic acid (GA), starch (ST) and tannic acid (TA).

Table 2: summary of the most significant results derived from physico-chemical characterization

		NANOTEXTURE	Ag-DEPOSITS		OH	Notes
TREATMENT			MICRO	NANO		
CT - GLU+Ag	Ti-cp	Good	Yes	Yes	++	
	Ti6Al4V	Good	Few	Yes	+++	
CT - GA+Ag	Ti-cp	Good	Yes	Yes	+	
	Ti6Al4V	Good	No	Yes	+++	
CT - PVA+Ag	Ti-cp	Good	Yes	Yes	+	
	Ti6Al4V	Good	No	Yes	+++	
CT - GA+PVA+Ag	Ti-cp	Good	Few	Yes	+	
	Ti6Al4V	Good	No	Yes	+++	
CT - ST+Ag	Ti-cp	Good	Yes	Yes	+	Organic deposits
	Ti6Al4V	Good	Yes	Yes	++	Organic deposits
CT - ST+GA+Ag	Ti-cp	Good	Yes	Yes	+	Organic deposits
	Ti6Al4V	Good	Yes	Yes	+++	Organic deposits
CT - TA+Ag	Ti-cp	Poor, few cracks	Few	Yes	+	Ag nps in solution
	Ti6Al4V	Good, few cracks	No	Yes (few)	+++	Ag nps in solution

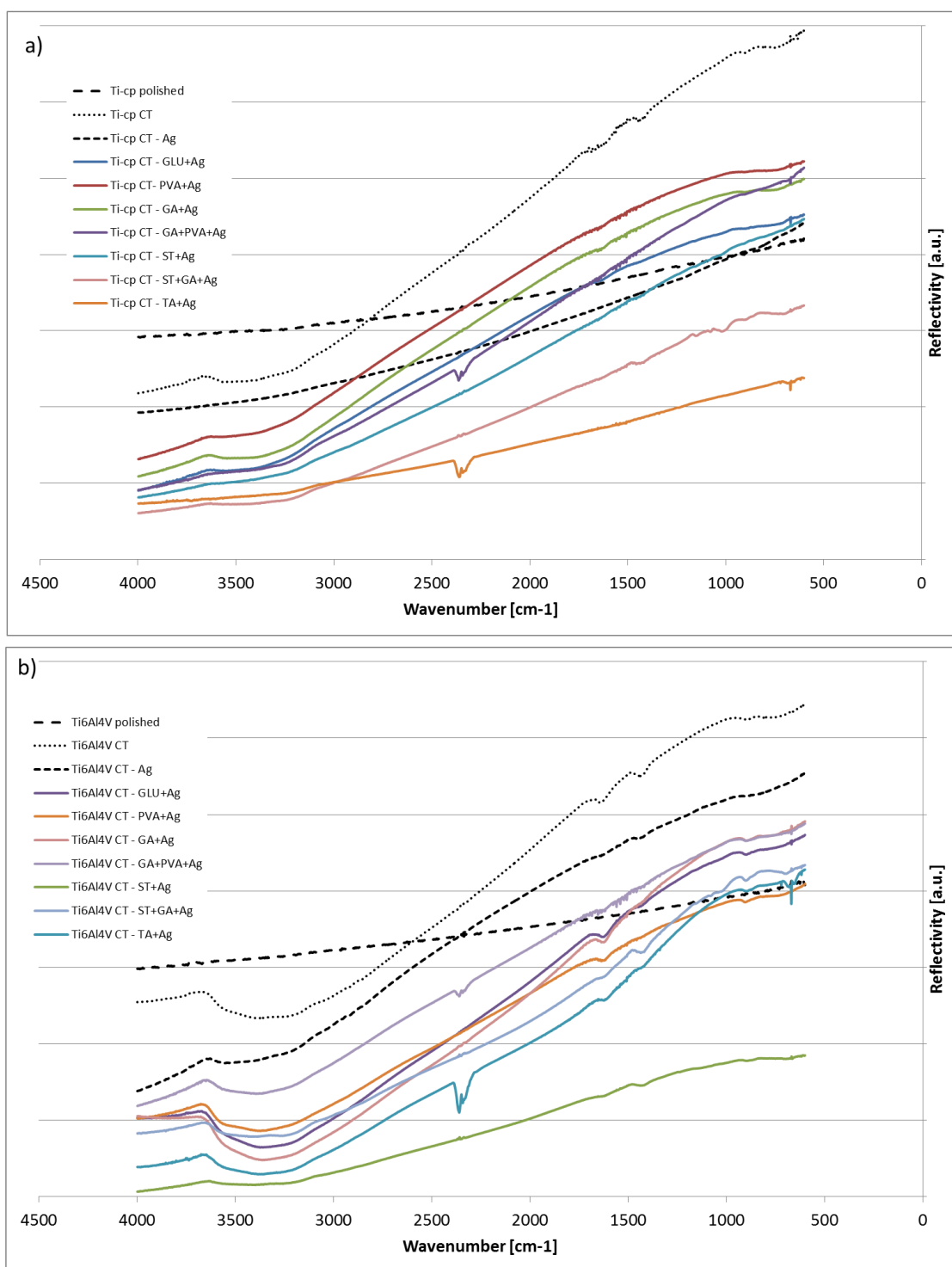


Figure 3: FTIR spectra of a) Ti-cp and b) Ti6Al4V treated samples (chemical treatment in hydrogen peroxide with the addition of silver ions and various additives: glucose (GLU), poly vinyl alcohol (PVA), gallic acid (GA), starch (ST) and tannic acid (TA)).

3.2 FTIR analyses

FTIR spectra of the treated surfaces are reported in Figure 3. Hydroxyl groups, evidenced by a broad absorption band between 3000 and 3600 cm^{-1} [11,12,33, 34, 35], are present on all the samples (except for

the untreated polished ones), but some differences can be noted depending on the substrate and the additive employed in the treatment.

A first difference can be highlighted between Ti-cp and Ti6Al4V: the OH signal is always more intense on the alloy. As previously evidenced by the authors [11] this result can be explained considering both the thickness of the oxide layer on the two substrates and the FTIR penetration depth. In fact, it was previously observed that the nanotextured oxide layer is thinner on Ti-cp (at about 80 nm) than on Ti6Al4V (at about 200 nm) [11] and both the thickness are completely overcome by FTIR penetration depth (at about 1 μm). The addition of silver and additives does not alter this trend.

The introduction of additives does not generally interfere with the hydroxylation degree of the nanotextured oxide layer. A certain reduction in the OH signal intensity can be registered on the Ti-cp sample treated by TA addition. This result can be correlated with the initial corrosion of the titanium surfaces in TA-containing solution previously observed by means of FESEM. A decrease in the OH signal intensity has been observed also in the case of the Ti6Al4V sample treated by ST addition.

No signals attributable to surface contamination by additives have been registered by means of FTIR analyses.

Taken together, FESEM and FTIR results point out that commercially pure titanium substrates fail to completely reach two of the required features set as the main goal of the research independently from the additive employed in the treatment: precipitation of silver micro-deposits is not avoidable and development of high density of OH groups is not completely fulfilled. For this reason, Ti-cp substrate was excluded from further characterizations.

As far as the different additives are concerned, ST (both alone and in combination with PVA) caused surface contamination with organic deposits (FESEM observations) and limited surface hydroxylation: for this reason, it was excluded from further investigations.

3.3 XPS analyses

XPS was employed for the determination of the surface chemical composition (survey analysis) as well as for the investigation of the chemical state of elements (high resolution spectra of the element of interest).

Table 3: Surface chemical composition (at%) of Ti6Al4V surfaces from XPS survey analyses

	CT - Ag	CT – GLU+Ag	CT – PVA+Ag	CT – GA+Ag	CT – GA+PVA+Ag	CT – TA+Ag
O	51.4	49.4	48.1	50.5	51.1	53.8
C	27.1	35.0	29.5	23.6	23.8	19.8
Ti	18.0	13.0	16.8	17.5	17.6	18.9
Ag	3.5	1.4	4.9	4.9	5.3	4.1
N		1.1	0.7	3.5	2.2	3.4

The chemical composition of the analyzed surfaces is reported in Table 3. It can be observed that silver has been introduced through all the considered procedures in a similar amount, except for the samples treated by addition of GLU, which present a lower silver quantity compared to the others.

The high resolution spectra of carbon, oxygen and silver are reported in Figure 4.

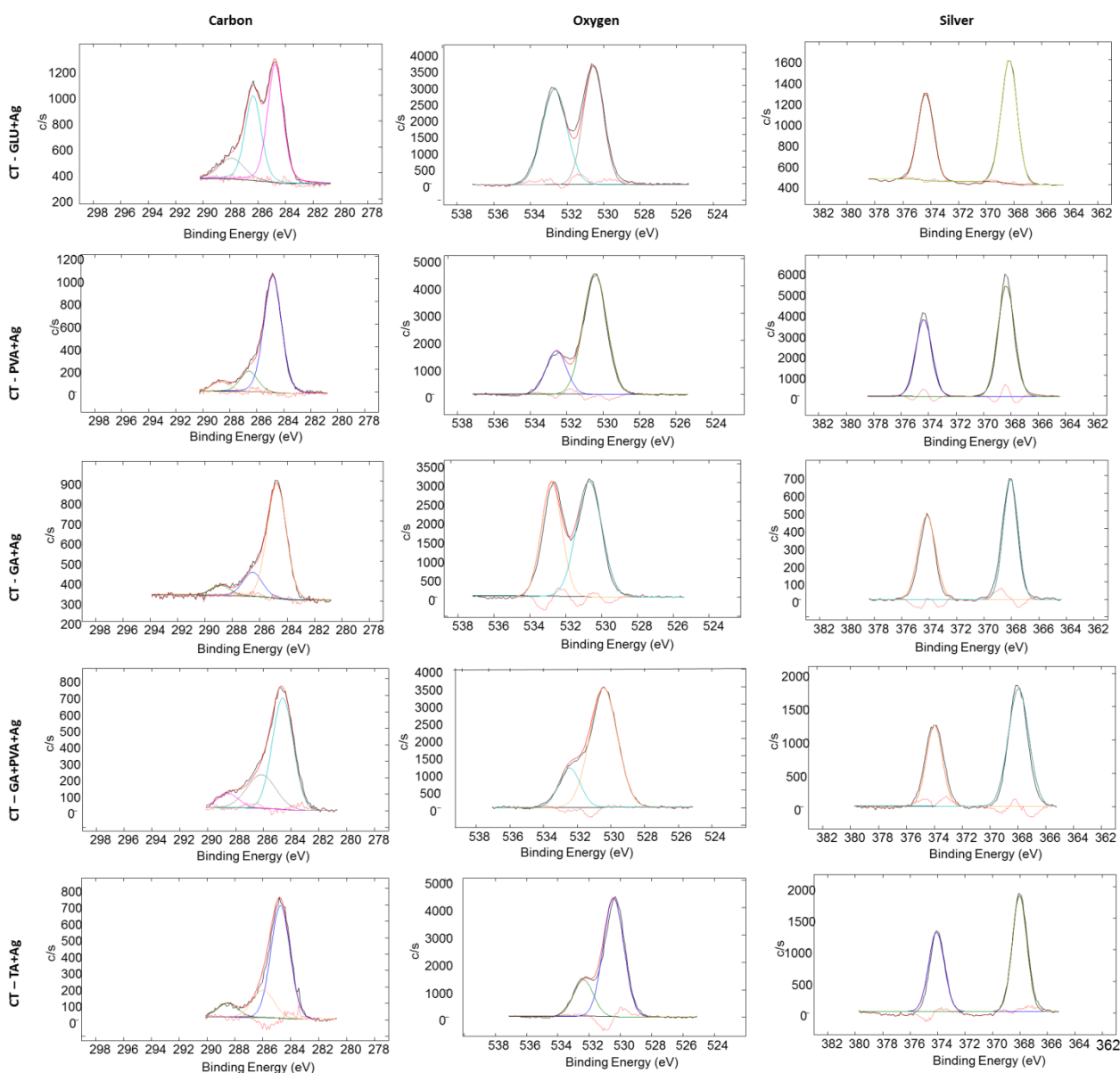


Figure 4: The XPS high resolution spectra of carbon, oxygen and silver regions for Ti6Al4V samples treated by using different additives.

The high resolution spectra of the carbon region (Figure 4, first column) evidences three contributions at about 284, 286 and 288 eV. The first one can be attributed to C-C and C-H bonds and it is usually related to surface hydrocarbon contaminants from the atmosphere, which are particularly frequent onto reactive titanium surfaces [36, 37]. The other two signals can be attributed to respectively C-O and C=O bonds [38, 39, 40]. Their presence may be associated both to additives and contaminants from the atmosphere. Their intensity is very small, except for the sample CT – GLU+Ag: it can be concluded that contamination of the treated surfaces from additives can be considered negligible, with the exception of the case of GLU.

Two contributions can be evidenced in the oxygen region (Figure 4, second column), one at about 530 eV and the other at about 532 eV. The first one can be attributed to Ti-O bonds of titanium oxide, while the second one can be ascribed to Ti-OH bonds [11,12, 36, 41, 42] and it confirms high surface hydroxylation

as previously observed by FTIR (section 3.2 of the present paper). The hydroxyl signal is significantly higher on the CT – GLU+Ag and CT – GA+Ag samples: a correlation to the permanence of a certain amount of additive on the surface cannot be excluded mainly in the case of the CT-GLU+Ag sample.

A doublet at 368 and 374 eV can be observed on the spectra of all the samples in the silver region (Figure 4, last column) and it is attributed to metallic silver [43, 44, 45]: the presence of metal silver nanoparticles on all the treated surfaces is confirmed.

Considering that the CT – GLU+Ag samples show the lower surface silver content (Table 2) and evidence of GLU contamination on the treated surface (Figure 4, high resolution spectrum of the carbon region), this additive was excluded from further characterizations.

3.4 Silver release

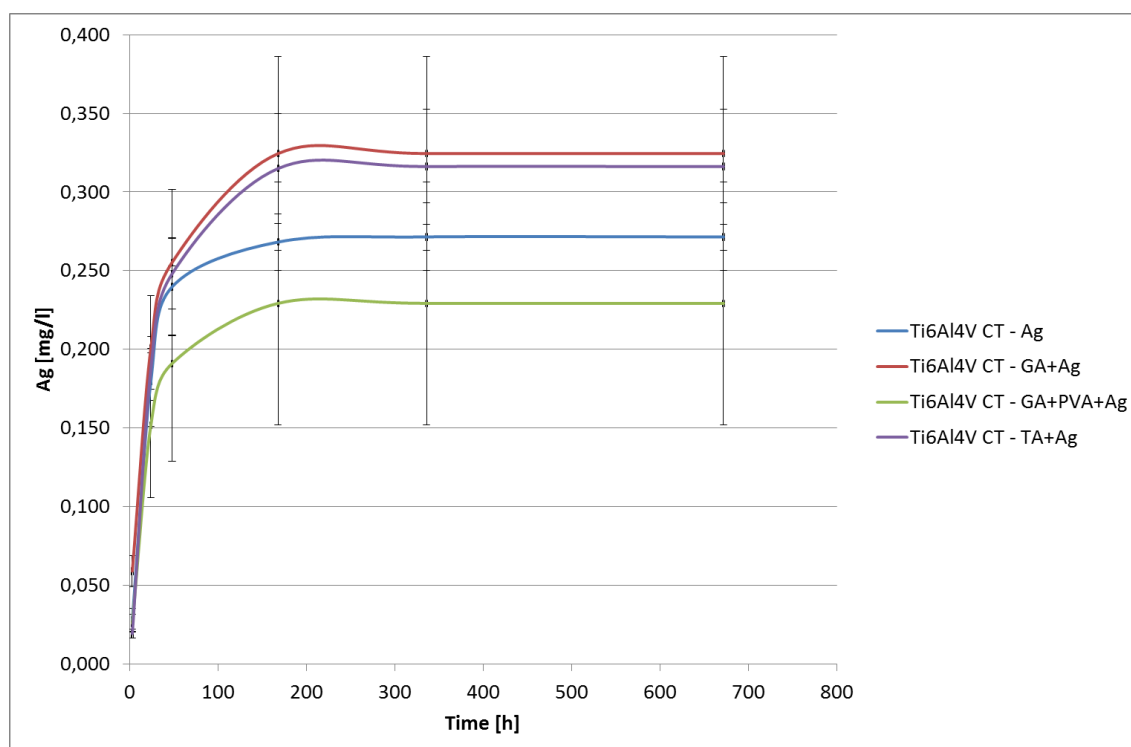


Figure 5: Silver release from the surface of the Ti6Al4V treated samples prepared by using different additives: poly vinyl alcohol (PVA), gallic acid (GA) and tannic acid (TA)

The amount of silver ions released in water is reported in Figure 5: It can be seen from the data in Figure 5 that all the samples are able to release silver ions up to 1 week and that the maximum release is reached at 24 h. As it can be seen from Table 4, the release of silver is almost complete, in fact the silver amount on all the surfaces (from XPS survey analyses after 28 days of soaking in water) is reduced to values comprised between 0.1 and 0.4%at. The amount of released silver is comparable with the one obtained in a previous work of the authors without the addition of additives [11]: it is higher than the minimum bactericidal concentration [11] and it is lower than the cytotoxicity limit reported for silver ions [46, 47]. It can thus be

hypothesized that the treated surfaces are antibacterial and biocompatible: experimental evidence of antibacterial behavior is reported in the last paragraph of this paper, while cytocompatibility will be tested in a future work. Unfortunately, the ion release is limited to 1 week and a certain data dispersion, already observed by the authors in absence of reducing/stabilizing agents [11], is still present despite of the addition of additives. Ion release limited to few hours/days is typical of silver modified titanium surfaces [48], but it represents one of the major obstacle to the clinical application of these surfaces, especially in the dental field, where long-lasting antibacterial activity is required. Further research should be undertaken to investigate the possibility to optimize the treatment to obtain a longer release and reduce data dispersion.

Table 4: Chemical compositions (at%) from XPS analyses of the treated Ti6Al4V samples after 28 days of soaking in water

	CT - +Ag	CT - +GA+Ag	CT - +GA+PVA+Ag	CT - +TA+Ag
C	26.0	25.3	28.1	24.4
O	52.8	53.3	49.3	50.7
Ti	20.1	21.3	17.3	18.7
N	1.0		2.3	1.2
Ca	0.6		0.7	
Ag	0.1	0.1	0.5	0.4
Other			1.9	4.6

3.5 *In vitro* bioactivity

In order to investigate the ability of the treated surfaces to induce hydroxyapatite precipitation, some samples were soaked in SBF up to 2 weeks and then observed by means of FESEM-EDS and analyzed by XPS.

The measured pH values during the soaking period fluctuate between 7.2 and 7.6 in the case of all the samples, thus pH does not deviate from the physiological tolerability range.

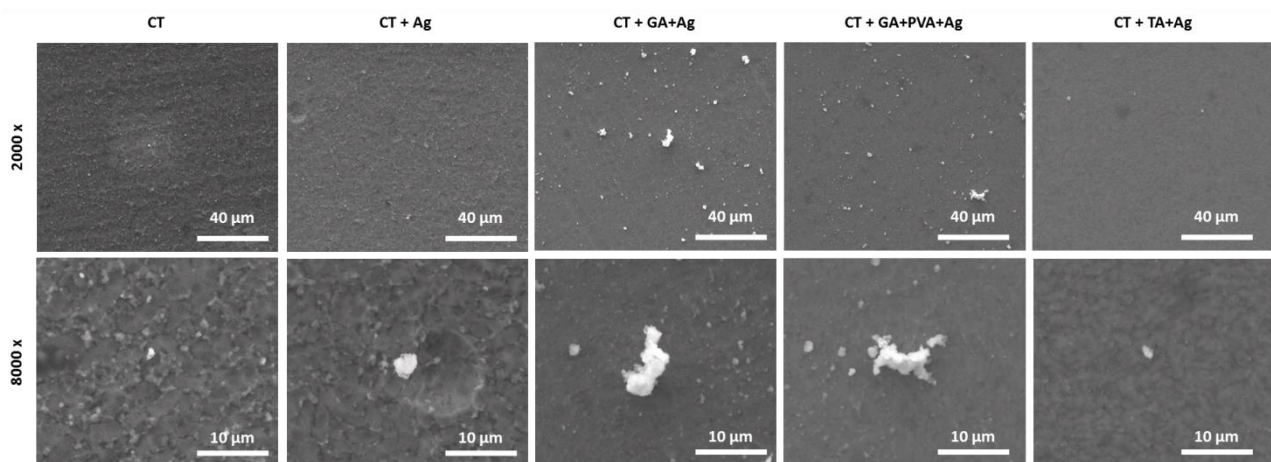


Figure 6: FESEM images of the Ti6Al4V treated surfaces by using different additives ([poly vinyl alcohol (PVA), gallic acid (GA) and tannic acid (TA)] after 15 days of soaking in SBF

From the FESEM images reported in Figure 6, it is quite evident that some particles precipitate during the soaking in SBF: they have the typical morphology of hydroxyapatite. EDS analyses (not reported) confirm that these precipitates are made of calcium (Ca) and phosphorous (P), as well as a certain surface enrichment in these elements also on the surface outside the precipitates (Ca 0.39 – 0.66 %at, P 0.04 – 0.13 %at). The adsorption of Ca and P is confirmed by the XPS analyses on the samples after SBF soaking (Table 5).

A higher amount, as well as higher dimensions, can be noticed for the Ca-P precipitates on CT - +GA+Ag and CT+GA+PVA+Ag samples (Figure 6), however nor EDS (not reported) nor XPS (Table 5) were able to point out differences in the surface Ca and P content between the samples treated by using different additives.

The XPS analyses performed on the samples after SBF soaking (Table 5), in accordance with the XPS analyses performed on the samples after release test (water soaking, Table 4), confirm that all the samples released almost the whole loaded silver: in fact the silver amount is negligible on all the surfaces after 15 days of soaking in SBF.

Table 5: Chemical compositions (at%) from the XPS analyses on the treated Ti6Al4V samples after 15 days of soaking in SBF

	CT	CT - +Ag	CT - +GA+Ag	CT +GA+PVA+Ag	CT - +TA+Ag
C	47.9	23.9	28.3	30.7	33.1
O	40.2	55.9	51.5	51.2	49.5
Ti	6.1	13.5	10.3	11.4	10.7
Ca	2.3	3.1	2.3	2.0	1.8
P	2.1	2.2	1.7	0.7	1.2
Al	1.1	<0.1	0.4	0.4	0.0
N	0.3	1.3	3.0	3.5	3.5
Ag	0.0	0.1	0.0	0.1	0.1
Si	0.0	0.0	2.5	0.0	0.0
Ca/P	1.1	1.4	1.4	2.9	1.5
(Ca+P)/Ti	0.7	0.4	0.4	0.2	0.3

3.6 Zeta potential measurements

Zeta potential measurements were performed on the Ti6Al4V CT – GA+PVA+Ag samples because they resulted the most promising samples, according to the required features defined at the beginning of the results section (Table 2). As reference materials, the Ti6Al4V CT and pure bulk metallic silver were also tested. As far as Ti6Al4V CT and Ti6Al4V CT – GA+PVA+Ag samples are concerned, the measurements were repeated, only in the basic range, after SBF soaking, to better evidence the surface modifications occurring during the bioactivity test (Figure 7).

What stands out in Figure 7a is the difference between the zeta potential curves of Ti6Al4V and Ti6Al4V CT samples, already evidenced by the authors [49]. Ti6Al4V shows the isoelectric point at pH= 4.7, in accordance with the data reported in literature for untreated Ti surfaces [50, 51, 52]; the chemical treatment induces a shift of the isoelectric point to more acidic values (close to pH=2) and the appearance of a plateau in the basic region, attributable to the presence on the surface of homogeneous functional

groups with acidic behavior [53]. These observations can be correlated to the surface hydroxylation upon the chemical treatment already evidenced, in the present paper, by means of FTIR and XPS analyses. On the other hand, no significant differences in the surface charge/isoelectric point can be evidenced by the addition of silver and additives (GA and PVA). Also this result is in accordance with the XPS and FTIR data; in fact the signal of hydroxyl groups is almost unchanged on the Ti6Al4V CT – GA+PVA+Ag samples compared to the Ti6Al4V CT ones. Finally, it can be observed that silver shows the isoelectric point at pH no far from 2, as the Ti6Al4V CT samples, thus its presence is not expected to alter the surface isoelectric point.

Two changes in the slope of the zeta potential titration curve can be observed in the range between pH 3 and 4.5 in the case of the Ti6Al4V CT sample, while the shape of curve in the same pH range is slightly different in the case of the Ti6Al4V CT GA+PVA+Ag sample. A cascade of slope changes and small plateau in the titration curve derives from the presence of OH groups with different acidic behavior (much more acidic the lower the pH value is) [54]. The exact meaning of the small differences between the titration curves of the Ti6Al4V CT and Ti6Al4V CT GA+PVA+Ag samples must be investigated much more in details, but a slight difference in the acidic reactivity of the OH groups can be hypothesized.

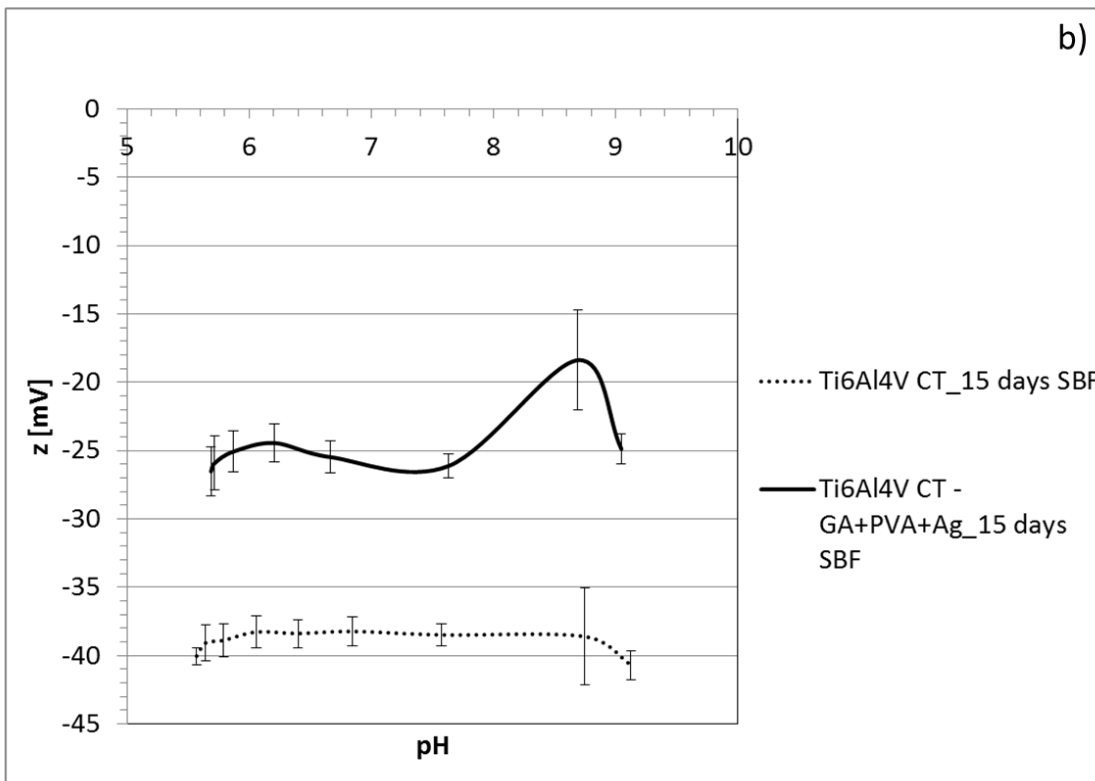
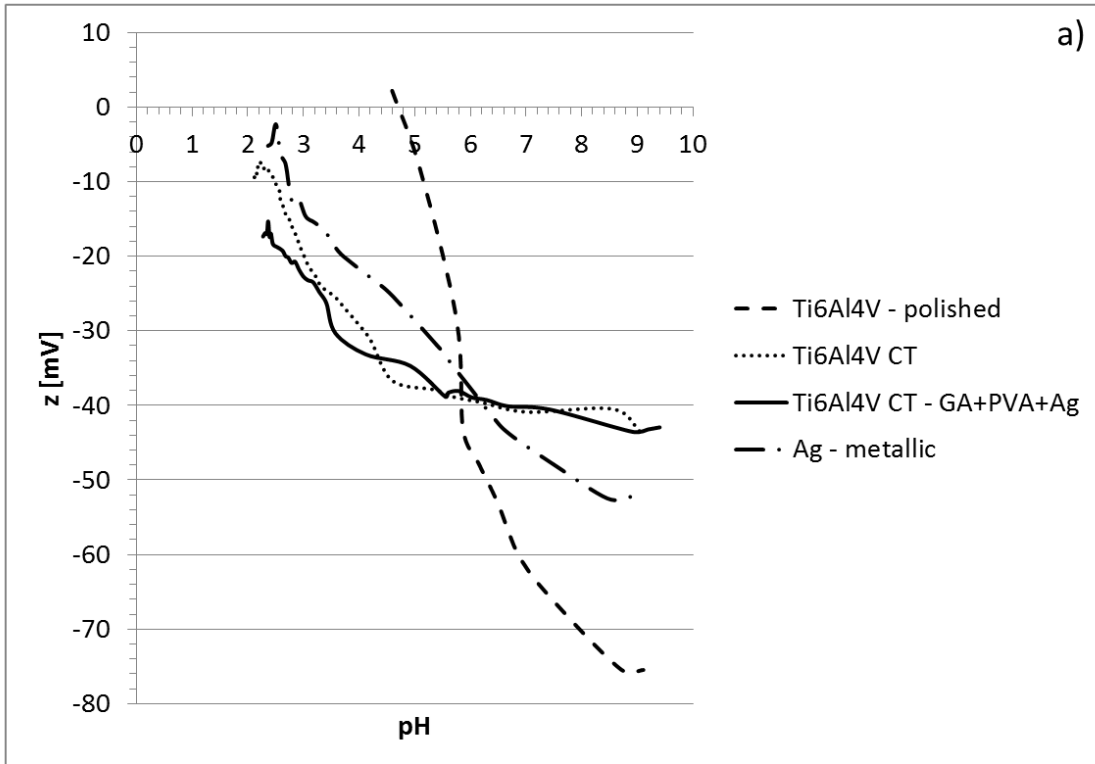


Figure 7: Zeta potential measurements: a) as prepared samples (Ti6Al4V CT , Ti6Al4V CT GA+PVA+Ag samples and reference untreated materials), b) samples after SBF soaking (15 days)

A shift of zeta potential towards less negative values, can be evidenced in the case of Ti6Al4V CT – GA+PVA+Ag after 15 days of soaking in SBF compared to the Ti6Al4V CT sample tested after the same

soaking time (Figure 7b); the curves were almost overlapped before SBF soaking and the surface charge of Ti6Al4V – CT does not change so much after soaking in SBF. A possible explanation for this might be that the Ti6Al4V CT – GA+PVA+Ag samples are able to adsorb a higher amount of Ca from SBF during soaking in SBF. This hypothesis can support the previous observation (Section 3.5 of the present paper) that the Ti6Al4V CT – GA+PVA+Ag and Ti6Al4V CT – GA+Ag samples seem to induce the precipitation of a higher amount of Ca-P precipitates.

The different kinetic of hydroxyapatite precipitation can be hypothesized to be related to the different acidic reactivity of the OH groups exposed on the surface. A possible explanation could also deal with the ability of GA to interact with Ca^{2+} ions favoring surface adsorption [55, 56, 57]. Although no significant evidence of GA permanence on the surface was demonstrated, a certain effect cannot be completely excluded.

3.7 Tape adhesion test

The tape test was applied to the Ti6Al4V CT and Ti6Al4V CT – GA+PVA+Ag samples (Figure 8):

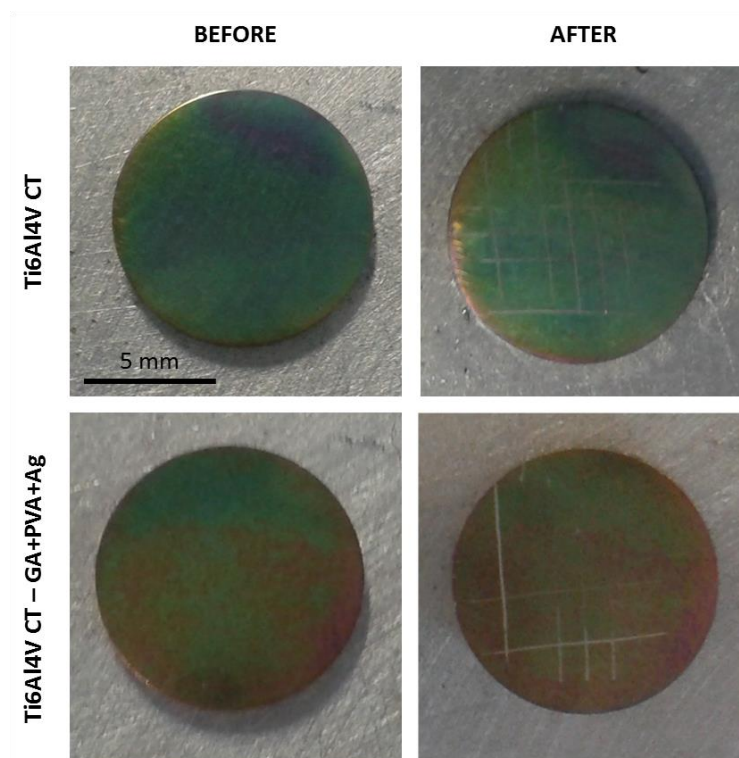


Figure 8: Tape adhesion test on the Ti6Al4V CT and Ti6Al4V CT – GA+PVA+Ag samples

it is evident that no damages to the surface oxide layer occur (both inside and outside the grid) after the application and removal of the tape. In addition, no traces of the oxide have been detected on the tape at the end of the test. These results lead to the classification of both surfaces as 5B (highest degree of adhesion) according to ASTM D 3359. These findings are in accordance with the good adhesion results previously obtained by the authors on the Ti6Al4V CT samples by means of scratch tests [12] and implant simulation tests [15]. Moreover, the obtained results suggest that the introduction of silver and additives in the chemical treatment do not alter the adhesion of the nanotextured oxide layer to the substrate. This finding has important implications for developing bioactive and antibacterial surfaces suitable for clinical applications.

3.8 Antibacterial tests

Antibacterial tests were performed on the Ti6Al4V CT - Ag, Ti6Al4V CT- TA+Ag, Ti6Al4V CT GA+Ag and Ti6Al4V CT – GA+PVA+Ag samples, considering the Ti6Al4V CT and Ti6Al4V polished untreated samples as control materials (Figure 9). All the treated samples containing silver on the surface are able to produce a significant inhibition halo (Figure 9a, 3-3.5 mm).

Focusing the attention on the results of the adhesion test, it can be surprisingly observed that a moderate, no significant reduction in the staphylococcal adhesion (33%) can be reached on the chemically treated surface without introduction of silver ions in the process (Figure 9b - Ti6Al4V CT). This finding was unexpected and suggests that the nanotextured surface produced by the chemical treatment (controlled surface oxidation in hydrogen peroxide) not only shows high wettability, protein absorption ability and bioactive behavior (ability to induce hydroxyapatite precipitation) [12, 13,14, 15], but it does not increase, even slightly reduces, bacterial adhesion. These features are extremely promising for the clinical application of the proposed treatment. A first explanation of this result is that the roughness of the chemically treated surface is on the nanoscale that means Ra value lower than 0.2 μm (0.06 μm , measured by contact profilometry), namely below the threshold for a roughness-dependent increase of bacterial adhesion [58, 59, 60]. The mechanism of bacteria adhesion on this surface will be carefully investigated to clarify the origin of the registered slight decrease of bacteria contamination. The addition of silver induces an active, significant ($p < 0.05$) anti-staphylococcal activity of the surfaces with a maximum reduction of 97% of the bacterial adhesion for Ti6Al4V CT – Ag (Figure 9b); the use of additives does not involve a statistically significant difference in the CFU count. A moderate, significant ($p < 0.05$) reduction of CFUs can be reached in the whole incubation broth (Figure 9c, up to 60% for Ti6Al4V CT – Ag) and it is not dependent from the type of the employed additive. Further experiments to explain the difference in the various actions of silver on the adherent and non- adherent bacteria will be necessary.

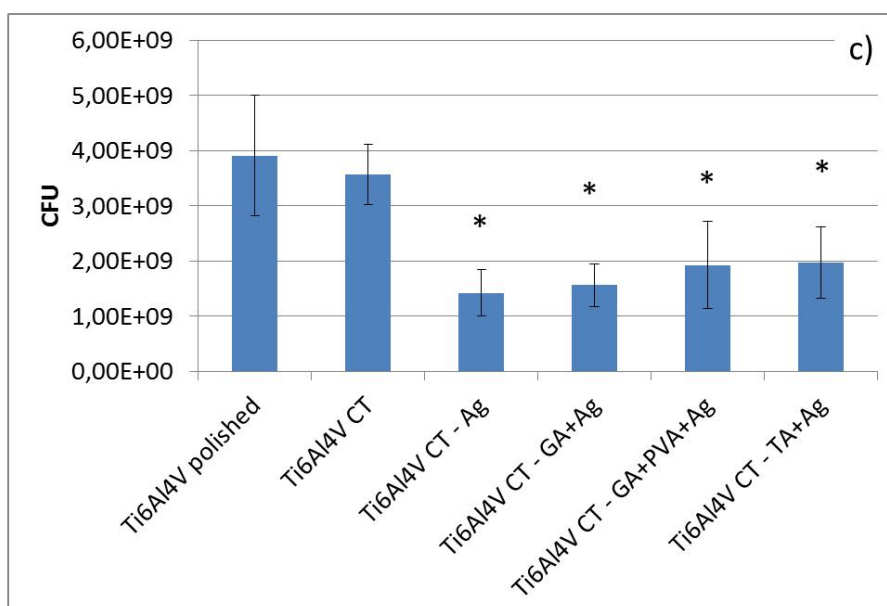
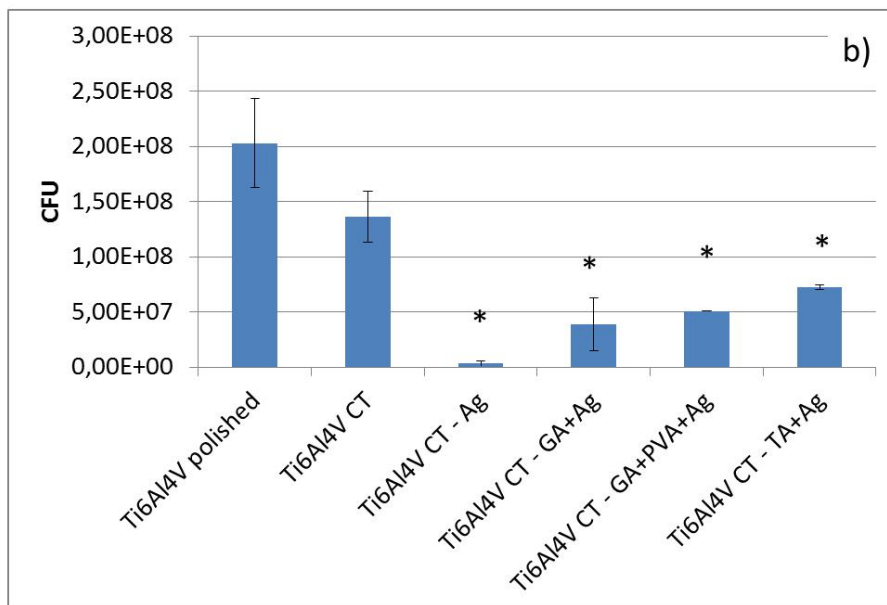
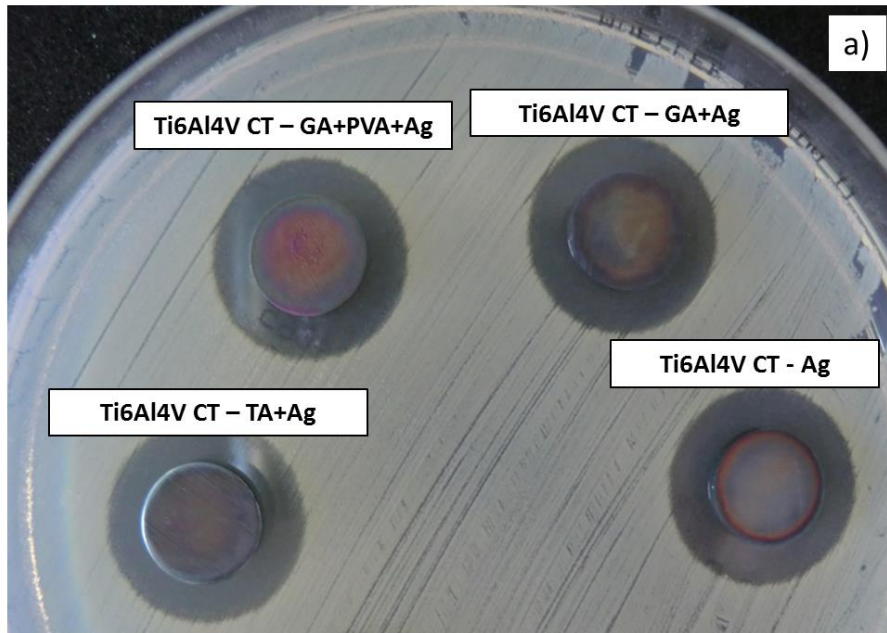


Figure 9: Antibacterial tests on the Ti6Al4V samples treated by using different additives: a) inhibition halo, b) adhered bacteria and c) non-adhered bacteria . * $p < 0.05$ significantly different from the control Ti6Al4V CT.

Conclusions

In conclusion, the aim of the research was reached, considering that surfaces with the required features were obtained:

- nanotextured oxide layer with functionalities suitable for osseointegration: the morphology (nanotexture) of the surface oxide layer is independent from the use of the additives and it can be obtained by using all the tested chemical mixtures, a part from TA causing some corrosion cracks. The samples treated by adding GA and PVA showed good bioactivity, negative surface charge and exposition of a single functional surface group as already evidenced on the surface treated without additives;
- high density of hydroxyl groups: all the treated Ti6Al4V surfaces show a high density of hydroxyl groups, while the chemical process is less effective on the Ti-cp substrate.
- silver nanoparticles embedded in the surface oxide layer and silver ion release able to induce antibacterial behavior: the highest amount and most homogenous distribution of nanoparticles has been obtained on the Ti6Al4V CT – GA+PVA+Ag samples. The use of GLU as additive induces the presence of a less amount of silver on the surface compared to the other additives. In the case of the surfaces treated by adding GA and PVA, the completely release silver ions up to 1 week, with a maximum release at 24 h and the amount of released silver is comparable with the data obtained without the addition of additives: it is higher than the minimum bactericidal concentration and it is lower than the cytotoxicity limit. A detailed investigation of the mechanism of the antibacterial behaviour will be performed in a future work;
- absence of silver micro-aggregates: none of the considered additives was able to avoid the precipitation of silver micro-aggregates on Ti-cp, while micro-precipitates are visible only on the Ti6Al4V samples treated with the addition of ST and GLU. Conversely, TA, GA and PVA (used alone or in combination) are effective to avoid precipitation of micro sized silver aggregates;
- absence of contamination of the surface by the additives at the end of the process: some evident additive residues on the surface have been observed on the surfaces treated with ST and GLU.
- unchanged adhesion of the surface oxide layer: the surfaces treated by GA and PVA showed the highest degree of adhesion by tape test.

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