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Surface functionalization of Ti6Al4V with an extract of polyphenols from red grape pomace / Riccucci, Giacomo; Cazzola, Martina; Ferraris, Sara; Alessandra Gobbo, Virginia; Guaita, Massimo; Spriano, Silvia. - In: MATERIALS & DESIGN. - ISSN 1873-4197. - ELETTRONICO. - 206:(2021). [10.1016/j.matdes.2021.109776]

Availability:
This version is available at: 11583/2910712 since: 2021-07-02T11:22:31Z

Publisher:
Elsevier

Published
DOI:10.1016/j.matdes.2021.109776

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http://dx.doi.org/10.1016/j.matdes.2021.109776

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03 May 2024

Materials & Design 206 (2021) 109776

Contents lists available at ScienceDirect

Materials & Design

journal homepage: www.elsevier.com/locate/matdes

Surface functionalization of Ti6Al4V with an extract of polyphenols from red grape pomace

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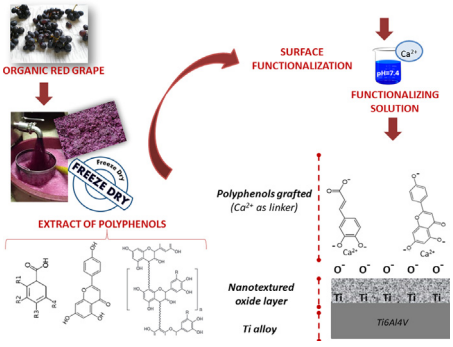
HIGHLIGHTS

- Chemically treated Ti6Al4V can be surface functionalized with polyphenols from red grape pomace.
- The grafting link is between deprotonated OH surface groups and Ca2+ ions forming a complex with the polyphenols.
- The grafted polyphenols have redox chemical and radical scavenging ability on the metallic surface.
- The polyphenols are released in aqueous media.
- The functionalized surfaces can be sterilized by gamma irradiation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 February 2021
Revised 9 April 2021
Accepted 26 April 2021
Available online 30 April 2021

Keywords:
Titanium alloys
Surface treatment
Functionalization
Polyphenols
Natural molecules
Grafting

ABSTRACT

The focus of the paper is on effectiveness of a functionalization process with natural polyphenols on a chemically treated and bioactive Ti6Al4V alloy, on the grafting mechanism, and the redox/scavenging activity of the grafted biomolecules.

Polyphenols (phenolic acids, flavonoids, and condensed tannins) are extracted from organic red grape pomace. The functionalization process is performed at pH = 7.4 with the addition of calcium ions, which act as a bridge between the substrate and polyphenols. The presence, amount (semi-quantitative), distribution, release, and type of bonding to the surface of the grafted polyphenols have been assessed. The functionalized samples have a homogeneous distribution of polyphenols as a continuous layer and micro-sized agglomerates. The grafted polyphenols maintain redox chemical and radical scavenging ability. A fraction of polyphenols is released into water in one day, while a firmly grafted layer remains on the surface even after four weeks. A complete release can occur in case of an environment with pH of 4–5 (e.g. inflammation). The functionalized surfaces can be sterilized by gamma irradiation without significant damage of the grafted polyphenols.

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1. Introduction

Polyphenols constitute an interesting class of biomolecules, which are known from Ancient Greece, but are methodically studied only in the last years. Extracted from numerous types of plants, they have a series of interesting properties and synergic actions, which could be exploited for different applications: antioxidant,

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https://doi.org/10.1016/j.matdes.2021.109776
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antibacterial and anti-inflammatory actions are only the main ones.

This research is focused on polyphenols, extracted from red grape (Barbera), belonging to different classes: hydroxycinnamic acids (belonging to phenolic acids class), isoflavones, flavanols (such as condensed tannins) and anthocyanins (appertaining to flavonoid class). Hydroxycinnamic acids are a subclass of phenolic acids, whose structure, called C_6—C_3, is composed by a hydroxyl-substituted benzene ring at which a chain constituted by three carbon atoms is bound. The main sources of hydroxycinnamic acids are the skin and the vacuoles of red grape. The overall content increases proportionally to the dimension of the fruit itself, but it has been observed that the concentration decreases in the grape during maturation [1]. Flavonoids – the main class of polyphenols – have in common a basic structure, formed by a diphenilpropane skeleton, a structure also known as C_6—C_3—C_6. It is constituted by two aromatic rings (A, B) and a third ring (C), which is the cyclization of a three carbon atoms chain: A and C rings are bonded together, while B and C rings are connected by a three carbon atoms chain [2]. While C ring is a pyran ring – that is a heterocyclic ring in which there is an oxygen atom – B ring often exposes different substituent OH groups: the position and the number of these groups determine the different polyphenolic subclasses [3]. Isoflavones are exploited for clinical application because of their chemopreventive effects and their weak behavior as estrogen agents. Flavanols are also extracted from the skin of red and white grapes and from their seeds. They constitute an important subclass of flavonoids because of their ability to reduce arteries pressure and their anti-inflammatory properties, exploitable for cardiovascular applications [4,5]. Flavanols often aggregate in oligomers and polymers and, in this case, they are called pro-antocyanidins (condensed tannins). Pro-antocyanidins can be found in nature in two different configurations: catechins, characterized by the trans- configuration, and epicatechins, characterized by the cis- one. Catechins are not particularly bioavailable when assumed through food, while epicatechins are easier to be adsorbed in the intestinal tract [6,7]. Then, anthocyanins constitute the glycosylated form of anthocyanidins, a class of flavonoids which are fundamental in fruit pigmentation and whose color is pH dependent [8]. Concerning clinical applications, this study evaluates the possibility to include these biomolecules in medical devices through surface functionalization. Bypassing the intestinal tract, polyphenols could be significantly more bioavailable and chemically stable, locally exercising their properties. One of the possible clinical applications could be in the orthopedic and dental fields, functionalizing biomaterials for bone contact [9].

By grafting polyphenols on a bioactive surface, it is possible to combine the mechanical and chemical properties of the substrate and the beneficial action of the biomolecules, obtaining a biocompatible and bioactive material, also having important antioxidant and radical scavenging properties (conferred by polyphenols), through which it can exercise anti-inflammatory and antimicrobial actions. These actions can be exercised by different mechanisms [10]. The grafted polyphenols, in fact, can be released, after the implantation of the functionalized material, in two different ways: a bulk release, which consists in a fast and abrupt release that has place along few hours, and a slow release, which is characterized by slower and spread in time diffusive phenomena, whose effects can be observed till different days or weeks. Both these release mechanisms permit to the polyphenols to exercise their properties not only on the first cell layers around the implant, but also in farther tissues, thanks to diffusion. The grafted polyphenols can be also firmly bound to the surface, so that a direct contact with the surrounding bone cells is possible: the strongly grafted biomolecules can locally exercise their properties for a longer time, ideally during the entire life of the implant. Release phenomena and strong grafting can coexist, and the grafted biomolecules can beneficially act both on the local environment, continuously and for a long time, and on the farther surrounding tissues, for the shorter time of the inflammation process [11].

Different materials are currently studied at this purpose. This research is focused on Ti6Al4V alloy, that is the main metal used for bone implants because of the relatively low Young modulus, high fatigue resistance and proved biocompatibility. The untreated Ti6Al4V surface is not able to be osteoinductive neither to promote hydroxyapatite precipitation from the physiological fluids. To optimize the biological response of this already effective biomaterial, a chemical treatment has been used [12,13] to obtain a nanoporous oxide layer, characterized by multi-scale roughness, close to morphology of the extra-cellular matrix of bone tissue, and bioactive behavior, that is ability to induce hydroxyapatite precipitation when put in contact with physiological fluids. Finally, thanks to the chemical treatment, a dense exposition of hydroxyl groups on the surface of the biomaterial is induced. So, the elevate surface-to-volume ratio and the presence of OH groups allow a more efficient functionalization with polyphenols than the one obtainable on the native oxide. The results reported in this paper are part of a larger research work where bioactivity and biological response of the functionalized surface will be tested.

2. Materials and methods

2.1. Samples preparation and chemical treatment

A standard titanium-aluminum-vanadium alloy (Ti6Al4V, ASTM B348, Gr5, Titanium Consulting and Trading) was used as substrate. The samples are disks 10 mm in diameter and 2 mm thick, which were polished to get a smooth surface (SiC papers from 320 to 4000 grit). The samples were washed firstly in acetone for 5 min and then twice in ultra-pure water for 10 min. Each step was done in an ultrasonic bath. After drying under a hood, the chemical treatment was carried out, following a patented protocol previously implemented [11,12,14]. The treatment involves a first removal of native oxide through hydrofluoric acid etching, followed by a controlled oxidation with hydrogen peroxide, forming a nanoporous oxide of titanium rich in hydroxyl groups. Before the storage, the samples were dried under a hood.

2.2. Surface activation

The activation of the surface was carried out to improve the reactivity of surface through the exposition of the hydroxyl groups, while eventual contaminations from the atmosphere are removed. The activation process involves an exposition of the samples surface to UV irradiation for 1 h (UV-C 40 W; 253.7 nm). [15]. The samples of titanium alloy after the chemical treatment and irradiation take the name of CT (Chemically Treated titanium alloy) in the present article (unless otherwise noted).

2.3. Polyphenols extraction

The polyphenols were extracted from grape pomaces of Barbera type. The protocol explained in a previous study was followed and here it is briefly summarized [16]. The pomace of organic Barbera grapes was sampled at Tre Secoli winery (Mombaruzzo, AT, Italy) at racking off, after soft pressing (0.5 bar). The grape residues were so separated: pulp and stalk were excluded, while seeds and peel were dried in ventilated oven (48 h, 35 °C) and successively milled (coffee grinder, 60 s). A pomace flour was obtained at the end of this process: this powder is characterized by an elevate surface-to-volume ratio, so that polyphenols extraction is optimized. The

polyphenols extraction, according to a previous work [17], was implemented through ground pressing, exploiting the conventional solution (ethanol in water solution 50% v/v). The extraction ratio was imposed at 1:6 w/v flour/solvent (100 g flour in 600 ml solvent). The solution was shaken for 2 h through a magnetic stirrer at room temperature. After that, the precipitates were removed through a centrifugation process (20 min, 18 °C) carried at 28880g (Centrifuge 5810 R Eppendorf - Hamburg, Germany) and, finally, ethanol tracks were eliminated through evaporation. The substances obtained by the protocol were concentrated and freeze-dried. The efficacy of the process can be estimated considering that an amount of 23.9 g of pomace flour is needed to obtain 1 g of freeze-dried extract. The freeze-dried polyphenols (P) were stored in a dark environment to preserve polyphenols from degradation. As exposed in a previous work [16], the so-obtained polyphenols extract was analyzed by HPLC to evaluate its composition. The method consists of the phloroglucinolysis reaction (phloroglucinol + ascorbic acid in methanol/HCl 98:2 at 50 °C) and the HPLC analysis of the reaction products. Monomer flavan-3-ols ((+)-catechin and (−)-epicatechin) were determined excluding the reaction with phloroglucinol. The dry residue of the extracts was re-dissolved in 2% aqueous acetic acid and applied on a C-18 solid phase extraction cartridge (Sep-Pak tC18 1 g, Waters, Milford, MA, USA). Sugars and organic acids were eluted with 2% aqueous acetic acid, the phenolic compounds were recovered with methanol.

2.4. Surface functionalization

The aqueous solution, in which polyphenols were dissolved in order to prepare the functionalizing solution, was prepared by adding Tris(hydroxymethyl)aminomethane (TRIS), HCl and $CaCl_2$ to ultra-pure water. The name of this aqueous solution is TRIS/HCl + $CaCl_2$ for whole paper. TRIS and HCl have a buffer action and allow to obtain pH = 7.4. Calcium was added to the solution in order to exploit its ability to facilitate polyphenols grafting to titanium through the formation of polyphenols-calcium complexes, as previously described by the authors for gallic acid [13,18]. The TRIS/HCl + $CaCl_2$ solution was prepared dissolving in 1 L of ultra-pure water the following reagents: $CaCl_2$ (0.292 g), HCl (39 ml) and TRIS (6.118 g). Finally, the solution was stored in fridge at 5 °C and used within a month. For the preparation of the H_2O + P solution, the protocol - explained in a previous article - was followed, which involves dissolution of polyphenols for 1 h in stirring condition, performed through a magnetic bar [16]. This solution was used as a reference for some characterizations. The functionalizing solution has the previous solution as solvent with a concentration of 5 mg/ml of polyphenols and it will be called TRIS/HCl + $CaCl_2$ + P. It was magnetically stirred for 1 h. The process was done in dark condition to avoid exposure of the solution to the light, as polyphenols are photosensitive and they might be subjected to degradation or damaging. After that, each CT samples was put in a dark container with 5 ml of TRIS/HCl + $CaCl_2$ + P solution. The functionalization process was carried out in an incubator at 37 °C for 3 h. At the end, each sample was washed in ultra-pure water for twice, left to dry under a hood and stored in a multi-well plate in dark condition. The functionalized samples have the name of CT + P.

2.5. Release test

The test consists in soaking the functionalized samples (CT + P) for 28 days in 15 ml of ultra-pure water, which was left in dark condition in an incubator at 37 °C. After 1 day, 7 days, 14 days and 28 days the soaking liquid was changed, refilling the container with a new amount of ultra-pure water. At each experimental time, the pH value of the liquid used for the test was measured and the F&C test (see section 2.8) done, to check the eventual release of polyphenols. After 4 weeks, the F&C test was also done on the solid soaked samples by applying a modified protocol for the solid samples [19,20]. Moreover, the DPPH test, observations through fluorescence microscopy (see section 2.9) and UV–VIS spectroscopy were done on the soaked samples. The samples soaked for 4 weeks will be referred to as CT + P28.

2.6. Sterilization

The sterilization protocol that was followed for the functionalized samples (CT + P) is the standard one for medical devices. Each sample was packed and subjected to a γ radiation treatment at 25 kGy. The procedure was performed by Gammaton (Como, Italy). Then, the samples were kept in the sterilized packaging and shelf stored until the tests. The functionalized specimens undergone to sterilization have the acronym CT + P_S. The effect of sterilization was observed through fluorescence microscopy (see section 2.9) observations and UV–VIS spectroscopy, as well as the F&C test (see section 2.8), and compared with the results of the same tests on the freshly prepared samples.

2.7. UV–VIS spectroscopy

An UV-spectrophotometer (UV2600 Shimadzu) was employed to carry out the UV–VIS tests both on the solid and liquid samples. About the solid samples, an integration part (ISR-2600Plus, two-detector integrating sphere) was installed to capture the spectrum reflectance coming from the surface, which was pre-irradiated with different wavelengths. Through this method CT, CT + P, CT + P28 and CT + P_S were analyzed. Concerning the solutions, the instrument has two different modalities: spectrum or photometric. The spectrum modality was selected to analyze the whole absorbance spectrum (250–700 nm). The second modality was employed for the specific signals related to the colorimetric tests (as described below about the F&C and DPPH tests).

2.8. Folin and Ciocalteu (F&C) test

The test is used to quantify the total polyphenols content as Gallic Acid Equivalents (GAE) [19,20,21]. The followed protocol is explained in previous works [19,22].

The chemical reaction between the solutions to be tested and the reagent occurs in dark condition, because of photosensitivity of the polyphenols. After 2 h, the samples were analyzed with the photometric modality: this test was used to compare the solutions before and after the functionalization process (TRIS/HCl + $CaCl_2$ + P - functionalizing solution - and Uptake CT) and the liquid used for the release test.

For the analysis of the solid samples (CT, CT + P, CT + P28 and CT + P_S), the protocol was modified according to previous works [19]: the solid sample was used instead of test solution and the reaction occurred with the same reagents; to maintain the same volume of the final solution, extra volume of ultra-pure water was added to balance the one of the solution containing polyphenols [19,22]. The results are reported as GAE by referring to a calibration curve of acid gallic solutions of known concentration [19]. Considering that F&C test quantifies polyphenols by means of a redox reaction, this test is also a measurement of their redox activity.

2.9. Fluorescence microscopy

A confocal microscope (LSM 900, ZEISS) was used with a red filter and excitation wavelength (573 nm) to observe the red fluores-

cence emitted by the surface of the solid samples, setting 1 s of exposure time and with a magnification of 200X. Being the polyphenols naturally fluorescent, this observation was also carried out for the study of the distribution of the grafted molecules on the surface [23,24]. The total value of the red signal registered on the images (as arbitrary units – a.u.) has been used for a semi-quantitative evaluation of the grafted polyphenols. The images acquired by the confocal microscope have been analyzed through ImageJ to measure the diameter of the polyphenols agglomerates at least on 15 randomly-chosen agglomerates. The results are reported as [averaged diameter ± standard deviation]. The test was used to observe the surfaces of CT, CT + P, CT + P28 and CT + P_S.

2.10. Zeta potential titration curves

The zeta potential titration curves were used to study both the solid samples and the solution of the polyphenols. For the solid samples (before and after polyphenols grafting), the curves were obtained by an electrokinetic analyzer equipped with an adjustable gap cell (SurPASS, Anton Paar). The streaming potential technique is applied to determine the zeta potential when an electrolyte (0.001 M KCl) is forced to flow between two (equal) samples parallelly positioned at 100 μm of distance. The pH of the electrolyte is automatically titrated through the addition of 0.05 M HCl and 0.05 M NaOH, in the acid and basic range, respectively. The same set of samples underwent before the acid and later the basic range measurement. This technique was applied to CT and CT + P samples.

To proceed with the evaluation of zeta potential of liquid samples (Nanosizer Nano Z, Malvern Instrument, Malvern), utilizing an electrophoretic approach, it was necessary to prepare a KCl 0.001 M solution using, as the solvent, the solution to analyze. The pH was adjusted through HCl 0.05 M or NaOH 0.05 M. In this case, a light scattering system evaluated the electrophoretic velocities of the particles in the electrolyte, calculating the final zeta potential. The measure was carried out on the functionalizing solution: TRIS/HCl + $CaCl_2$ + P.

2.11. DPPH test (2,2-diphenyl-1-picrylhydrazyl)

To evaluate the radical scavenging ability of the functionalized biomaterials, the DPPH test was performed. The DPPH test protocol was developed following previous studies [25,26,27,28]. The DPPH solution was prepared dissolving 9.86 mg of DPPH (2,2-diphenyl-1-picrylhydrazyl, quality level 100%, Sigma-Aldrich) powder in 25 ml of ethanol, subsequently 20 min of sonication was performed in ultrasonic bath in dark condition. The solution was filtered through a 0.2 μm PTFE filter and, then, it was diluted 1:10 in ethanol. Before the test, the absorbance value of the DPPH solution was measured to verify the non-reaching of saturation conditions. Then, each sample was immersed in 3 ml of DPPH solution in a dark container and after 4 h and 24 h the measures are implemented. The intensity of the purple color characterizing the analyzed solution, where the samples were soaked, was compared to the intensity of the color of the control solution to calculate the radical scavenging activity (RSA). The absorbance of the DPPH solution (control) and the liquid samples were measured at 515 nm and the RSA parameter was calculated. The solid samples were soaked in the DPPH solution (following the protocol explained above) in a dark container for 4 and 24 h as described in [25,26,27,28]. The test was carried out on the CT, CT + P, CT + P28 samples.

2.12. X-ray Photoelectron spectroscopy (XPS)

X-ray Photoelectron Spectroscopy (XPS, PHI 5000 VERSAPROBE, PHYSICAL ELECTRONICS) was employed for the determination of the surface chemical composition and the presence of characteristic functional groups. For each sample a 400x400 μm area was analyzed. As illustrated in a precedent work [29], two modes were exploited: both survey spectra (0–1200 eV) and high-resolution ones (C, O, Ti and Ca regions) were acquired. All the spectra were referenced by setting the hydrocarbon C1s peak to 284.80 eV (in order to guarantee the charging effect compensation).

3. Results

The final aim of this research is functionalization of a chemically treated Ti6Al4V alloy with an extract of red grape pomace to couple the biological properties of polyphenols (antioxidant, antibacterial and anti-inflammatory) to a bioactive surface for a local action on the surrounding tissues. The substrate (a Chemically Treated Ti6Al4V alloy - CT) has been functionalized through a protocol involving a functionalizing aqueous solution buffered at pH = 7.4 with the successive addiction of Ca^{2+} ions. Both the substrate and the functionalized material have been characterized, as well as the functionalizing solution. Different techniques have been employed for the characterization: pH measurement, UV–VIS spectroscopy, fluorescence microscopy, zeta potential titration curves, DPPH and Folin and Ciocalteu (F&C) tests, XPS analysis.

To better characterize the polyphenol-biomaterial system, a test of release in water has been implemented, to study the kinetics of release of the polyphenols and the type of bond between the grafted molecules and the substrate.

The samples have also been sterilized by gamma irradiation and then newly characterized to evaluate if the functionalized material is feasible for industrial production and clinical applications.

3.1. HPLC characterization of the polyphenols extract and UV–VIS spectroscopy and F&C test of the functionalizing solution

The lyophilized polyphenols extracted from red grape pomace (P) have been analyzed. The exhaustive characterization of P is reported in previous works [16,17,30]. The total polyphenols content (as Gallic Acid Equivalents – GAE), the total anthocyanins content, the total flavonoids content and the flavans reacting with vanillin content (condensed tannins) are expressed in Table 1 as mg/g, referred to the freeze-dried extract (P). Then, HPLC has been exploited to evaluate the percentage of (-)-epigallocatechin (EGC), (+)-catechin (C), (-)-epicatechin (EC) and (-)-epicatechin-3-O-gallate (ECG) as principal monomeric units, divided into extention units and terminal units. Finally, the mean degree of polymerization (mDP) and the percentage of galloylation (G%) have also been measured [16,17,30].

The functionalizing solution, TRIS/HCl + $CaCl_2$ + P, has been analyzed through UV–VIS spectroscopy. As a reference, a solution of polyphenols in water without the addition of the buffer or $CaCl_2$ (H_2O + P) was also prepared and analyzed. The pH values of the TRIS/HCl + $CaCl_2$ + P and H_2O + P solutions are respectively 7.4, as expected because of the presence of the buffer, and 3.6, as expected because of the acidic nature of some polyphenols.

Because of the imposed buffering action and the presence of metal ions, it is possible to appreciate variations in the color of the solutions, going from purple in the case of the H_2O + P solution (almost the same color of the red grape pomace) to a darker color, almost black, with a blue nuance, in the case of the TRIS/HCl + $CaCl_2$ + P solution [18].

Table 1
Polyphenolic composition (average values ± standard deviation, n = 3) of the grape pomace extract (P), referred to the dry weight (DW) of P.

Total Polyphenols content – GAE (mg/g)	182 ± 16
Total anthocyanins (mg/g)	35 ± 3
Total flavonoids (mg/g)	200 ± 13
Flavans reacting with vanillin (mg/g)	61 ± 2
Total condensed tannins (mg/g)	95.0 ± 4.5
Flavan-3-ols (mg/g)	
C	1.72 ± 0.07
EC	1.97 ± 0.07
ECG	0.12 ± 0.03
Principal monomeric units (%)	
C	28.8 ± 0.6
EC	71.2 ± 0.6
Extention units (%)	
C	18.8 ± 0.5
EC	39.3 ± 0.7
ECG	14.9 ± 0.4
EGC	3.3 ± 0.2
Terminal units (%)	
C	10.0 ± 0.6
EC	10.2 ± 0.2
ECG	3.5 ± 0.1
mDP	4.20 ± 0.07
G%	18.4 ± 0.5

As illustrated in Fig. 1, the UV–VIS spectra obtained on both the solutions have in common two absorbance peaks in the band of the lowest analyzed wavelengths, while they have different behavior at the higher ones. Precisely, at 250 nm and at around 270–280 nm, two peaks are appreciable. For higher wavelengths, instead, the spectra of the solutions become different: H_2O + P solution has an absorbance peak in correspondence of 530 nm, while TRIS/HCl + $CaCl_2$ + P solution is characterized by two peaks, respectively at 450 and 600 nm.

The functionalizing solution (TRIS/HCl + $CaCl_2$ + P) has also been analyzed through the F&C test, for quantifying the total amount of the dissolved polyphenols and for a semi-quantitative evaluation of the redox activity of the solution itself: so, the value of GAE has been measured. As a reference, a solution of polyphenols without the addition of the buffer (TRIS + HCl) or $CaCl_2$ was also prepared and analyzed (H_2O + P). An eventual variation of the GAE value of the solution after the functionalizing process has also been considered, analyzing in the same way the CT uptake solution and comparing the results. The action of the buffer does not alter the redox activity (GAE) of the solution: the average GAE value of the H_2O + P solution is not statistically different from that of TRIS/HCl + $CaCl_2$ + P solution ($p > 0.05$, one-way ANOVA). As appreciable in Fig. 1, even the uptake solution has not any significant variation, maintaining a GAE value comparable to the one of the functionalizing solution (TRIS/HCl + $CaCl_2$ + P).

3.2. Fluorescence microscopy on the substrate and functionalized surfaces

In Fig. 2, the fluorescence microscopy observations on the CT samples before and after functionalization are reported; moreover, the total value of the red signal registered on the images (as arbitrary units – a.u) can be used for a semi-quantitative evaluation of the grafted polyphenols. Fig. 2 illustrates the surface of the substrate, on which the fluorescent signal is absent (total red signal equal to 4 a.u.). In Fig. 2, instead, the surface of the functionalized samples CT + P is reported. The uniform intensity, even if low, of the fluorescent signal emitted by the sample shows that the grafted polyphenols are characterized by a uniform distribution on the surface of the material forming a thin, but homogeneous, layer on the sample. Numerous agglomerates are distributed on all the surface. The diameter of these agglomerates is of some microns (diameter = 12.2 ± 5.1 μm); furthermore, they are homogeneously distributed on the surface of the functionalized material. The total red signal is equal to about 2000 a.u. Analyzing CT + P28, that is the functionalized sample after the release test in water, it is possible to appreciate a significant attenuation of the fluorescent signal (Fig. 2 - total red signal equal to about 600 a.u.): it means that the amount of the polyphenols grafted on the surface, after 28 days of soaking in water, is lower than the one of the as-prepared functionalized sample, but higher than the one of the bare sample. A very thin film, detectable by a signal with low intensity (600 a.uy. vs 4 a.u), but homogeneously distributed, is present on the analyzed sample, while the small agglomerates are not visible anymore after 28 days of soaking in water.

An image of the sterilized sample CT + P_S is reported in Fig. 2. The intensity of the fluorescent signal (about 1500 a.u.) and its uniformity on the surface, that is comparable to the one characterizing the as-prepared functionalized CT, reveals that the uniform layer of grafted polyphenols is not altered by gamma irradiation. A significant variation is instead appreciable concerning the dimension of the agglomerates: the dimension of the agglomerates averagely increases, and the statistical distribution of their diameter is less homogeneous (diameter = 21.1 ± 8.5 μm).

3.3. Zeta potential titration curves

Two types of zeta potential titration curves have been measured with different equipment: a first one on the polyphenols in aqueous solutions, both with and without the addition of the buffer (TRIS/HCl) and $CaCl_2$, and a second one on the solid samples, before and after functionalization. Even if they are obtained through different equipment, the parameters which influence the measure are maintained identical, thus the results of the two types of measurements are comparable. In the case of the solid samples, two sets of samples are respectively used for the acid and alkaline ranges of the titration curve as described in previous works [31,32]. In Fig. 3, it is possible to see the zeta potential titration curves of TRIS/HCl + $CaCl_2$ + P solution, CT and CT + P samples in function of pH. As a reference, a solution of polyphenols without the addition of the buffer and $CaCl_2$ (H_2O + P) is also reported. The isoelectric point, calculated as the intersection of the curve with the abscissa axis or by interpolation, corresponds to pH = 2.00 for the reference solution (H_2O + P), pH = 2.21 for the functionalizing solution (TRIS/HCl + $CaCl_2$ + P), pH = 2.80 for the CT sample, pH = 3.10 for the functionalized material (CT + P). Concerning the zeta potential titration curve of TRIS/HCl + $CaCl_2$ + P solution, the first significant aspect to highlight is the fact that polyphenols, when dissolved in an aqueous environment, are characterized by a negative zeta potential for the entire evaluated pH range. It is possible to see how the absolute value of the charge increases when pH increases. The trend of the curve is non-linear for all the explored pH range. In fact, two variations of the slope of the curve can be observed: the first one has place around pH = 4 and the second one is around pH = 7 and, in this case, a plateau is reached ($\zeta \sim -15$ mV). For the reference solution (H_2O + P), instead, the plateau is not reached, but the final slope is maintained till the last evaluated pH value (pH = 9).

Concerning instead the solid CT samples, it is possible to appreciate the chemical stability of the material in the whole range of explored pH, which is deduced by the low standard deviation of each point of the curve. The curve has a decreasing trend along the acid pH range and, around pH = 5, it reaches a plateau ($\zeta \sim -35$ mV). The functionalized CT sample is characterized by the same behavior at very low pHs (2–4) and by an analogue trend in correspondence of the alkaline ones; however, a significant difference is appreciable in the mildly acidic range: around pH ~ 5, the curve of

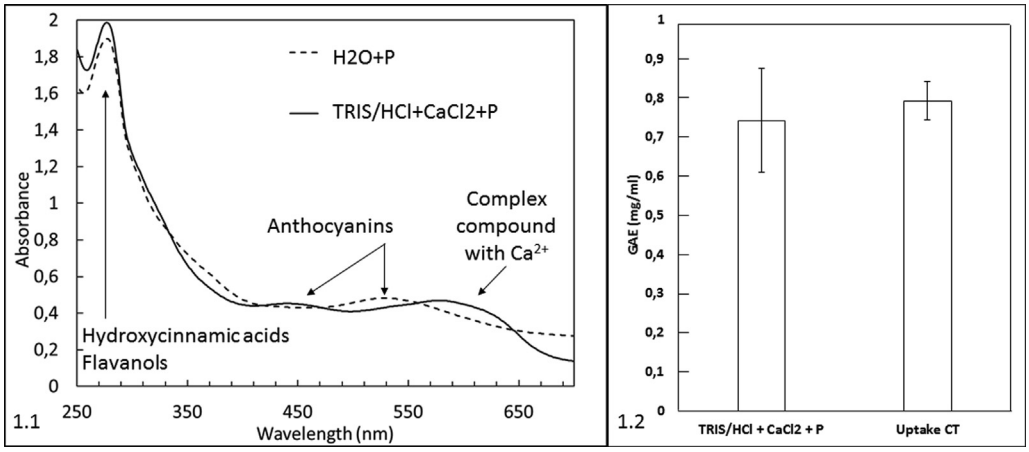

Fig. 1. UV–VIS spectra and F&C test. (Panel 1.1) UV–VIS spectrum of the functionalizing (TRIS/HCl + $CaCl_2$ + P – 5 mg/ml, pH = 7.4) and reference (H_2O + P – 5 mg/ml, pH = 3.6) solutions; (Panel 1.2) F&C test on the functionalizing (TRIS/HCl + $CaCl_2$ + P) and uptake (Uptake CT) solutions.

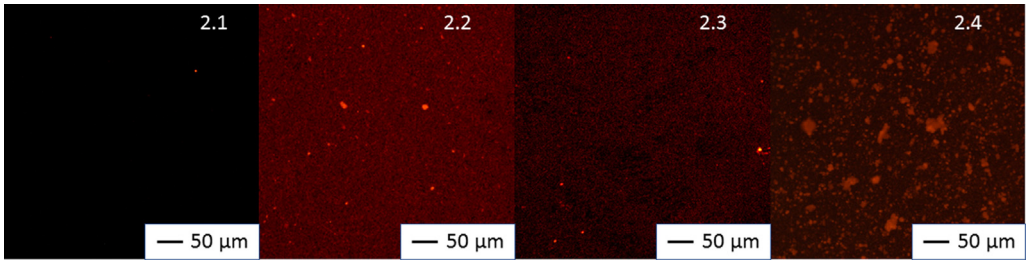

Fig. 2. Fluorescence microscopy observations on the surfaces of the (2.1) CT sample, (2.2) functionalized CT sample (CT + P), (2.3) functionalized CT sample after the release test in water (CT + P28), (2.4) functionalized CT sample after sterilization (CT + P_S).

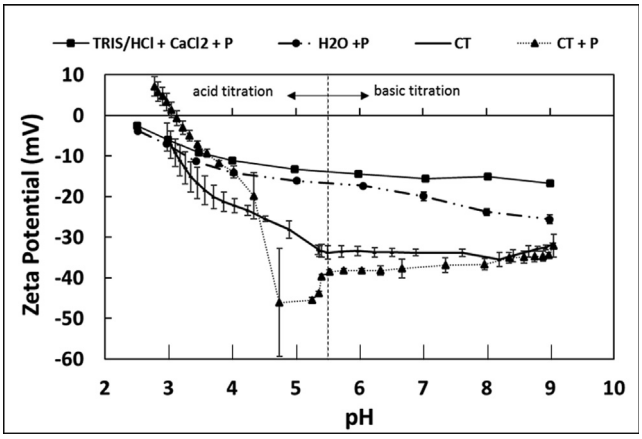

Fig. 3. Zeta potential titration curves of the TRIS/HCl + $CaCl_2$ + P solution and the CT, CT + P samples. The pH value at which the basic and acid titrations are measured from is evidenced with a line.

the CT + P samples has a significant step. For this pH value, the two titration curves of the CT and CT + P samples show a different behavior.

The two sets of the CT + P samples, extracted at the end of the measurements, both after the acidic range and alkaline range, have been observed through fluorescence microscopy. The obtained images show a negligible fluorescent signal, both for the samples used for the acidic range of the titration curve and for the ones used in the alkaline one; the intensity of the red signal is comparable with the CT sample (which has no functionalization) previously reported.

3.4. UV–VIS spectroscopy on the substrate and functionalized surfaces

The CT samples have been analyzed through UV–VIS spectroscopy, in reflectance modality, to compare the surface before and after functionalization, as well as after the release test in water or sterilization. First, as appreciable in Fig. 4, despite of the type of sample, all the spectra show multiple reflections. These reflections are characterized by local maxima, following a quasi-periodic trend: this trend can be described as a quasi-sinusoidal wave summed to an increasing ramp function, so that each peak is more intense than the previous one.

This phenomenon is particularly appreciable on the CT (substrate) and CT + P28 (functionalized and soaked in water for 28 days) samples. For both the materials, it is possible to see that the period increases at the increasing of the wavelength, going from around 100 nm for the first oscillations to around 200 nm for the last ones. These signals are due to multiple reflections of the light at the interface between the surface oxide layer, that is transparent, and the metal substrate [33]. Finally, it is possible to see that the reflections on the CT + P sample are significantly attenuated; the same behavior is maintained also after sterilization (CT + P_S). In both cases, the trend of the ramp function is clearly appreciable, while the oscillations are characterized by lower amplitudes and intensity.

3.5. F&C test on the solid samples

The protocol of the F&C test, usually used for solutions, is here adapted for the solid samples, as described in the paragraph 2.8. It is useful to test if the grafted polyphenols are still able to react as reducing compounds after grafting on the titanium substrate. Four

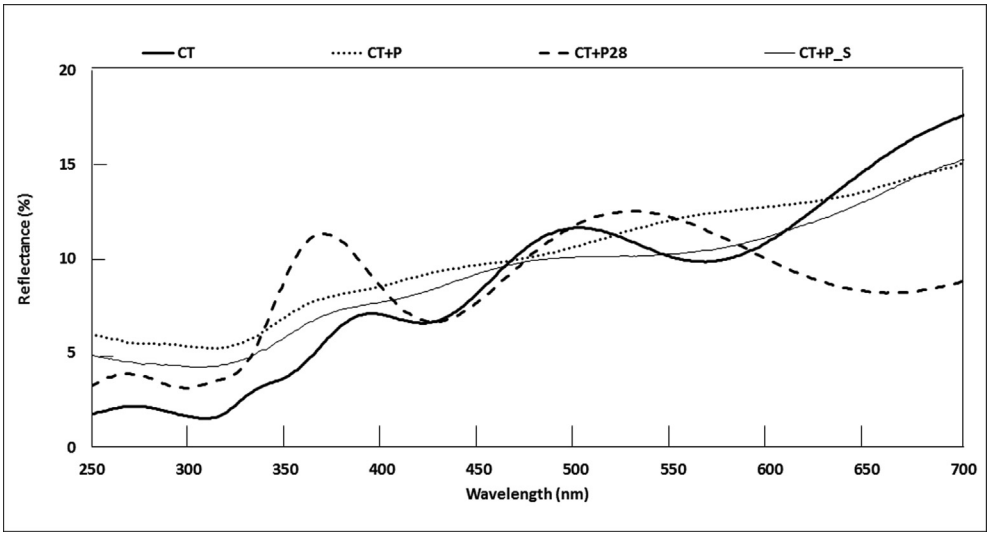

Fig. 4. UV–VIS spectra – reflectance modality – of the CT, functionalized CT (CT + P), functionalized CT after the release test in water (CT + P28) and functionalized CT after sterilization (CT + P_S) samples.

samples are studied, starting with a control sample, which is the substrate with no functionalization (CT).

On CT, the GAE is not zero (Fig. 5): the bare sample shows a weak capability of reduction of the F&C reagent. After polyphenols grafting, CT + P shows a higher GAE, with a significant increase even if there is also a marked increment of the standard deviation. CT + P28 demonstrates a significant decrement of the GAE value, returning to a value close to the one of the substrate (CT), but with a lower standard deviation. The sterilization does not alter the average amount of the polyphenols in terms of GAE, measured through the F&C test, and the standard deviation is comparable to the one of the CT + P samples.

The value of GAE was measured also in the soaking liquids collected during the release test at 4 experimental times, ergo after 1, 7, 14 and 28 days, respectively. The control is constituted by ultrapure water and, as expected, it does not reduce the F&C reagent for a null GAE value at any experimental time. The obtained results are here reported. CT + P immersed in water has a quick release of polyphenols during the first day, as it is deduced from the value of GAE registered in water after 1 day of soaking (0.0015 mg/ml), and a null release during the following experimental times. Furthermore, the pH of the extracted solution in which the samples were soaked was checked at each experimental time. It is in the range 6.8–7.8, that is the same one characterizing pure water used as control.

3.6. DPPH test

The radical scavenging activity of the functionalized surfaces is measured through the DPPH test to verify if the grafted polyphenols maintain their ability to stabilize free radicals; the spectroscopic analysis is performed after 4 and 24 h after the beginning of the test because different compounds may act in differing times. The measure is carried out on CT, CT + P and CT + P28; CT is used as a control sample.

The RSA measured on CT (Fig. 6) shows a minimum antioxidant activity of the substrate, that is consistent with the results of the F&C test analyzed in the previous paragraph (Paragraph 3.6). The close values of the RSA percentage of the un-functionalized sample at 4 and 24 h underline the constant antioxidant activity of the substrate surface over 24 h. The grafted polyphenols increase the value of RSA at 4 h and in a larger way at 24 h with a great ability to scavenge the radicals by the CT + P sample. After the release test in water, the DPPH test reveals a minimum variation of the antioxidant activity between CT + P and CT + P28, evidencing that the ability of polyphenols to scavenge the DPPH radicals has not decreased after soaking.

3.7. XPS analysis

The surface chemical composition of the as-extracted and freeze dried polyphenols (P), chemically treated Ti6Al4V (CT – before and after UV irradiation), and chemically treated and functionalized Ti6Al4V (CT + P) samples are reported in Table 2.

As expected, P is mainly constituted by C and O. Negligible amounts of Ca, P and N can be also detected, while no heavy metals have been noticed.

The CT surface has a certain amount of carbon and nitrogen, attributable to atmospheric and hydrocarbon contaminants always present onto titanium surfaces [34], but it is mainly constituted by Ti and O, peculiar elements of the titanium oxide layer. UV irradiation reduces the surface carbon content. After functionalization (CT + P), the carbon content on the surface significantly increases, while the titanium one drops close to zero and the oxygen one significantly decreases. These data suggest that the surface is completely covered by a layer of organic compounds. The presence of Ca can be associated with the use of a functionalizing solution which contains Ca^{2+} ions: grafting occurs through formation of polyphenols-calcium complexes [13,18,35,36].

The high-resolution spectra of the C and O regions of the different samples are reported in Fig. 7.

The high-resolution spectrum of the carbon region for the as-extracted and freeze dried polyphenols (P) evidences three main contributions at 284.75 eV, 286.28 eV and 287.60 eV which can be associated with the C—C or C—H, C—O and C═O bonds, respectively (Fig. 7a) [37]. The high-resolution spectrum of the oxygen region for the same sample (Fig. 7b) shows three signals at 531.39 eV, 532.66 eV and 533.39 eV, which can be attributed to aromatic C═O, O═C—OH and phenolic OH, respectively [38]. The high-resolution spectrum of the carbon region of the CT sample (Fig. 7c) has a lower intensity than P, in accordance with a lower C content (Table 2), and shows four main contributions at 284.92 eV, 286.70 eV, 288.14 eV and 289.33 which can be attributed to C—C/C—H, C—O, C═O bonds and to carbonates, respectively

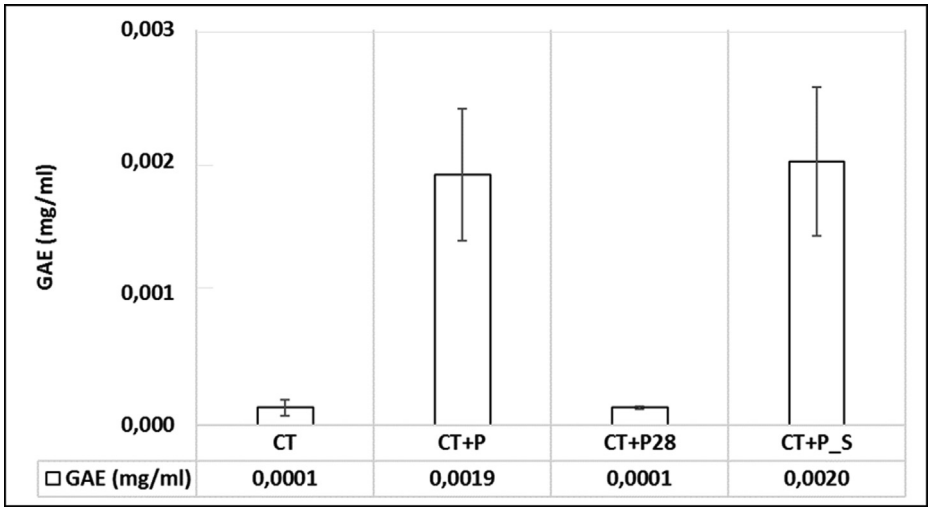

Fig. 5. GAE measures to compare the amount of polyphenols on the substrate (CT) and functionalized samples (CT + P), after the release test (CT + P28) and sterilization (CT + P_S).

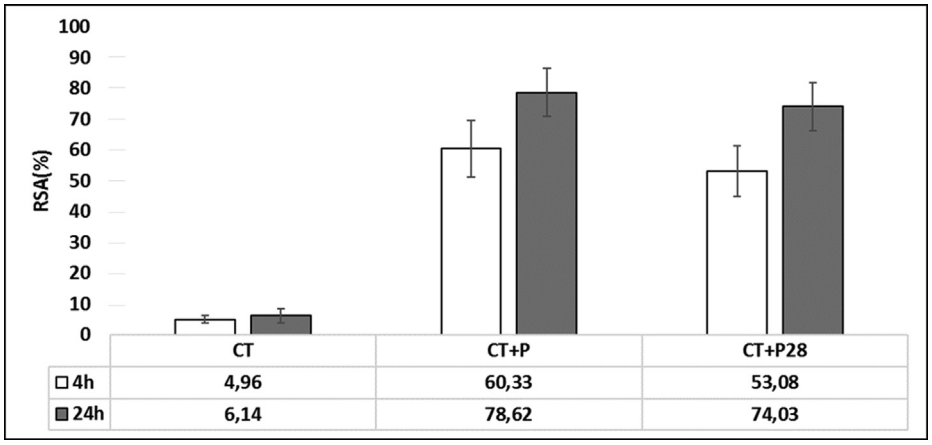

Fig. 6. Radical scavenging activity percentage (DPPH test at 4 and 24 h): control (CT), functionalized sample before (CT + P) and after the release test (CT + P28).

Table 2
Surface chemical composition (%at) from XPS survey analyses.

	C	O	Ca	Ti	N	P	Al	Si
P	69	28.7	0.2	–	1.5	0.7	–	–
CT (before UV irradiation)	24.3	56.5	–	15.8	3.4	–	–	–
CT (after UV irradiation)	12.1	62.9	–	21.0	–	–	2.4	1.6
CT + P	60.4	35.6	1.1	2.9	–	–	–	–

[37,38,39]. All these signals can be associated with carbon contaminants always present onto reactive titanium surfaces [35,36]. The carbon signal is reduced after UV irradiation (Fig. 7e) even if a certain amount of adventitious C is still present, with signals close to the ones of CT sample. In the high-resolution spectrum of the oxygen region of CT (Fig. 7d), four main contributions can be detected at 529.84 eV, 530.70 eV, 531.59 eV and 532.82 eV. The first one can be associated to the Ti-O bonds, while the second one to acidic OH groups (OHa), the third one to basic OH groups (OHb) and the fourth one to adsorbed water, as previously reported by the authors [40,41]. After UV irradiation (Fig. 7f) the signal of adsorbed water disappears and the signal of basic OH groups increases [15]. After polyphenols grafting (CT + P), the high-resolution spectrum of the carbon region has higher intensity (Fig. 7g), in accordance with the increase in the C content (Table S1) and shows two main contributions at 283.26 eV and 284.95 eV together with two lower signals at 287.43 eV and 290.19 eV. The signal at 284.95 eV can be attributed to the C—C and C—H bonds, that are abundant in polyphenols, while the ones at 287.43 eV and 290.19 eV can be associated with the C═O bonds and carbonates. The signal at 283.26 eV can be associated with the bonds between C and metals, as reported in [42,43,44]. The high-resolution spectrum of the oxygen region for the CT + P sample (Fig. 7h) shows two main contributions at 529.39 eV and 531.76 eV. The first one can be associated with oxygen bonding with metals (in this case, Ca within the polyphenol-calcium complex) [45] while the second one can be

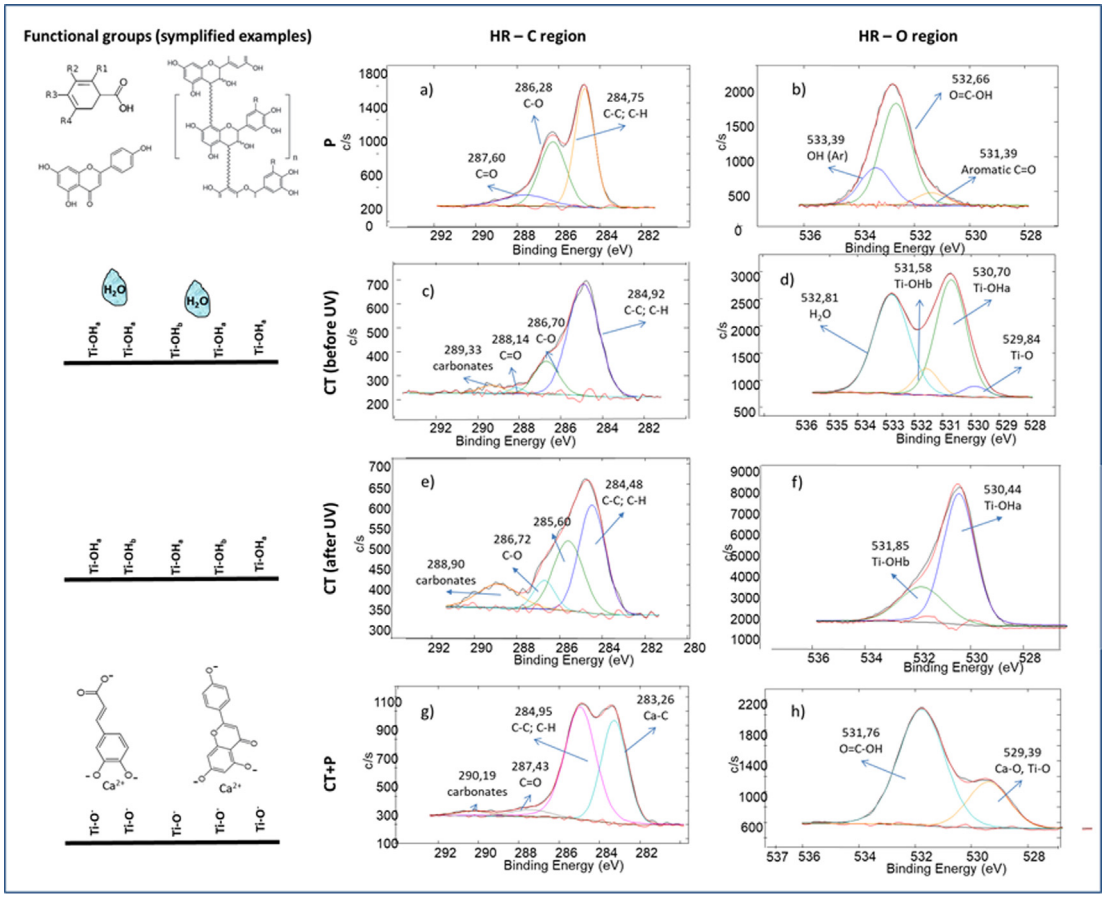

Fig. 7. Schematization of molecule/surface functional groups and high resolution spectra of the C (a, c, e, g) and O (b, d, f, h) regions for the P, CT, CT + UV and CT + P samples.

associated with the COOH groups with binding energy shifted due to the formation of polyphenols-metal ion complexes [46,47]. The high-resolution spectrum of the Ca region (reported in Supplementary Material) evidences two contributions at 346.42 eV and 350.02 eV, which can be associated with the Ca—O bonds, with slight lower binding energy due to the formation of Ca-polyphenols complex, as reported in [48,49] and already observed by the authors on hydroxyapatite functionalized with the same polyphenols [16].

The functionalized samples have been also characterized through FTIR-ATR spectroscopy: the results confirm the presence of the grafted layer and are described in Supplementary Material.

4. Discussion

The aim of the present research is to graft polyphenols on a chemically treated and bioactive Ti6Al4V alloy (CT samples) with the final goal to enhance the biological response of the surface The beneficial properties of these biomolecules, especially the antioxidant, antibacterial and anti-inflammatory ones, can be exploited with a local action to limit third body reaction and chronic inflammation in the surrounding tissues, as well as to fast osseointegration in case of implantation in a bad quality bone. In this paper, the focus is on effectiveness of the functionalization process, the grafting mechanism, and the redox/scavenging activities of the grafted biomolecules.

The chemical treatment on Ti6Al4V (CT samples) is aimed at formation of a nanoporous oxide layer rich in OH groups. As already shown by the authors, the obtained surface layer is modified in thickness, chemistry, and morphology with respect to the native surface oxide layer of Ti6Al4V. An oxide about 400 nm thick, with multi-scale roughness (micro- and nano-sized), high density of acidic OH groups and porosity at the nanoscale (about 10 nm) is obtained [11,12,13,40]. The chemically treated surface is biomimetic because porosity at the nanoscale is close to the one characterizing the extra-cellular matrix of the human bone tissue; then, it is bioactive because it induces hydroxyapatite precipitation in contact with the physiological fluids after implantation and it efficaciously interacts with osteoblasts and macrophages, contributing to the biochemical pathways that are involved in the physiological bone tissue regeneration [32]. The results here obtained on the substrate (CT samples) give complementary information to those already acquired and published by the authors on this surface. The DPPH and F&C test respectively evidence a slight radical scavenging and redox activity of the CT surface. The DPPH test performed at 4 and 24 h shows that the radical scavenging ability remains constant over this time range. Both the activities can be ascribed to the presence of a high density of OH groups on the surface of the CT samples. Analyzing the CT samples through UV–VIS spectroscopy in reflectance modality, it is possible to see multiple reflections, because of the transparency of the nanoporous titania layer at the visible light: reflection of the radiations occurs at the interface with the bulk metal underneath it [50,51].

The HPLC characterization of the polyphenol extract obtained from red organic grape (Barbera) evidences the presence of condensed tannins and monomer flavan-3-ols. According to literature [52], the monomer units composing the condensed tannins are (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-O-gallate and (-)-epigallocatechin.

Moving to functionalization of the CT surface, as first, the solution used for the process was analyzed (UV–VIS spectroscopy, zeta potential and F&C test). The main peaks obtained by UV–VIS spectroscopy characterize a wide range of polyphenols (isoflavones, catechins/epicatechins and flavanones), because intense absorbance in the range 250–280 nm is related to the presence in solution of polyphenols with an aromatic A ring and low conjugation between ring B; as expected, they are detectable both on the functionalizing solution (TRIS/HCl + $CaCl_2$ + P) and in a reference solution with the same concentration of polyphenols without the addition of the buffer and $CaCl_2$ (H_2O + P) [53]. Because the peaks at 270–280 nm are quite spread and partially overlapped, they appear as a broad and asymmetric peak: they can be attributed to hydroxycinnamic acids and flavanols, (catechins/epicatechins) [17,30,54,55]. It is also verified that the addition of the buffer and $CaCl_2$ does not significantly alter the molecular structure of the dissolved polyphenols. The higher intensity of the peak at 280 nm registered in the functionalizing solution (TRIS/HCl + $CaCl_2$ + P) with respect to the one detected on the reference solution (H_2O + P) is attributable to the signal due to π-system of the aromatic rings at pH > 7, which is added to the signal induced by the presence of anthocyanins [56]. In the functionalizing solution (TRIS/HCl + $CaCl_2$ + P), it is possible to see that the peak corresponding to anthocyanins is shifted from 530 nm (as it is in the reference solution H_2O + P) to 450 nm. It is due to the buffering action of TRIS, which carries pH from 3.6 to 7.4: anthocyanins are sensitive to pH and their color is pH-dependent. In fact, at neutral pH (pH = 7) deprotonation of the hydroxyl groups occurs, causing the observed shift of the absorbance peak of anthocyanins and a change in the color of the solution toward a darker nuance observable at a glance [57]. The observed variations in the color of the solution – going from purple at pH = 3.6 (color that is typical of red grape pomaces) to a darker color, almost black, with a blue nuance, at pH = 7.4 – are in agreement with the different responses to UV–VIS light of the two solutions. The number of hydroxyl groups bonded to the B ring induces a displacement in chromaticity, while pH increases [58]. A similar observation was made by the authors in the case of gallic acid (GA), a simple molecule belonging to the class of hydroxycinnamic acids and often used as a model of a generic polyphenolic compound; it is sensitive to the pH value characterizing the environment: because of deprotonation, it assumes a green color at pH = 7.4, while it is uncolored at acidic pH [18]. The alteration of the color of the TRIS/HCl + $CaCl_2$ + P solution is also caused by the formation of a coordination complex between the calcium ions Ca^{2+} and the polyphenolic compounds. Its presence can be observed through UV–VIS spectroscopy: a local absorbance maximum is clearly visible around 600 nm, which is attributed to this coordination complex. The formation of the complex is due to the attraction between the Ca^{2+} ions and dissociated hydroxyl groups. Ca^{2+} ions are derived from the dissociation of $CaCl_2$ in an aqueous environment [59]. In the case of GA, the dissociation of its carboxylic group is characterized by a pK_a value of 4.4, while deprotonation of the OH phenolic groups has a pK_a higher than 8.5 [60]. This means that GA and in general polyphenols are negatively charged in an aqueous environment at pH = 7.4 and easily interact with the calcium cations. The phenomenon of dissociation and formation of the molecular complex with Ca^{2+} was also deduced from the data obtained by the zeta potential titration curves, as discussed below. The zeta potential titration curve of the TRIS/HCl + $CaCl_2$ + P solution is negative for all the evaluated pH range with the isoelectric point registered at pH = 2.21 The polyphenols in the solution are negatively charged for all pH values higher than the isoelectric point with two variations of the slope, the first around pH = 4, the second around pH = 7: the first variation of the slope can be explained by dissociation of the OH groups of the carboxyl groups belonging to the phenolic acids (such as hydroxycinnamic acid), analogously to what reported for gallic acid (pK_a = 4.4 for the COOH group) [61];the second variation of slope, instead, can be explained through another dissociation process, which, in this case, involves the phenolic OH groups of catechins and anthocyanins, whose dissociation has place around pH = 7. The pK_a value of the phenolic OH groups of the extract utilized in this work is lower than the one of gallic acid (pK_a = 8.5) because of the presence of different classes of polyphenols in the extracted mixture and it changes in presence of the Ca^{2+} ions [57]. In fact, in the TRIS/HCl + $CaCl_2$ + P solution a plateau is reached around ζ = -15 mV, while it is absent in the reference solution (H_2O + P), where the slope is maintained constant till the end of the evaluated pH range, phenomenon explainable by the dissociation of these hydroxyl groups that has place progressively when pH increases [62]. It can be deduced that, because of the presence of the calcium ions in the TRIS/HCl + $CaCl_2$ + P solution and the formation of the complex compounds, the chemical reactivity of the phenolic OH groups of catechins and anthocyanins is changed, and they dissociate simultaneously, once reached a certain pH value (pH ~ 7) [62,63].

As expected, the polyphenols in solution have redox chemical ability as observed by the GAE value detected through the F&C test. The Uptake CT, that is the solution at the end of the functionalizing process, has been analyzed through F&C test and compared with the as-prepared functionalizing solution (TRIS/HCl + $CaCl_2$ + P). There is not any relevant difference concerning the polyphenols content before and after functionalization: the averaged GAE values of the uptake and functionalizing solutions are comparable and no alterations are appreciable after the functionalization process. This phenomenon is associated to the limited amount of the grafted polyphenols, compared to the initial high concentration in the functionalizing solution.

Then, the surface of the functionalized samples has been characterized through different techniques (UV–VIS spectroscopy, , fluorescence microscopy, zeta potential titration curves, F&C and DPPH tests, XPS), in order to verify presence, amount, distribution, release and type of bonding with the surface of the grafted polyphenols. As first, XPS chemical analysis shows that no toxic element or heavy metal is detected on the surface of the functionalized surfaces confirming the biocompatibility of the surface. A homogeneous distribution of polyphenols can be observed on CT + P through fluorescence microscopy with formation of a continuous layer and some agglomerates. The presence of a continuous layer of polyphenols is in agreement with the UV–VIS spectra of the CT + P samples (reflectance modality): the multiple reflections due to transparency of the nanoporous titania layer on CT surface and reflection at the interface with the bulk are strongly limited after surface functionalization because of the shielding effect exercised by the grafted polyphenols which are able to absorb a non-negligible fraction of the light emitted by the source. The formation of agglomerates can be due to entrapment of biomolecules in the porosity of the surface during functionalization.

The presence of the grafted polyphenols on CT + P is confirmed by the XPS analysis, as described below.

The zeta potential titration curves have been performed also on the solid samples, to compare the substrate and the functionalized surfaces. The isoelectric points, measured as the intersection of the curves with the abscissa axis or calculated by interpolation of the curves, correspond respectively to pH = 2.80 and to pH = 3.10, both in the acidic field. The curve of the CT substrate reaches a plateau (at about −35 mV) with onset around pH = 5: above this pH value, the complete dissociation of the OH groups exposed on the surface of the substrate occurs [64]. For this reason, the considered CT surface is negatively charged in the imposed pH conditions of functionalization (pH = 7.4). The chemical stability of the substrate

(CT), both in the acidic and alkaline ranges, is confirmed by the low standard deviation of each point of the measurement on the CT sample [65]. The zeta potential titration curve of the functionalized sample (CT + P) is close to the curve of the substrate for the alkaline and highly acid pH values. A significant difference between the substrate and functionalized surface is appreciable around pH = 5: in correspondence of this value, a step characterizes the CT + P samples curve. It could be explained considering that the mechanism of grafting is due to the formation of a complex compound in the solution with a bond between the OH groups of the polyphenols and calcium ions in the solution: the polyphenols are grafted to the surface because of the electrostatic attraction of the calcium ions to the negatively charged surface of the CT substrate due to the dissociated hydroxyl groups on it. The step registered at pH = 5 on CT + P, corresponds to detachment of the polyphenols from the functionalized surface. For lower pH values, the OH groups of the substrate are no more dissociated and protonation, of the phenolic OH groups of the polyphenols, induces the instability of the bond with the surface [62], as it can be induced from the step registered in this range of pH and from the high standard deviation registered around this pH value. After detachment of the biomolecules, the titration curve becomes close to that of the substrate. This hypothesis is confirmed by the absence of polyphenols on the surface of the samples used for the measurement of the titration curves, as observed by fluorescence microscopy. Complementary characterization is needed in order to confirm this hypothesis about the grafting mechanism. If this hypothesis would be confirmed, it would be of great interest because polyphenols would be released in a smart way from the material when pH goes down to 4–5, that means when an inflammatory event occurs. Surface grafting of polyphenols through the formation of a polyphenol-calcium complex at the surface is supported also by the XPS results. At first, the XPS data evidence the presence of a polyphenols layer almost completely covering the titanium substrate: increase in the carbon content, decrease of the Ti and O signals and presence of the characteristic functional groups, such as C—C, C—H, C═O, COOH, support this explanation. Concerning the attribution of the high resolution fitting, the authors suggest the following explanation according to the literature. The phenolic OH groups (present in the P high resolution spectrum of the oxygen region) are not visible on the CT + P one, supporting their involvement in the formation of a polyphenol-calcium complex, as reported in the literature [66]. Moreover, a peak at around 283 eV appears in the carbon region, which can be associated with the metal–carbon bonds [44] and in this specific case it is reasonable to suppose that it is Ca—C, as suggested in the grafting scheme of Fig. 7 In addition, the spectrum of calcium evidences the signals ascribable to the Ca—O bonds, with slight lower binding energy due to the formation of a Ca-polyphenols complex, as reported in [45,49] and already observed by the authors on hydroxyapatite functionalized with the same polyphenols [16], in agreement with this hypothesis Finally, in the oxygen region, the signal at 531.76 eV can be associated with COOH groups shifted respect to the polyphenol extract (532.66 eV) due to the formation of the polyphenols metal ion complex and the alteration of the chemical environment of the COOH group [47]. All these data suggest the formation of the polyphenols-calcium complexes on the titanium surfaces, exploiting mainly the phenolic OH groups (no more visible at XPS after grafting), with the consequent exposition of the carboxylic groups of the phenolic acids, with different chemical around compared to the polyphenol extract. The high chemical affinity between the calcium ions and the surface oxide layer of CT, attracted because of the negative charge on the CT surface, is of great interest both because it has a role in the grafting mechanism of the polyphenols and it induces an enrichment of calcium ions at the interface between the material and the host tissue. The presence of these cations could induce an osteoinductive effect, implementing hydroxyapatite precipitation *in vivo* after the implantation (bioactivity); simultaneously, the polyphenols, present at the interface as grafted molecules or locally released (as explained below), could act as antioxidants, decreasing the probability of third body reaction or chronic inflammation, thanks to the high bioavailability that is possible through this system of local action.

The DPPH and F&C test carry out a complementary role in the study of chemical reactivity of the grafted polyphenols. Simple phenols react with the F&C reagent, although they are not radical scavenging molecules, so that the F&C and DPPH tests could not have correlated results. The different polyphenols in the extract have different scavenging kinetic [67], making two measures necessary: the first at 4 h and the second at 24 h. The reaction between DPPH molecules and polyphenols has been studied in a previous work [68], demonstrating that the principal step of the reaction consists of an electron transfer process from the anions to DPPH. The DPPH test performed on the CT + P samples shows that a consistent amount of polyphenols are grafted on the surface and they have different kinetics of scavenging activity. The measure at 4 h of the RSA percentage shows a strong scavenging activity of the phenolic compounds: it means both that the grafted compounds expose a high number of reactive OH groups and that they are chemically stable [69]. After 24 h, the RSA value registered on CT + P increases, but the most part of the grafted polyphenols reacts during the first 4 h.

Concerning the test of release in water (pH≈7), the results of several techniques (UV–VIS spectroscopy, F&C test and fluorescence microscopy) agree that the amount of the grafted polyphenols on the surface after 28 days of soaking is significantly lower than the one of the as-prepared functionalized sample. UV–VIS spectroscopy reveals that the layer of polyphenols, that is thick enough to shield the UV light on the CT + P sample, is no more present on CT + P28.

The F&C test of the water used for soaking the CT + P28 samples (applied at different experimental times) shows a quick release at 24 h and then no more release phenomena occur. On the other side, a weak and uniformly distributed fluorescent signal is present on CT + P28, revealing that a layer of grafted polyphenols is maintained, also if subjected to a significant thinning, while the agglomerates are not present anymore. As shown by the DPPH test, the RSA percentage characterizing CT + P28 has not a significant decrement respect to CT + P. The high value of RSA confirms the presence of the polyphenols grafted on the surface and shows that they act as radical scavengers even after a long soaking time. Furthermore, no redox ability of the surface is detected by the F&C test after the release test: this is explainable considering that F&C test (modified for the solid samples) is sensitive to the grafted polyphenols dissolved from the surface into the solution during the test, while DPPH detects the scavenging activity of the polyphenols firmly grafted to the surface. The result of the F&C test can be explained considering that, after 28 days of soaking in water, CT + P28 does not release any other polyphenol after this test. On the other side, the result of the DPPH test shows that after soaking in water there is still an amount of grafted polyphenols which have the same scavenging ability of the just freshly functionalized samples. Considering all these data, it has been supposed that an important fraction of the total amount of the initial polyphenols – present on the CT surface after the functionalization process – is released along 28 days, but a thin layer of them is still present on the analyzed surface at the end of the test. It has been hypothesized that the released polyphenols correspond to the fraction of them with a weaker chemical interaction with the surface, such as the aggregates linked through physical adsorption in the porosity of the surface, while the polyphenols chemically grafted through

strong electrostatic interaction are firmly bound to the substrate, with a stable biomolecule-surface interface. This agrees with the stability of the pH values characterizing the release solutions. Despite of non-significant oscillations, the average value is maintained around pH = 7, that is a condition, as induced by the considerations about zeta potential titration curves, in which the firmly grafted polyphenols are not released. This explains the presence of a residual grafted layer, that is maintained after soaking, detectable through fluorescence microscopy and the DPPH test at the end of the release test. Further studies are needed to explore if the released polyphenols will be stable and chemically active in a biological environment; some interesting results at this purpose are reported in [70].

Analyzing the sterilized CT + P_S samples through fluorescent microscopy, F&C test and UV–VIS spectroscopy, it is possible to observe that the uniform layer of the grafted polyphenols is not altered during sterilization. Fluorescence microscopy shows that it is homogeneous and the grafted polyphenols are well-distributed, as before the treatment. This is in agreement with the response to UV–VIS spectroscopy, where the attenuation of the multiple reflections typical of the as-prepared functionalized surface is still observable after sterilization. The total amount of the grafted polyphenols on CT + P, able to exercise a shielding effect to UV light, is not significantly altered by the sterilization treatment, after which the efficiency of functionalization is maintained. The sterilization process does not alter the redox ability of the functionalized surface: in fact, CT + P_S shows a high value of GAE concentration, that is comparable to CT + P one. The similar value confirms that sterilization by gamma irradiation, carried out through the common protocol used for the biomedical devices, does not alter the chemical reactivity of the grafted polyphenols [71]. A significant variation is instead appreciable concerning the dimension of the agglomerates: it averagely increases and the distribution of their diameters is ampler after sterilization. It is probably due to a polymerization process implemented by gamma radiations: if irradiated with low doses – as 25 kGy, imposed by the Medical Device Directive, is – the polyphenols are induced to polymerize because of the formation of reactive species, which induces the biomolecules to react together combining each other to product higher molecular mass compounds, increasing the total antioxidant activity of the polyphenols themselves [72,73]. The gamma-induced polymerization does not alter the UV shielding efficacy of the functionalized material.

5. Conclusions

Polyphenols (phenolic acids, flavonoids and condensed tannins) from an extract of organic grape pomace have been successfully grafted on a chemically treated and bioactive Ti6Al4V alloy adding antioxidant and radical scavenging properties. The final aim of the research is to optimize the biological response of the surface in contact with bone. The results reported in this paper deal with effectiveness of the functionalization process in an aqueous solution of the extract, buffered at pH 7.4, and enriched with calcium ions in order to exploit the calcium mediation role in grafting. The chemical treated surface has a nanoporous oxide layer rich in OH groups, with acid chemical behavior, suitable for the functionalization process. The presence, amount, distribution, release and type of bonding with the surface of the grafted polyphenols have been assessed. A homogeneous distribution of polyphenols as a continuous layer with micro-sized agglomerates is formed. The grafted polyphenols maintain redox chemical and radical scavenging ability on the metallic surface. A fraction of polyphenols is released in water in less than one day, while a second fraction is firmly grafted on the surface even after a soaking time of some weeks. The functionalized surfaces can be sterilized by gamma irradiation without any significant damage of the grafted polyphenols.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

EU Commission is acknowledged for funding NAT4MORE project (M.ERA-NET 2016)

Eng. C. Tonda Turo is acknowledged for freeze-drying of the polyphenols extract.

Dr. A. Bosso is acknowledged for discussion of HPLC and UV-VIS data.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.matdes.2021.109776.

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