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Coupling of keratin with titanium: a physico-chemical characterization of functionalized or coated surfaces

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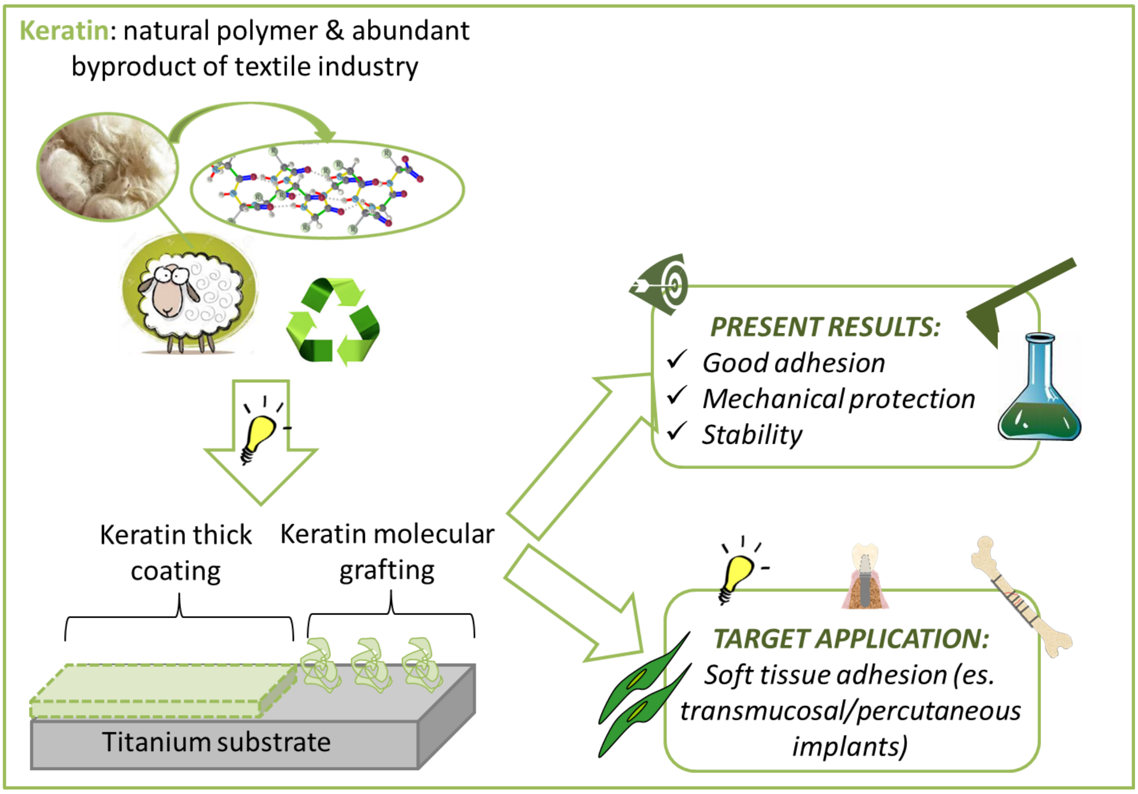
Abstract

Coupling of keratin with titanium surfaces is here realized both through thick coatings and molecular functionalization. This coupling has the final aim both of chemical and mechanical protection of the titanium surfaces and of improving interaction of titanium implants with soft tissues in transmucosal and percutaneous implants. Wool-derived keratin has been used and plasma decontamination/activation has been tested as possible strategy for the improvement of keratin grafting ability and adhesion. Coated/functionalized surfaces have been characterized by means of X-ray photoelectron spectroscopy (XPS) and Scanning Electron Microscopy (SEM), contact angle measurements X-Ray Diffraction (XRD), zeta potential and adhesion (tape and scratch) tests. These analyses evidenced that keratin was successfully coupled to titanium surfaces both in the form of molecular grafting and thick coating. In both cases, plasma surface activation improves keratin wetting and bonding ability for the substrates. The obtained coatings showed good adhesion and mechanical stability.

Graphical abstract



Keywords: titanium, keratin, functionalization, coating

Introduction

The aim of the present research is the combination of titanium, which is the material of choice for dental and orthopedic implants, with keratin, which can effectively stimulate fibroblast adhesion and growth. The goal application of keratin coated/functionalized titanium is the portion of transmucosal or percutaneous implants in contact with soft tissues. Keratin can be obtained from abundant byproducts of the textile industry, with a circular economy approach, and used with high added value for applications in the biomedical field.

Keratin is a naturally abundant protein, which can be found in the tissues of humans and animals and in particular in feathers, hair, nails, horns, hooves, bones, furs, claws, hides, bird beaks, skin, wool, scales, and bristle [1]. The abundance of this protein in nature and in industrial byproducts (e.g. textile industry) increases the interest in its application, considering a sustainable use of the resources.

Keratin can favor the adhesion of several types of cells and has already been proposed as a possible coating for the cell culture dishes [2, 3]. In particular, keratin is known for its ability to support fibroblast cells growth [4, 5, 6] and has been previously studied by the authors, in the form of submicrometric electrospun fibers, as a possible coating for the dental implant collar to support soft tissue growth and gum sealing [7, 8, 9]. Recently, keratin coatings have been also proposed to support soft tissue growth in percutaneous implants [10] and, in the form of keratin hydrogel as possible coating (to be applied directly in surgical operations) for the improvement of bone integration in orthopedic and dental implants [11]. Moreover, an anti-inflammatory action of keratin has been reported in the literature [12]. Finally, due to its strong ability to bind metal ions, keratin can be doped with antibacterial metal ions to confer antibacterial properties to the implants, as previously shown by the authors [9].

The above cited properties make keratin extremely interesting in applications facing soft tissues and with high risk of inflammation/infection development such as transmucosal dental implants and percutaneous implants. These implants are usually made of titanium and a suitable surface modification able to improve titanium interaction with soft tissue can be of particular interest.

On the basis of the previous results on keratin fibers and considering the promising properties of this molecule in contact with soft tissues, in the present research work, for the first time, keratin has been coupled with titanium, in the form of a continuous thick coating or as a molecular grafted layer (functionalization). Differently from the few works reported in the literature [10, 11], keratin has been coupled to titanium without any synthetic spacer and as coating/functionalized layer on the metallic material before the implantation (with the idea to be performed at the production stage of medical implants). Moreover, differently from previous works of the authors [7,8,9], in the present research keratin has been considered as a molecular layer (functionalization) or thick continuous coating instead of submicrometric fibers. The first strategy completely covers the metallic substrate with a smooth continuous coating which hides the substrate (topography and chemistry), while the second one foresees the grafting of a thin layer of molecules with exposition of the substrate topography and eventually of its chemistry, depending on the entity of grafting. This last approach is of interest when surface topography is designed for a specific purpose, such as for cell contact guidance which is promising for fibroblasts as already shown by the authors [7,8]. On the other hand, the case of a smooth a continuous coating of keratin is interesting when a reduction of bacterial adhesion is needed without compromising fibroblast colonization of the surface, because the bacteria, unlike the fibroblasts, preferentially adhere to rough surfaces [13]. The importance of a proper combination of surface roughness, chemistry and wettability in order to modulate the biological response is widely evidenced in the literature [14, 15].

The main issues in keratin coupling with biomaterials are its mechanical adhesion and its long term stability [10]. The effect of a plasma surface pre-treatment of titanium has been considered in this work in order to enhance keratin-titanium bonding. Moreover, the thermal cross-linking of keratin [7] has been applied in order to improve its biological stability.

Plasma surface treatment of titanium is widely applied in the decontamination of dental implants [16], due to its ability to reduce hydrocarbon contaminations and to improve the biological response of the implants. Removal of carbon contaminants, together with exposition of the hydroxyl groups [17, 18], can favor the coupling with keratin (both through molecular functionalization and coating strategies) and improve its adhesion. Moreover plasma based techniques have been successfully used for the combination of titanium oxide nanoparticles and keratin fabrics [19, 20].

Finally, the availability of plasma treatments facilities in the majority of the companies producing dental implants can facilitate the industrial scale up of the process.

Wool keratin has been used in the present research for the functionalization/coating of commercially pure titanium substrates with/without plasma activation. The coated or functionalized samples have been characterized by means of X-ray Photoelectron Spectroscopy (XPS), Scanning Electron Microscopy equipped with Energy Dispersive Spectroscopy (SEM-EDS), wettability, contact profiler measurements, confocal microscopy, zeta potential and adhesion (tape and scratch) tests.

2. Materials and Methods

2.1 Samples preparation

Commercially pure titanium (Ti grade 2, ASTMB348,Gr2, Titanium Consulting and Trading) was used as substrate. Discs (10 mm diameter and 2 mm thickness) were obtained from cylindrical bars and polished with abrasive papers up to 4000 grit. Polished samples were washed once in acetone (5 min) and twice in ultrapure water (10 min each). Polished and washed samples will be named Ti-MP from now on.

2.2 Surface activation

Part of the samples was treated with an air plasma (10min, 100W, TUCANO2, Gambetti Kenologia Srl) in order to remove surface carbon contaminants and expose OH groups and improve keratin bonding ability to the metallic surface [17, 18]. Surfaces were plasma treated on Al foils and suddenly packed at the end of the treatment in order to avoid contamination. Plasma treated samples will be named Ti-MP-PL from now on.

2.3 Keratin extraction, solutions preparation and surface modification

Keratin was extracted from discarded wool by sulfitolysis with sodium metabisulfite, purified and freeze-dried in powder as reported elsewhere [7], considering the possibility to transform an abundant byproduct of the textile industry in a high added value resource for biomedicine. Keratin powder was dissolved in ultrapure water at different concentrations:

- 7% wt considering the maximum solubility of keratin in water
- 3% wt in order to maintain high concentration but reducing solution viscosity and increasing its homogeneity
- 1% wt in order to prepare diluted solution

The first two solutions were used for the preparation of keratin coatings while the last one (1% wt) was used for the surface functionalization.

For thick coating preparation a drop (few hundreds µl) of keratin solution (7 or 3% wt) was deposited on the titanium surface and spread with the help of the micropipette tip.

For molecular functionalization, each titanium sample was soaked in 5 ml of the 1% keratin solution for 1h at room temperature and then gently washed with ultrapure water in order to remove unbounded molecules.

After drying both coated and functionalized samples were thermally treated 2h at 180°C in order to stabilize the keratin layer [7, 21].

2.4 Surface characterization

The morphology and semi-quantitative chemical composition of coated and functionalized surfaces was investigated by means of Scanning Electron Microscopy equipped with Energy Dispersive Spectroscopy (SEM-EDS, JEOL, JCM 6000 plus and JED 2300).

Surface chemical composition and the presence of specific functional groups were investigated by means of X-ray Photoelectron spectroscopy (XPS, Kratos, Axis Ultra^{DLD}). Both survey spectra and high resolution ones (for C, O, N, S) were acquired, using a monochromatic Al kα source operated at 20 mA and 15 kV. Survey spectra were acquired at pass energy of 160 eV, energy step of 1 eV and over an analysis area of 300 x 700 µm. High resolution spectra were acquired on the same are, at pass energy of 10 eV and with an energy step of 0.1 eV. The Kratos charge neutralizer system was used on all samples; binding energy scale calibration was performed by setting the position of the main C 1s component at 284.8 eV (for C-C bonds).

Surface wettability was measured through contact angle measurements (sessile drop method, DSA-100, KRÜSS GmbH, Hamburg, Germany) with ultrapure water on Ti-MP and Ti-MP-PL-ker3% samples. A drop of ultrapure water (5 µl) was deposited on the surface and the contact angle measured by the instrument software. Moreover the same technique was used in order to determine the wettability of titanium surfaces (before and after plasma treatment) by keratin solution. In this case, a drop (5 µl) of keratin (3% wt in water) was used and the contact angle measured as described above.

The thickness of the keratin coatings (keratin 3% wt) was measured through a contact profiler (Taylor-Hobson, Form Talysurf 120L) and by confocal microscope (Zeiss model LSM900 reflection laser scanning confocal microscope).

For the contact profiler measurements, the step was generated by forming the coating only on a half of the sample and the height of the step was measured by a contact profiler. In the case of confocal microscopy, the thickness of a full coated sample was evaluated through a laser confocal profiler used in the line-scanning mode to measure a cross-section profile through a transparent coating (according to the method described in [22]) and considering the refractive index of keratin as 1.55 according to [23].

Both the techniques are suitable for the measurement of the coatings and are not suitable in case of molecular (nanometric) layers, so these measurements were not performed on the functionalized samples.

The crystalline structure of keratin was investigated by means of X-Ray Diffraction analyses (XRD, PANalytical X'Pert Pro PW 3040160 Philips). Measurements were performed on Ti-MP-PL-ker3% sample as well as on free standing keratin films prepared from the same keratin solution.

Surface charge in function of pH was analyzed by means of zeta potential electrokinetic measurements (SurPASS, Anton Paar) in 0.001M KCl electrolyte [7]. An adjustable gap cell was used for the measurements. For each measurement, two titanium disks were attached to the sample holders creating a gap between them in which the electrolyte is forced to flow. Movement of the electrical charges is induced by the electrolyte flow on the samples surfaces developing a potential difference which is measured at the edges of the cells and used for the calculation of the zeta potential [24].

Coating adhesion to the substrate was investigated by means of tape and scratch tests. These measurements were performed only on the optimized thick coatings (Ti-MP-PL-ker3%) because they are not suitable for molecular functionalization. Tape tests were performed according to ASTM D 3359 standard [25]: a grid of parallel cuts was prepared on the sample and followed by application and removal of a pressure sensitive adhesive tape. Coating adhesion was estimated by visual observation of the surfaces of the sample and of the tape. Scratch tests were performed, on Ti-MP-PL-ker3% sample, in Revetest mode (CSM, Revetest machine) with a diamond indenter with 200 µm tip radius, with a progressive load increasing up to 10 N, as reported in [26] for polymeric films, a loading rate of 50 N/min and a track length of 4.91 mm.

3. Results and discussion

3.1 Visual inspection

The visual appearance of the coated samples is shown in the first row of Figure 1. The keratin coatings obtained from 7% wt keratin solution (Ti-MP-ker7% andTi-MP-PL-ker7%) are not homogeneous and present bubbles, on the other hand, the keratin coatings obtained from 3% wt keratin solution (Ti-MP-ker3% andTi-MP-PL-ker3%) appear as a homogeneous film on the titanium surface with yellow gold appearance. This difference can be explained considering the high viscosity of the solution with 7% wt of keratin. According to these results, a solution of 3% wt of keratin was selected for the preparation of the coated samples used for the chemical and physical characterization reported in the following. The functionalized samples are not reported in Figure 1 because they are identical to the polished ones. In fact, as expected when a thick coating is absent and when molecules used for grafting are not colored, no visual alteration are introduced by the surface functionalization.

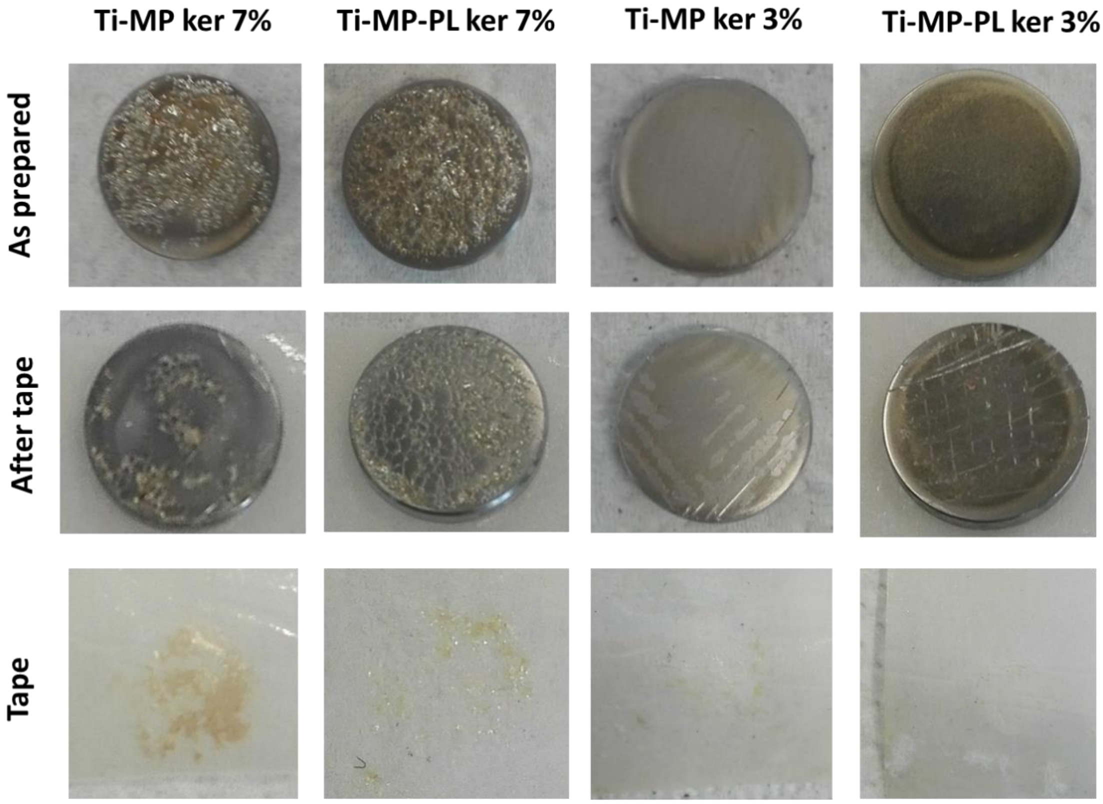


Figure 1: Visual appearance and tape resistance of the keratin coatings. First row: visual appearance of the as-prepared samples; Second row: visual appearance of the samples after tape removal; Third row: visual appearance of tape after removal from the samples surfaces.

3.2 SEM-EDS observations

SEM images and related EDS analyses of the functionalized (Ti-MP-PL-ker1%) and coated (Ti-MP-PL-ker3 %,) surfaces are reported in Figure 2.

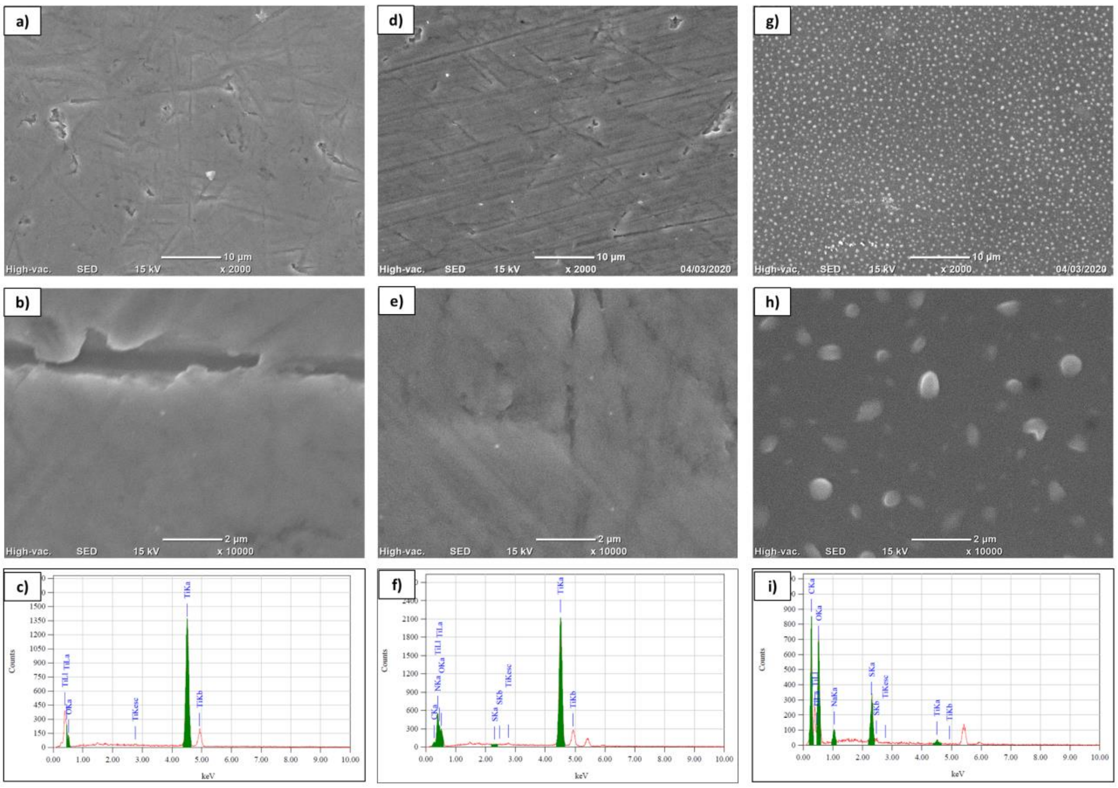


Figure 2: a) low magnification (2000x) SEM image of Ti-MP-PL, b) high magnification (10000x) SEM image of Ti-MP-PL, c) EDS spectrum of Ti-MP-PL,d) low magnification (2000x) SEM image of Ti-MP-PL-ker1%, e) high magnification (10000x) SEM image of Ti-MP-PL-ker1%, f) EDS spectrum of Ti-MP-PL-ker1%,g) low magnification (2000x) SEM image of Ti-MP-PL-ker3%, h) high magnification (10000x) SEM image of Ti-MP-PL-ker3%, i) EDS spectrum of Ti-MP-PL-ker3%

The surface morphology of the substrate (Ti-MP-PL) (Figure 2a, 2b) evidences only some defects associated with mechanical polishing, as usually observed on Ti surfaces, the appearance of the functionalized Ti-MP-PL-ker1% sample is analogous (Figure 2d, 2e) and no signs of an organic coating are visible. This observation confirms that, as expected, surface functionalization does not alter the surface topography of the metallic substrate, but only its chemistry. Consequently, this process can combine the surface topographic features of the substrate with the biochemical ones associated to molecular grafting.

On the other hand, the morphology of the Ti-MP-PL-ker3% sample (Figure 2g, 2h) is significantly different and shows the presence of a continuous layer, with sub-micrometric spot-like features, which completely hidden the topography of the metallic substrate. The coating has not any micrometric roughness which cans obstacle fibroblast adhesion and increase bacterial contamination. As expected, the formation of a coating completely covers the surface conferring to it a new smooth topography. Both topography and chemistry resulted modified by means of this process, in a way which is promising for soft tissue contact with limited infection risk.

As for the morphology, EDS analysis of the Ti-MP-PL-ker1% sample (Figure 2f) shows mainly the substrate signal (Ti), similarly to the EDS spectrum of the Ti-MP-PL sample (Figure 2c), with negligible contribution from the keratin characteristic elements (C, N, S, O). A certain amount of oxygen can be related to the titanium surface native oxide layer. On the other hand, Ti is almost completely covered in the case of the coated Ti-MP-PL-ker3% sample and the EDS spectrum (Figure 2i) evidences the presence of the keratin constituents (mainly C, N, S, O). These results can be explained considering EDS penetration depth (micron range) which is significantly higher than the one of the functionalized layer (molecular layer), but comparable with the one of the coating (micron range, as discussed in the following).

3.3 XPS analyses

The chemical composition of the coated (Ti-MP-PL-ker3%) and functionalized (Ti-MP-ker1% and Ti-MP-PL-ker1%) surfaces, as well as of the bare and plasma treated substrates (Ti-MP and Ti-MP-PL) obtained from the XPS survey analyses, is presented in Table 1. The Ti-MP-PL-Ker3% and Ti-MP-Ker3% are both covered by a thick coating of keratin and it is not expected any difference in their chemical composition of the outermost layer analyzed by XPS: this is why only one of these two surfaces was analyzed by this technique.

Table 1: Surface chemical composition (at%) from XPS survey analyses

	SUBSTRATES		**FUNCTIONALIZED SAMPLES**		**COATED SAMPLE**
	Ti-MP	**Ti-MP-PL**	**Ti-MP-ker1%**	**Ti-MP-PL-Ker1%**	**Ti-MP-PL-Ker3%**
O	45.1	52.0	32.7	17.2	19.1
C	32.7	18.6	48.4	67.2	63.6
N	1.2	1.0	7.1	13.0	11.5
Ti	18.8	20.0	8.4	0.0	0.0
S			0.6	2.0	3.2
Al		1.8			
others	2.2	6.6	2.8	0.6	2.6

The analysis of the Ti substrates (Ti-MP and Ti-MP-PL) revealed a reduction in the carbon content, attributable to contaminations, on the plasma treated ones with respect to on the polished samples, confirming the effectiveness of plasma in surface cleaning. On the other hand, the appearance of a not negligible Al amount, on the Ti-MP-PL sample, can be noticed and associated with the transfer of Al from the Al foil (used as sample holder during the treatment) to the sample during the plasma treatment. Moreover, a negligible amount of contaminants (such as Na, F, Zn, Si and Ca) have been detected on the various samples and have been grouped in the “others” line in Table 1. This contamination is completely removed after coating/functionalization.

After functionalization and coating (Ti-MP-ker1%, Ti-MP-PL-ker1% and Ti-MP-PL-ker3%), a significant increase in the surface carbon and nitrogen content (much higher than the one attributable to surface contaminants) can be detected together with the appearance of sulphur. These elements are present in the keratin molecule and can be correlated with the effectiveness of coating/functionalization. Higher amounts of carbon, nitrogen and sulphur can be observed on the samples functionalized after the plasma pre-treatment (compared to the untreated one), suggesting an increase in keratin grafting ability on plasma treated titanium.

On the other hand, titanium content is strongly reduced (because of significant surface coverage) after surface coating or functionalization with keratin according to the successful outcome of the processes and it becomes absent (because of complete surface coverage) if a plasma pre-treatment is applied before functionalization (Ti-MP-PL- ker1%) or, as expected, a thick coating is present (Ti-MP-PL- ker3%).

As expected, the oxygen content decreases on all the coated/functionalized surfaces with respect to the substrate because it is relatively higher in the titanium native oxide layer than in the keratin molecules even if the amino-acids of keratin contain oxygen. Considering this, a certain amount of oxygen is expected also on the keratin coated/functionalized samples; according to the registered amount of Ti, it can be supposed that the detected oxygen is due only to the keratin molecules on Ti-MP-PL-Ker1% and Ti-MP-PL-Ker3% while a contribution from the titanium oxide derives on Ti-MP-ker1% revealing a thinner layer of keratin.

The ability of XPS to detect the keratin presence even after functionalization (Ti-MP-ker1% and Ti-MP-PL-ker1%), compared to EDS, can be explained considering that XPS sampling depth is comparable with the thickness of the functionalized layer. At the same time, the fact that on Ti-MP-PL- ker1% and Ti-MP-PL-ker3% samples the signals of the elements of the substrate are not visible indicates that in those cases the thickness of the layer is higher than the sampling depth of the technique. According to the inelastic electron mean free paths calculated from the TPP-2M formula [27], electrons emitted from Ti 2p levels could travel through organic materials for distances up to approx. 10 nm (sampling depth, usually calculated as three times the inelastic mean free path). Therefore, on both cases where the plasma pre-treatment was applied, we could set a lower limit to the keratin layer thickness at 10 nm. This value is in accordance with the dimension of keratin molecules and aggregates, which are reported (for wool keratin) up to 80 nm [28].

High-resolution XPS spectra of the oxygen and carbon regions are reported in figure 3.

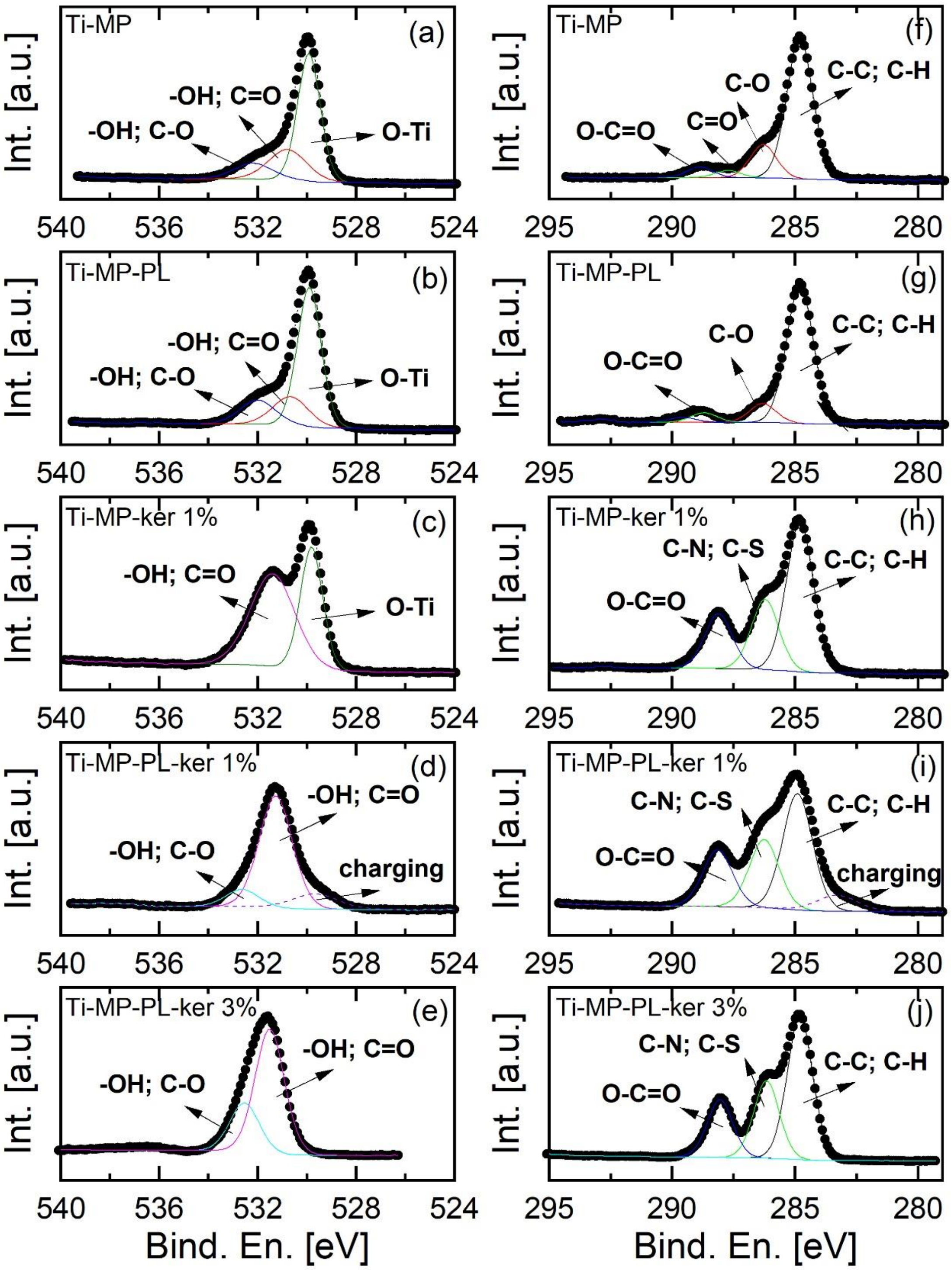


Figure 3: High-resolution XPS spectra of the oxygen (panels a-e) and carbon (panels f-j) regions.

The O 1s spectrum of the polished substrate surface (Ti-MP, Figure 3a) has a predominant peak at about 530 eV, attributable to the Ti-O bonds of the native titanium oxide layer [29] together with small signals at about 531 and 532 eV, which can be associated with basic and acidic OH groups as well as to C=O and C-O bonds respectively [29]. After plasma treatment (Ti-MP-PL, Figure 3b), the situation is similar, but a certain increase in the signal related to basic OH groups (532 eV) can be noticed.

The polished titanium surfaces functionalized with keratin (Ti-MP-ker1%, Figure 3c) still show a signal attributable to Ti-O bonds, however the peak related to acidic OH/C=O significantly increases and can be associated with the presence of keratin and of the COOH acidic group. When surface functionalization with keratin is performed on plasma treated titanium (Ti-MP-PL-ker1%, Figure 3d), the peak related to acidic OH/C=O becomes predominant, suggesting a conspicuous keratin grafting. For this sample, the observed signal at approx. 530 eV is not attributed to Ti-O bonds, given the fact that the intensity of the Ti signal in the survey analysis (Table 1) is reduced to zero. As visible also on all the other spectra collected on this sample (see C 1s in fig. 3i, as well as N 1s and S 2p spectra in figure 4), a low binding energy peak is always present and it is most likely due to a non-homogeneous/not fully effective charge neutralization. This could indicate either a high roughness of the keratin layer or the presence of patches with different thicknesses, thus giving rise to different charging phenomena in the investigated area.

Finally, when the keratin coatings are prepared on the plasma pre-treated surfaces (Ti-MP-PL-ker3%), the Ti-O peak completely disappears (in accordance with the absence of Ti signal in the survey analysis) and only OH/C=O and OH/C-O contribution can be observed, suggesting a homogeneous surface coverage (as indicated by visual inspection, Figure 1, first row).

A remarkable signal (around 532 eV), attributable to OH basic groups, is observable on all the surfaces pre-treated with plasma and it is interesting to note that this functional group is preserved after the functionalization or coating procedures, with a contribution of amino acids that have OH group side chains like serine, threonine and tyrosine with a concentration of about 10, 6 and 3 % mol, respectively, as observed in wool keratin films by means of HPLC in [4].

Concerning the carbon signals (panels f-j of Figure 3), the spectrum of the polished titanium (Ti-MP, Figure 3f) shows a prevalent contribution at 284.8 eV, which is related to C-C and C-H bonds attributable to unavoidable hydrocarbon contaminants from the atmosphere, always reported for reactive titanium surfaces [30, 31]. The other signals can be also related to carbon contaminants due to the high affinity of titanium for carbon compounds. After plasma cleaning (Ti-MP-PL, Figure 3g), the main shape of the spectrum is not changed, as expected because no new functional group is added to the surface. On the other hand, a certain reduction in the signals (reduction of the C-O contribution and reduction of the signal amplitude) can be noticed, in accordance with the decrease in the surface carbon content evidenced in the survey spectra (Table 1). After keratin grafting or coating (Ti-MP-ker1%, Ti-MP-PL-ker1% and Ti-MP-PL-ker3%, Figure 3h, i, j), three contributions at about 284.8, 286 and 288 eV, attributable respectively to C-C/C-H, C-O/C-N/C-S, and O-C=O bonds, become clearly defined and can be associated with the presence of keratin [32], in accordance with the above discussed oxygen spectra.

The high-resolution spectra of nitrogen and sulphur are shown in Figure 4. These signals are significant only on the keratin functionalized and coated surfaces and consequently have been reported only for those.

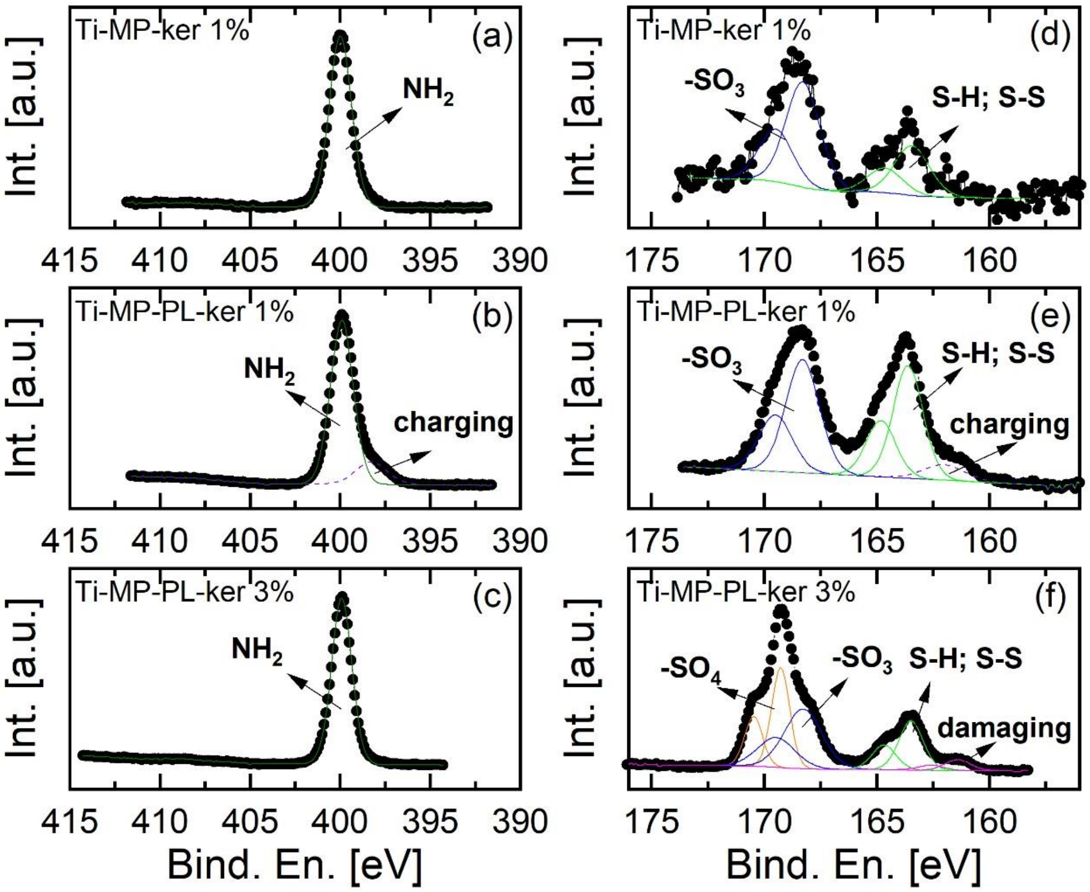


Figure 4: High-resolution XPS spectra of nitrogen and sulphur for keratin functionalized/coated samples.

In the nitrogen region, a single contribution at about 399.9 eV can be observed and attributed to NH_2 groups in the keratin molecule. As in the case of the oxygen and carbon spectra, the N 1s signal of the Ti-MP-PL- ker1% sample shows an additional component at the low binding energy side of the main peak, due to charging. In the sulphur region, due to spin-orbit coupling, each chemical state is represented by a doublet of peaks, having the same width, an area ratio of 2:1 and an energy separation of 1.2 eV. All the samples show the presence of a doublet with main component at about 163.5 eV (green peaks) ascribable to disulfide bridges or thiol moieties of the keratin molecules. [33] Also, a doublet with main component at 168.3 eV (blue peaks) is present for all the investigated samples. This S doublet could be assigned to the formation of sulphonates in the keratin extraction process [34]. For the Ti-MP-PL- ker1% sample, also the S 2p spectrum shows the charging effect already discussed for the other elements. In the case of the Ti-MP-PL- ker3% sample, the disulphides and the sulphonates doublets are accompanied by other two doublets. One is at higher binding energies (orange peaks, main component at 169.3 eV) and could be assigned to the presence of sulfates, probably related to sodium dodecyl sulfate and side product of metabisulfite in keratin extraction; the other is at low binding energies (magenta peaks, main component at 161.4 eV). In this case, we did not assign this signal to charging, since there are no similar effects on the spectra collected on oxygen, carbon and nitrogen. Most likely, this signal could be related to the effect of X-rays on the keratin molecules, inducing C–S bond breaking and resulting in the presence of atomic Sulphur at the surface of the sample [35].

3.4 Wettability measurements

Two types of wettability measurements were performed: one with a drop of keratin solution in order to test its chemical affinity for the substrates and one with a drop of water in order to test the hydrophilic/hydrophobic behavior of the coated surfaces. The wettability of the coated/functionalized samples is of interest because it can affect the surface interaction with water based media and consequently the biological performance. In fact, biological fluids are water based and the first interaction of a surface with the biological environment is based on water and proteins absorption [36].

The contact angle measurements made with a drop of keratin solution on the polished and plasma pre-treated titanium surfaces are reported in Figure 5a. It can be observed that the plasma pre-treatment reduces the contact angle (66%) of the keratin solution on the titanium surfaces, thus evidencing a higher chemical affinity of the pre-treated surfaces. This phenomenon can be associated with the reduction of carbon contamination and with the exposition of the basic OH groups due to the plasma cleaning, as discussed in 3.3 Section (XPS results).

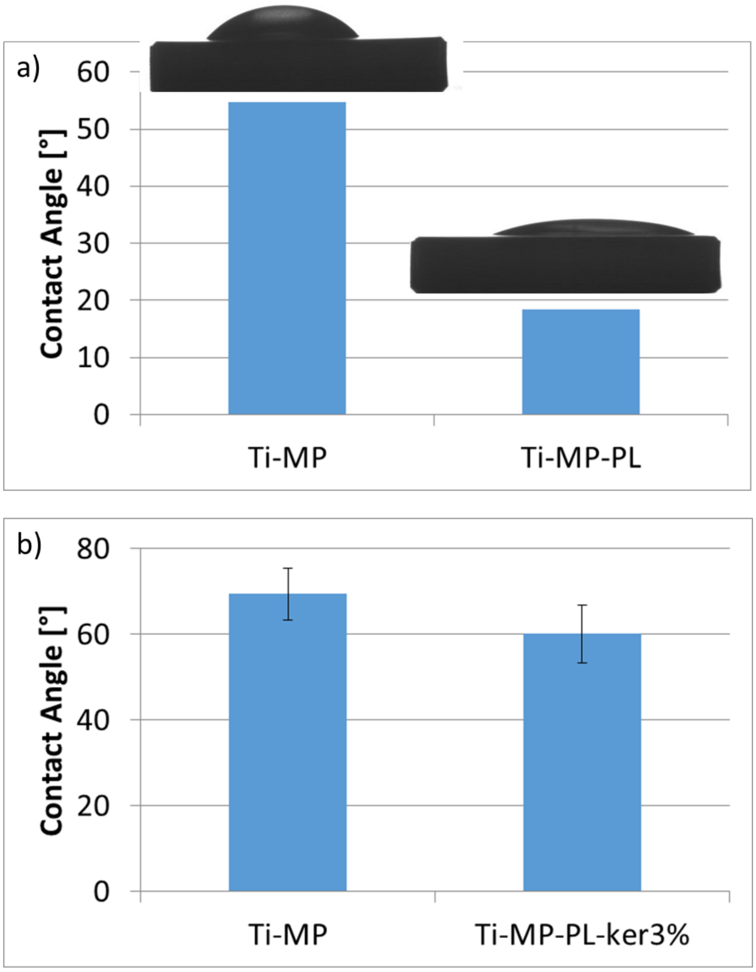


Figure 5: Wettability of titanium surfaces with respect to keratin solutions (a) and wettability of bare and keratin coated surfaces with respect to water (b).

Figure 5b shows the contact angle measurements made with a drop of ultrapure water on the bare (Ti-MP) and keratin coated (Ti-MP-PL-ker3%) titanium surfaces. Ti-MP-PL-ker3% was considered for these measurements as the best coating in term of adhesion and uniformity. A 13% reduction of the contact angle can be evidenced after the coating and can be associated with the hydrophilic properties of keratin reported in the literature [37]. An opposite behavior was observed by the authors when keratin was deposited onto titanium substrates in the form of sub-micrometric fibers, in this case the peculiar topography associated to the fibers induces a decrease in surface wettability (compared to the uncoated samples) as reported in reference [7]. A strict relationship between surface topography and wettability has been widely reported in the literature, especially for submicrometric textures [14, 15].

3.5 XRD measurements

The XRD measurements on the coatings are reported in Figure 6.

In the XRD spectrum of a free standing keratin film (obtained by using same aqueous keratin solution used for coating and functionalization) two peaks at 9 and 19° can be observed and attributed to keratin β-sheets, as reported in the literature for keratin regenerated from wool [38]. The same signal can be noticed also on the keratin coated titanium substrates, but the intensity is lower due to the important diffraction of the substrate (signals at 35, 38, 40, 52, 62, 70 and 77° can be associated with the titanium substrate, reference pattern 01-089-2762). This phenomenon can be associated with XRD penetration depth, which is close to the coating thickness (micron range). No measurements were performed on the functionalized samples because the modified layer is too thin (nanometer range, as previously discussed) to be analyzed by this technique.

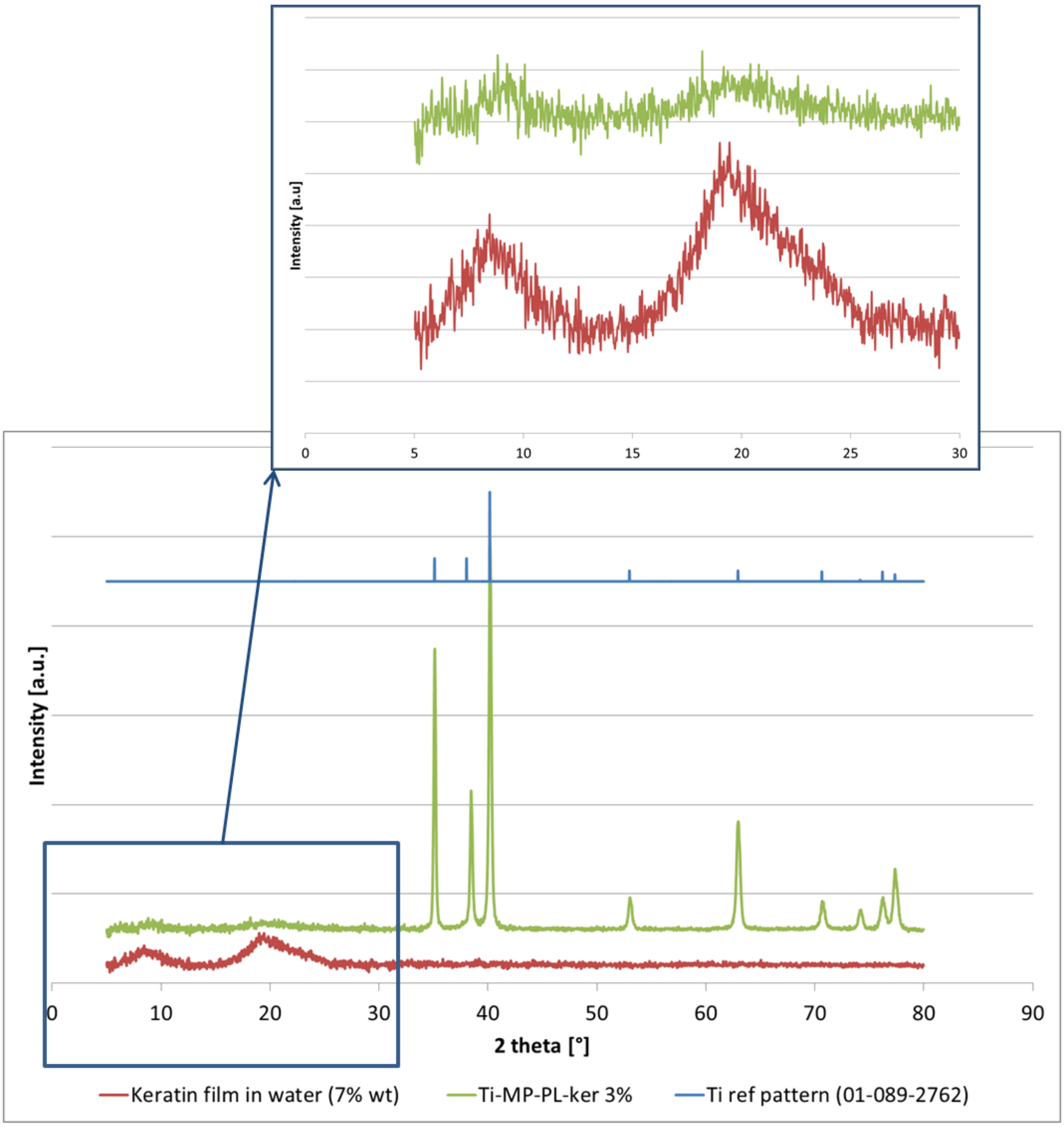


Figure 6: XRD measurements

3.6 Coating thickness estimation

The thickness of the keratin coating has been estimated by means of contact profiler measurements (Figure 7a) and confocal microscopy (Figure 7b). Contact profiler gave a thickness of 11 µm, while confocal microscopy a thickness of 6 µm. Despite of the different experimental techniques, both the measurements gave results of the same order of magnitude. The variability can be associated with the manual procedure used for the preparation of the coatings in the present work. But the data can be considered consistent and both techniques suitable for coating thickness estimation. The results evidence that the prepared keratin coatings are some microns thick and they can be properly considered as belonging to the thick coatings category.

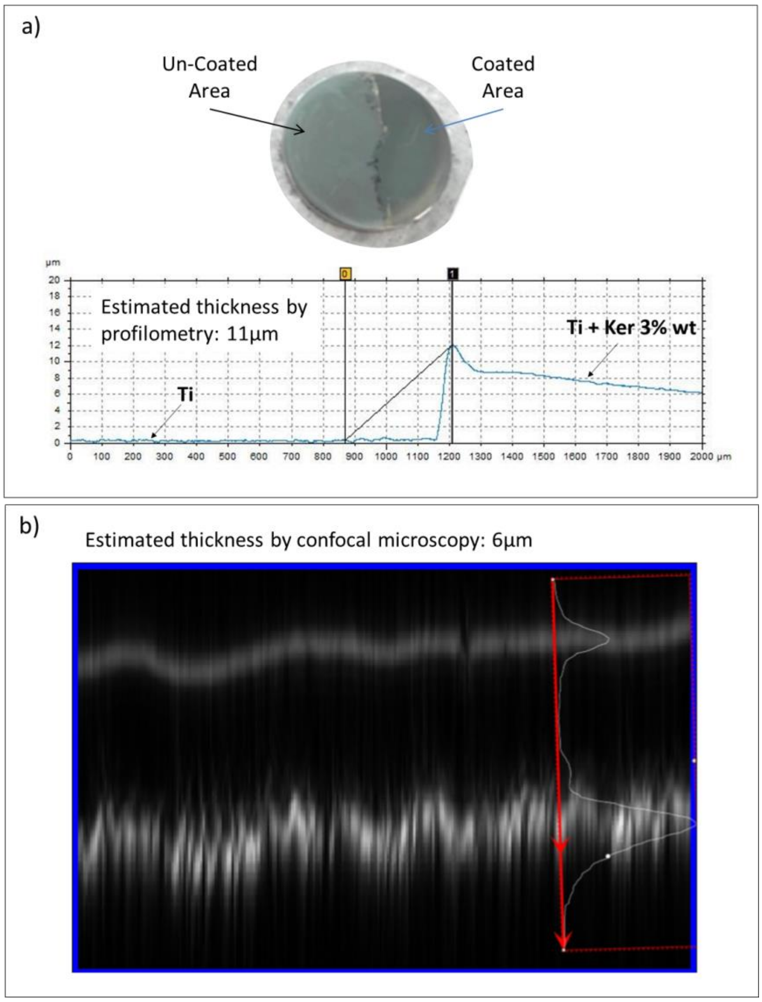


Figure 7: Coating thickness evaluation: (a) Contact profiler, (b) Confocal microscopy.

3.7 Zeta potential measurements

Zeta potential measurements can give information about the surface charge developed by a material upon contact with an aqueous electrolyte. From the titration curves, the value of the isoelecteric point, surface charge at a specific pH (such as the physiological one) and presence of acid/basic functional groups can be determined. Moreover, information on chemical/mechanical stability of a surface in contact with a flow of an aqueous medium at different pHs can be deduced. In the present research, this information can evidence the eventual surface modifications occurring after plasma treatment and keratin coating. Moreover, information about the surface charge can be useful in case of any further surface modification and to explain the biological response of the tested surfaces (surface charge can affect both cellular and bacterial adhesion).

The zeta potential titration curves of the titanium substrates (Ti-MP and Ti-MP-PL) and of the keratin coating (Ti-MP-PL-ker3%, selected as the best thick coating for homogeneity and adhesion) are reported in Figure 8.

The isoelectric point (IEP) of titanium is 4, according to the data reported in the literature [39, 40, 41]. After the plasma pre-treatment, the IEP is shifted through more basic values (at about 5.6), according to the above discussed exposition of basic OH (see section 3.3). After the keratin coating, the isoelectric point is shifted again to more acidic values (close to 3.4), according to the previously published measurements reported by the authors on keratin fibers and films [7]. Moreover, the appearance of a plateau in the basic region can be observed for the keratin coated samples, it can be attributed to the presence of functional groups with acidic behavior (COOH in the keratin molecule) [7, 24]. Standard deviation of zeta potential measurements on the keratin-coated samples is very limited (except at pH 3.7). The standard deviation of the zeta potential in the titration curves has been previously correlated by the authors with the chemical and mechanical stability of the surfaces and coatings at the different pHs [42]. A small (few mVs) standard deviation in a wide pH range can be considered an index of surface stability. This point is crucial for the applicability of the keratin coating here proposed; in fact one of the main issues that actually hamper clinical application of polymer coatings on titanium is their chemical and mechanical stability [10].

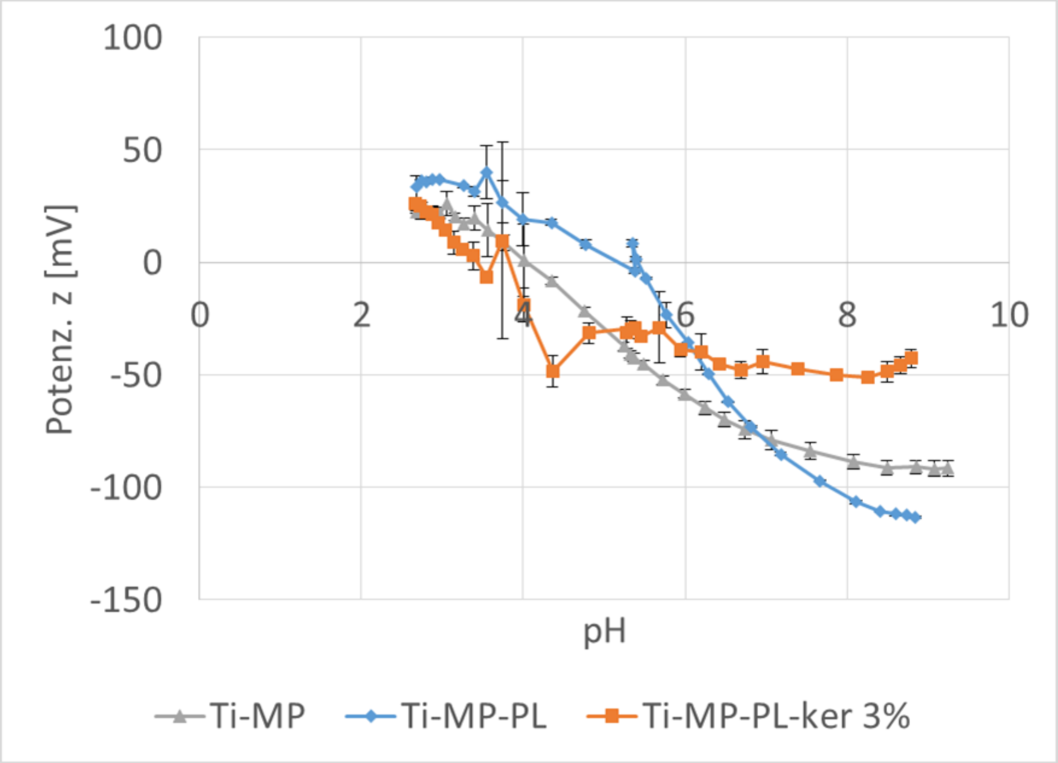


Figure 8: Zeta potential titration curves

3.8 Tape adhesion test

Tape adhesion test is aimed at the estimation of the adhesion of thick coatings to the substrate, for this reason it has not been performed on the functionalized samples.

The results of the tape test are reported in Figure 1 (second and third rows). The adhesion of the keratin coating prepared from a solution of keratin 7%wt on Ti-MP (Ti-MP-ker7%) is poor, more than the 50% of the coating is removed during the test and it can be observed on the tape. A certain improvement on the adhesion can be obtained by the plasma pre-treatment (Ti-MP-PL-ker7%), however the inhomogeneity of the coating induces in any case a non-optimal adhesion and lead to the detachment of a part of the coating during the test. The keratin coating prepared from a solution of keratin 3%wt (Ti-MP-ker3%) presents a better adhesion even on the Ti-MP substrates, but a certain detachment can be observed, particularly near to the cuts. An optimal coating adhesion (0% detachment) can be observed for the keratin coating prepared from a keratin solution 3%wt on a plasma pre-treated titanium substrate (Ti-MP-PL-ker3%). The improvement of keratin adhesion after plasma pre-treatment can be associated with the previously discussed surface cleaning (reduction of carbon contaminations), OH exposition and increase of wettability by the keratin solution induced by the plasma pre-treatment. This is an important result, in fact, adhesion and stability of keratin are ones of the factors limiting the clinical application of it as an implant coating [10], as discussed in the introduction. A good adhesion to the substrate can guarantee a coated devise to be implanted without coating detachment, which is a crucial point for applicability in transmucosal or percutaneous implants.

3.9 Scratch test

Scratch test is aimed at the evaluation of adhesion and mechanical stability of thick coatings, for this reason it has not been performed on the functionalized samples.

The scratch test results are reported in Figure 9. The substrate and the coating prepared from a solution of keratin 3% wt (Ti-MP and Ti-MP-PL-ker3%) were selected for the test according to the previous results.

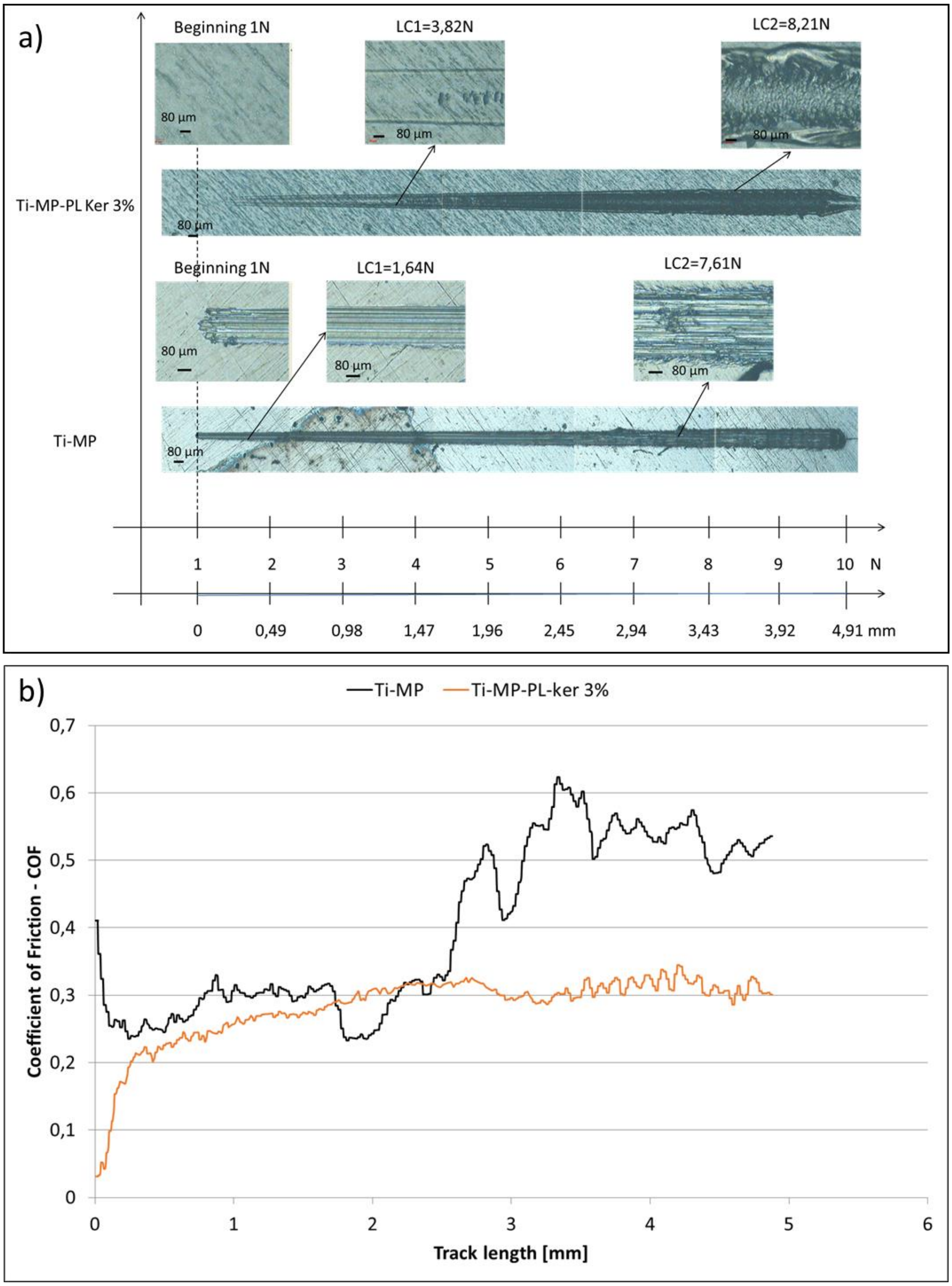


Figure 9: Scratch test – a) optical observations of the scratch tracks, b) coefficient of friction estimation in the scratch test

The scratch induces plastic deformation and surface damage on the uncoated titanium substrate (Ti-MP), as previously observed by the authors on steel substrates [43]. On the other hand, despite of unavoidable plastic deformation of the substrate, no surface damage can be observed on the keratin coated titanium

(Ti-MP-PL-3%ker). Even if the keratin coating is spread by the scratch tip, it remains on the substrate and seems to protect it from the damage. As discussed for the tape tests, this is a confirmation of good coating adhesion, which is a fundamental characteristic for a safe application in transmucosal and percutaneous implants which have to sustain friction during the implantation surgical procedure. Moreover, it can be observed that the coefficient of friction of Ti-MP-PL-ker 3%coat is lower than the one of the uncoated titanium substrate (Ti-MP) and that this behavior is maintained up to the end of the scratch track. This result confirms the permanence of the keratin coating for the whole scratch track (up to 10N) and its protection action for the metallic substrate. Similar results were obtained by the authors with peppermint essential oil coatings [43] and can be correlated with a certain lubrication action of these organic coatings. Moreover, the lower friction coefficient for the sample coated with Keratin can be due to the lower generation of micro cracks around the scratch channel, as can be noticed from the magnification reported (beginning and Lc1). This result is extremely interesting and is worth of further investigations.

Conclusion

Improved interaction of the soft tissues with titanium is a topic of interest for the percutaneous and transmucosal implants, however, it is still poorly explored. Keratin is a promising molecule for stimulation of adhesion and growth of fibroblasts, as reported in the literature and in previous works from the authors. Moreover, keratin is abundant in the byproducts of the textile industry and obtainable through a sustainable use of the resources. The main problems for its application are poor adhesion and stability when coupled to titanium. Looking at this context, the aim of this research is to exploit keratin extracted from discarded wool to improve the surface properties of commercially pure titanium intended for applications in contact with soft tissues. Wool keratin has been successfully coupled to commercially pure titanium (grade 2) both as a molecular layer (functionalization) and as a coating (6-11 µm thick). The presence of keratin was verified by means of SEM-EDS, XPS, XRD and zeta potential measurements. The tape test, the scratch test and the zeta potential titration curves evidence good adhesion and stability (mechanical and chemical) for keratin thick coatings. An oxygen plasma pre-treatment of the titanium substrate significantly enhances keratin grafting ability both as grafted molecules and as a coating and increases adhesion in the case of a coating.

Considering that keratin can effectively stimulate fibroblast adhesion and growth (as previously reported by the authors) and that the here reported coatings and functionalization are mechanically and chemically stable, titanium surfaces functionalized or coated with wool derived keratin are extremely promising for the improvement of soft tissue integration of transmucosal and percutaneous titanium implants, overcoming the actual main issues in keratin application.

The here proposed approaches can be explored in the future respectively to combine some topographical stimuli for fibroblasts of a titanium substrate, such as grooves with contact guide effect, with the chemical ones of the keratin molecules (because functionalization does not alter the surface morphology of the substrate) or to create a smooth and homogeneous coatings suitable for fibroblast adhesion and limitation of bacterial contamination.

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