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Abstract

Silicon-based materials present a high potential for dental implant applications, since silicon has been proven necessary for the correct bone formation in animals and humans. Moreover, the addition of silicon is effective to increase the bioactivity of hydroxyapatite and other biomaterials, and silicon-based thin films are proposed for the surface modification of titanium implants. In this work, the interaction of hydrogenated amorphous silicon (a-Si:H) coatings with human keratinocytes and fibroblasts is investigated and compared with the behavior of titanium surfaces. Films with different levels of hydrogen incorporation were produced by plasma enhanced chemical vapor deposition (PECVD) on titanium substrates and their surface composition and hydrogen content were analyzed by means of X-ray photoelectron spectroscopy (XPS) and Fourier-transform infrared spectrometry (FTIR) respectively. The surface energy and roughness were measured through optical contact angle analysis (OCA) and high-resolution mechanical profilometry respectively. Coated surfaces showed a slightly lower roughness, compared to bare titanium samples, regardless of the hydrogen content. Films with lower hydrogen content showed a surface energy comparable to the titanium surfaces. However, films with higher hydrogen incorporation showed a lower surface oxidation and a considerably lower surface energy, compared to the less hydrogenated samples. Moreover, hydrogen-rich films increased manifolds the adhesion of keratinocytes, but not of fibroblasts, suggesting a selective biological effect.

Keywords

Hydrogenated amorphous silicon, keratinocytes, fibroblasts, surface energy, XPS, FTIR, roughness.

1 Introduction

Dental implants represent a fundamental option to treat edentulism, whether it be partial or complete. Since Brånemark's first studies, research focused on the interaction between bone and titanium fixtures [1]. However, to be clinically used, implants must be connected to an intra-oral prosthesis, which implies the presence of a transmucosal component interfacing also with fibroblasts and epithelial cells [2]. Unwanted, yet frequent, clinical responses such as gingival recession and the so-called peri-implantitis have recently highlighted the importance of the soft tissue around the implant as a possible barrier to bacterial penetration along the fixture [3,4].

Although the extra-osseous part of an implant may be designed to reduce plaque accumulation, several limitations remain as for the soft tissue surrounding the implant. Indeed, the connective tissue and epithelium attach to the cemental root surface of a natural tooth [5], but act differently around implants [6,7]. To this end, numerous surface modifications have been recently proposed [8–11].

Interestingly, controversial data are available as regards the use of roughened surfaces [12–16] in enhancing soft tissue healing. Additive surface modifications are thus increasingly studied as possible means to drive selective adhesion of the two main cell types within the gums: epithelial cells and fibroblasts. Among the most promising options, silicon-based coatings, such as amorphous silicon (a-Si), as well as silicon-oxygen, silicon-nitrogen and silicon carbon amorphous thin film alloys (a-SiO_x, a-SiN_x, a-SiC_x), play an important role. These materials are similar to bioglass pertaining to hardness, mechanical resistance and optical properties, i.e. transparency, and may display antibacterial properties and peculiar features as cell interface [17–20]. These thin-film coatings have attracted a strong interest for medical applications, especially owing to their silicon content, the role of which has been evidenced in the interaction between inorganic surfaces and living tissues since the work of Carlisle in 1970 [21]. The presence of silicon traces in the diet is necessary to achieve a normal growth in chickens [22] and to stimulate cartilage synthesis in ovariectomized

rats [23]. Moreover, the dietary intake of Si is proposed to be correlated with bone mineral density in humans [24]. Unsurprisingly, titanium oxide nanostructures have also shown an improvement of their biological interaction after their surface was implanted by Si ions [25], while the use of nitrogen-incorporated a-SiO_x coatings on metal implants accelerates the bone healing process, thanks to their ability to release Si^{4+} ions [26].

While the role of silicon in the enhancement of the interaction with biological environment has been already demonstrated in the literature, a synergistic role of silicon and hydrogen has been suggested, possibly owing to the ability of hydrogen to tune the hydrophilic/hydrophobic properties of silicon-rich surfaces [27]. Indeed, the hydrogenation of crystalline Si samples improves their interaction with osteoblasts [28]. Hydrogenated amorphous silicon (a-Si:H) layers enchance osseointegration, evidencing their ability to promote apatite formation in simulated body fluid [29]. Despite these interesting results, the effective role of hydrogen in the biological interaction of silicon-base surfaces is still not completely clear, due to the limited number of works on the subject. In addition, most studies concentrate on the osseointegration process, while results regarding the interaction of silicon-based surfaces with soft tissues, such as the gums, are lacking. The present work aims to expand the knowledge of the role exerted by hydrogen in the biological interaction of silicon-based materials, comparing two different hydrogenated amorphous silicon coatings, with different hydrogen content, as means to enhance soft tissue cell adhesion. To accomplish this task, the early cell response of human keratinocytes and fibroblasts was tested on the above mentioned surface modifications.

2 Material and methods

2.1 Sample preparation

Ti-Al-V samples were machined to obtain 8 mm × 3 mm cylinders (2r × h) (Titanmed s.r.l., Milan, Italy). Crystalline silicon samples of approximately rectangular shape and dimensions of about 10 mm × 20 mm, cut from p-type (100) wafers of about 50 Ohm cm resistivity, were also prepared. All the samples were cleaned in an acetone ultrasonic bath for 10 min, immersed in isopropanol for 10 min, then rinsed in deionized water and finally dried by blowing them with dry nitrogen gas. Films grown on silicon were used to allow the FT-IR analysis in transmission mode, since the metallic Ti-Al-V alloy absorbs the IR radiation, while the high-resistivity crystalline Si is IR-transparent. In the following, the prefix 'Ti' in the sample nomenclature refers to films grown on Ti-Al-V substrates, while the prefix 'Si' identifies the films grown on silicon substrates. Moreover, the terms "titanium" or "machined titanium" are used in the following, for simplicity, to refer to the uncoated Ti-Al-V alloy samples.

Two different amorphous silicon (a-Si) coatings (named a-Si_90 and a-Si_350), showing different amount of hydrogen incorporation, were grown by *radio frequency - plasma enhanced chemical vapor deposition* (RF-PECVD) technique using silane (SiH_4) as the Si precursor on both titanium and Si substrates. The hydrogen content was tuned by modifying the substrate temperature, exploiting the enhanced diffusion of H atoms in the films at high temperature [30,31]. The growth reactor consisted of a capacitively coupled PECVD system, composed of two parallel electrodes of 12 cm x 12 cm area each, located at a relative distance of 20 mm. The substrates were coated on the whole surface in order to perform the biological tests. The precursor gas was injected inside the reactor through a gas manifold, using a *mass flow controller* (MFC) to control the flow rate. The reaction byproducts and exhausted gases were removed through a pumping system, consisting of a turbo-molecular pump connected to a downstream mechanical pump. The plasma discharge was ignited by means of a RF generator (RFPP–RF5S), working at a fixed frequency of 13.56 MHz, and an impedance matching

network (Huttinger - FR 1500A) was used to tune the load impedance. All the process parameters, except the substrate temperature, were kept fixed for all the deposition runs and are summarized in Table 1. The heater temperature was measured by a KF thermocouple fixed on the heating unit and the substrate temperature, which was calculated by means of a calibration curve, was varied between 90 °C and 350 °C. The deposition time was fixed at 30 min, resulting in an approximate film thickness of 300 nm for all the films.

Sample Name	$T_{Substrate}$	Gas Flow Rate	Gas Pressure	RF Power Density
	°C	sccm	mTorr	W / m^2
a-Si_90 °C	90	30	450	208
a-Si_350 °C (CVD669)	350	30	450	208

Table 1. *Set of the deposition parameters used to grow the a-Si coatings by PECVD.*

A representative number of samples underwent physical and chemical characterization before the biological experiments. Pristine titanium samples were also kept as unmodified controls during further experiments

2.2 X-ray Photoelectron Spectroscopy

X-ray Photoelectron Spectroscopy (XPS) was carried out by using a PHI 5000 VersaProbe (Physical Electronics) system. The usual surface pre-cleaning of the samples by means of Ar^+ ion bombardment was not intentionally performed so as not to alter the surface chemical composition that was present at the moment of the biological assays. The X-ray source was a monochromatic Al Kα radiation. The binding energy scale was calibrated by assigning a binding energy value of 284.8 eV to the main C1s contribution.

XPS signals were collected using a SPECS (Phoibos MCD 150) X-ray photoelectron spectrometer, with Mg Kα radiation (1253.6 eV) as X-ray source, having a power of 150 W (12 mA, 12.5 kV). The

spot size of the analyzed region was 7 mm x 20 mm. The emission of photoelectrons from the sample was analyzed at a take-off angle of 90° under UHV conditions. No charge compensation was applied during the acquisition. After collection, the binding energies were calibrated using as reference the adventitious carbon C1s peak. The accuracy of the reported binding energies (BEs) can be estimated to be ± 0.1 eV. The XPS peak areas were determined after subtraction of a background. The atomic ratio calculations were performed after normalization using the Scofield factor of each element. All spectrum processing was carried out using the Casa XPS v2.3.13 software (Casa Software Ltd.) package and Origin 7.1 (Origin Laboratory Corp.). The spectral decomposition was performed by using Gaussian-Lorentzian (70%/30%) functions, and the FWHM was fixed for each given peak.

2.3 Roughness and thickness

A stylus profiler (Tencor P-10 from KLA-Tencor, Milipitas, California) was used to measure the surface roughness and the coating thickness. For roughness determination, performed on titanium coated and uncoated samples, seven measurements of 3 mm length were conducted for each of the samples according to the R_a and R_q amplitude parameters. R_a is defined as the arithmetic average of the absolute differences between the measured heights and their mean value [30]. R_q (formerly called root-mean-square or RMS) is the square root of the arithmetic average of the squared differences between the measured heights and their mean value. The R_q parameter, which is equivalent to the empirical standard deviation of the height distribution, is more sensitive to occasional heights and lows, making it a valuable complement to R_a.[30] The R_a and R_q values were calculated from the signal after the application of a highpass gaussian filter, with a cutoff length of 250 ìm, to remove the waviness component of the signal [31].

The coating thickness was evaluated on silicon substrates masking a selected area of the substrate before deposition and then measuring the height of the step between the uncoated and coated areas.

2.4 FTIR Spectrophotometry

Films grown on silicon substrates were used for transmission FT-IR spectrophotometry characterisation. Measurements were carried out by means of a Perkin Elmer Spectrum One spectrophotometer, by performing 32 scans for each sample.

2.5 Contact angle and surface energy evaluation

The wetting properties were investigated on titanium uncoated and coated samples by means of optical contact angle (OCA) measurements with the sessile drop technique, using an OCAH 200 (DataPhysic Instruments GmbH). Two different liquid probes were used: water (dH_2O) and diiodomethane (CH_2I_2). Each liquid drop (1 µl in volume) was dispensed and the image of the drop on the sample was acquired with the integrated high-resolution camera. The drop profiles were extracted and fitted with a dedicated software (SCA20) through the Young–Laplace method and, indeed, contact angles, at the liquid–solid interface, between fitted function and base line were calculated. For each sample and each liquid probe, the contact angle measurement was repeated five times on different areas. The surface energy, divided in the polar and dispersive components, was then estimated by applying the Owens Wendt method [32], starting from the average contact angle estimated for each of the two different liquid probes.

2.6 Cell culture

To characterize the biological response *in vitro*, human keratinocytes (HaCaT) and human dermal fibroblasts (NHDF) (ECACC, Salisbury, UK) were used. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Milan, Italy), 100 U/ml penicillin, 100 µg/ml streptomycin, under a humidified atmosphere of 5% CO_2 in air, at 37°C. Cells were always passaged at subconfluence to prevent contact inhibition.

2.6.1 Cell adhesion

Cell adhesion was evaluated on titanium uncoated and coated samples using a 48-well plate (BD, Milan Italy). Cells were detached using trypsin for 3 minutes, carefully counted and seeded at 3000 cells/well on the disks with different roughness. The 48-well plates were kept at 37 °C, 0.5 % CO_2. Before and after fixation in 4% paraformaldehyde in PBS for 15 min at room temperature, cells were washed two times with PBS and then stained with 1μM DAPI (Molecular Probes, Eugene, California, USA) for 15' at 37°C to stain cell nuclei. Images were acquired using a Nikon Eclipse T-E microscope with a Nikon Plan 10X [33]. Cell nuclei were counted using the 'Analyze particles' tool of ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).

2.6.2 Cell viability

Cells were plated at a density of 2000 cells/well in 48-well culture dishes and the viability was assessed by Cell Titer GLO (Promega, Milan, Italy) according to the manufacturer's protocol at 48h [34].

2.6.3 Cell morphology and focal adhesion quantification

Cells were seeded at a concentration of 2000 cells/well in a 48-well plate. After 24 h, cells were fixed in 4% paraformaldehyde in Phosphate Buffer Saline (PBS) and stained with Rhodamine-Phalloidin and DAPI (Life Technologies, Milan, Italy) to stain actin network and nuclei, respectively. Focal adhesions were specifically detected by an anti-Paxillin N-Term 04-581 antibody from Millipore (Merk, Darmstadt, Germany). Images were acquired with a Nikon Eclipse Ti-E microscope using a Nikon Plan Fluor 20X (Nikon Instruments, Amsterdam, Netherlands) [35,36]. Cell spreading and focal adhesion density were quantified with ImageJ software.

2.7 Statistical analysis

Data were analyzed by GraphPad Prism6 (GraphPad Software, Inc., La Jolla, CA, USA). Each experiment was repeated at least three times. Statistical analysis was performed by using the Student t-test and Wilcoxon Mann Whitney test. A p-value lower than 0.05 was considered significant

3 Results

3.1 Chemical composition analysis

3.1.1 *X-ray photoelectron spectroscopy (XPS)*

XPS was carried out to study the chemical composition of the surface for the amorphous silicon (a-Si) coatings. Figure 1A shows the wide-scan XPS spectra collected from the amorphous Si coatings. The emitting lines characteristic of silicon (Si), oxygen (O) and carbon (C) were detected and witness that these are the main elements composing the surface of the investigated samples. Both the a-Si coatings show a similar chemical composition at their surface, where Si is the most abundant element (around 50 at.%). The relevant presence of oxygen (around 35 at.%) is due to native silicon oxide that spontaneously forms when Si is exposed to ambient conditions. The huge amount of C (around 15 at.%) is due to contaminants adsorbed onto the sample's surface. These were not removed since pre-cleaning of the investigated samples was not performed, as mentioned in the experimental section. Figure 1B, C shows the high-resolution (HR) Si2p spectra collected from the a-Si coatings. In both cases the raw data can be fitted by different peaks, each one corresponding to a proper chemical phase involving the Si element. The two peaks positioned at 99.3 eV and 99.9 eV are due to the closely spaced Si2p3/2 and Si2p1/2 spin-orbit components. The other contributions placed at 100.2 eV and 103.1 eV are ascribed to the native silicon oxide [37]. By considering the area below each deconvolution peak it is visible that only a small fraction is effectively involved in the formation of the native oxide, while most of Si participates to the formation of amorphous Si—Si compounds.

[Figure1]

3.1.2 FTIR analysis

The FTIR analysis was performed in the range 500–3800 cm^{-1}, mainly to investigate on the amount of hydrogen incorporated in the a-Si:H films grown at different temperatures. The results of this analysis are shown in figure 2 as transmittance spectra, where the presence of a stronger absorption is evidenced as a reduction of the intensity of radiation transmitted by the sample. Indeed, the IR

absorption spectra shown on figure 2 reveal a considerable difference between the H content of a-Si:H films grown at 90 °C and 350 °C. In fact, films grown at the higher temperature show only two main absorption peaks: one at about 630 cm^{-1}, which is usually attributed to rocking vibrations of Si-H bonds [38], and another one at about 2000 cm^{-1}, usually attributed to stretching vibrations of the same bonds [38]. On the other hand, films grown at 90 °C show a richer absorption spectrum, which is characterised by a much stronger peak at 630 cm^{-1} and the presence of peaks at 845, 880 and 2090 cm^{-1}, which are not visible for the samples grown at the higher temperature. These peaks are usually attributed respectively to wagging, bending-scissoring and stretching vibrations of SiH_2 and $(SiH_2)_n$ bonds [38]. The peak at 2000 cm^{-1} is not visible for the sample grown at lower temperature, possibly because it is masked by the one at 2090 cm^{-1}. The stronger intensity of the peak at 630 cm^{-1} is a signal of a higher hydrogen content in low temperature grown a-Si:H thin films, while the presence of SiH_2 and $(SiH_2)_n$ peaks accounts for additional hydrogen incorporation paths for samples grown at 90 °C respect to the ones deposited at 350 °C.

[Figure2]

3.2 Roughness analysis

Results of roughness analysis are shown in figure 3. The measured R_q values are higher than R_a ones for all samples, as is usually expected [30]. A 9% reduction of the R_q value can be observed, after the deposition of the a-Si:H coatings, regardless of their hydrogen content. The R_a (Fig. 3A) value is also reduced by 9% after the deposition of the more hydrogenated film, while a R_a reduction of 7% is observed after the deposition of the coating with low H content. The values of the R_q/R_a ratio have also been calculated for bare titanium and coated samples and are reported as insets in figure 3B. All the measured values are compatible, considering the experimental uncertainties, with the R_q/R_a value of 1.25, which is expected for a gaussian distribution of the heights [30].

[Figure 3]

Measurements were repeated on 7 different tracks for each sample and error bars represent the standard error of the mean. Calculated values of R_q/R_a ratio are also shown as insets (Fig 3B).

Wetting properties

3.3 Wetting properties

The wetting properties of the control titanium (Mac) sample and the a-Si coatings were evaluated by optical contact angle (OCA) measurements. The uncoated titanium control (Mac) shows a quite hydrophilic behaviour, with an average contact angle (CA) value of θ 35° and θ 40°, for water and diiodomethane (CH_2I_2), respectively (Fig. 4A, B). The a-Si coating was able to impart the surface two opposite behaviours, according to the difference in the growth temperature of the film.

A transition toward the hydrophobic regime was found when a-Si thin films were grown at low temperature (sample a-Si_90), with an average CA of 80° and 49° for H_2O and CH_2I_2, respectively. On the other hand, the wetting behaviour of the high temperature grown a-Si coating (sample a-Si_350) was comparable with the one observed for the controls (Mac). The average CA for water and CH_2I_2 were 49° and 43°. Figure 4 shows the water and CH_2I_2 drops spreading on the surface of samples a-Si_90 (Fig. 4C, D) and a-Si_350 (Fig. 4E, F).

[Figure4]

The surface free energy (SFE) together with its polar and dispersive components were computed starting from the CA values returned for the water and CH_2I_2 liquid probes, by applying the Owens-Wendt theory. The results are reported in Table 2.

SAMPLE	*H_2O CA (°)*	*CH_2I_2 CA (°)*	*SE (mN·m)*	*SE-Dispersive (mN·m)*	*SE-Polar (mN·m)*
Ti	34.7±6.8	39.6±1.6	61.6	26.7	34.9
a-Si_90	79.4±4.0	45.4±1.8	37.9	32.6	5.3
a-Si_350	49.1±4.3	42.3±4.4	52.6	25.0	27.6

Table 2. *Total contribution, dispersive and polar components of surface energy, calculated on diiodomethane and water contact angles measured on bare titanium (Ti) substrate and on Ti coated with amorphous silicon grown by PECVD at 90°C and 350°C substrate temperature (a-Si_90 and a-Si_350 respectively).*

3.4 Biological evaluations

3.4.1 Cell adhesion

As depicted in Figure 5, the thin film grown at lower temperature, a-Si_90, increased manifolds the adhesion of HaCaT cells when compared to the pristine Ti controls (Mac) and the a-Si_350 coating at 2 h (Fig. 5A). This pattern was still present, albeit attenuated, at 24 h (Fig. 5C). No such effects could be detected on the NHDF cells: a-Si_350 resulted the least adhesive condition at both 2 and 24 h (Fig. 5B, D).

[Figure5]

3.4.2 Cell viability

Cell viability was assessed at 48 h. The a-Si_90 coating significantly sustained better HaCaT cell viability than control samples (Mac), as shown in figure 6A. On the other hand, on a-Si_350 coating,

HaCaT cells proliferated less than control condition. Regarding NHDF cells, the viability was significantly lower than control condition for both coatings, as shown in figure 6B.

[Figure6]

3.4.3 Cell morphology and focal adhesion quantification

To further investigate the biological effects of a-Si coatings on HaCaT and NHDF cells, we studied cell morphology and cell adhesion. The cytoskeleton organization and focal adhesion distribution are shown in figure 7.

[Figure7]

A number of relevant cell shape descriptors were analyzed, namely: cell area, perimeter and polarization (Fig.8). Both HaCaT and NHDF cells seeded on a-Si coatings were smaller than control conditions (Fig. 8A, D, E). Moreover, a-Si_90 coating slightly influenced HaCaT cells, not affecting cell perimeter and polarization (Fig.8A, B, C). These data are in accordance with the highly conserved morphology of these cells, characterized by a marked roundness [39,40]. Nevertheless, it is interesting to observe that a-Si_90 coating reduced NHDF cells area and perimeter (Fig.8D, E) and induced a more rounded shape (Fig.8F).

[Figure8]

To better understand the correlation between cell morphology and the biological results on cell adhesion, focal adhesions were analyzed. As shown in figure 9A, C, no significant differences were observed in HaCaT cells. However, a-Si_90 increased the trend for both the number of focal adhesions per cell and focal adhesion density (Fig. 9A, C). Interestingly, the number of focal adhesions in NHDF cells was significantly lower for both a-Si coatings (Fig.9B). Moreover, the correlation between the number of focal adhesions and the cell area revealed a significantly higher focal adhesion density, suggesting a stronger interaction between cells and substrate for a-Si coatings (Figure 9D).

[Figure9]

4 Discussion

Cells-substrate interactions are phenomena mainly dependent on the material interface, where the first 10-100 nanometers play the uttermost role during the early cell adhesion [41]. Therefore, the composition of the coating surface is more relevant than its bulk counterpart, as the coating surface is more effectively involved in the interaction with the cultured cells. Due to its sampling depth lower than 10 nm, XPS is extremely sensitive to the chemical composition of the sample surface. Here the authors could demonstrate that Si was the most abundant element (around 50 at.%), followed by oxygen (around 35 at.%), in the PECVD-grown a-Si:H coatings. High-resolution (HR) spectra revealed that Si participated mostly to the formation of amorphous Si—Si compounds, whilst only a small fraction was effectively involved in the formation of the native oxide. To investigate the hydrogen content of the thin films the authors recurred to FTIR analysis. A considerably different hydrogen incorporation was found in the form of Si-H and $Si-H_2$ bonds, as well as $(Si-H_2)_n$ groups. Namely, the hydrogen content was higher in the sample grown at 90 °C (a-Si_90) than in the one deposited at 350 °C (a-Si_350), which is consistent with the widely accepted concept that hydrogen content in PECVD amorphous silicon is considerably reduced at a higher deposition temperature [42,43]. This is usually addressed to the increase of the hydrogen diffusion rate inside the amorphous matrix, allowing the removal of the H atoms that are incorporated during the deposition process [42,43].

Based on the optical contact angle (OCA) measurements, the behaviour of the samples was overall hydrophilic both for coated and uncoated titanium, although the a-Si coatings were able to impart the surface two slightly different features. A transition toward the hydrophobic regime was found for a-Si_90, the more hydrogenated coating, while the wetting of a-Si_350 was comparable to the pristine titanium control. Importantly, as the roughness analysis detected only a negligible difference between the samples coated by films with low and high hydrogen content, the deriving difference in surface

energy between the amorphous silicon coatings was not apparently related to the surface morphology. It seems then reasonable to attribute this discrepancy of contact angles to the different chemical composition of the films. Since a higher surface hydrophilicity of silicon is usually related to surface oxidation [27], the higher water contact angle measured on a-Si_90 could be due to a lower oxide presence, caused by a lower reactivity of its surface with atmospheric oxygen. This hypothesis is supported by the XPS survey analysis, which evidenced a lower oxygen content on a-Si_90, and by the high resolution analysis of the Si_{2p} XPS peak, which showed a lower contribution by Si-O bonds for this coating.

The peculiarity of the surface chemistry of these silicon coatings had clear biological effects *in vitro*. Here, a-Si_90 increased significantly the adhesion of the keratinocytes (HaCaT) when compared to the pristine titanium control and the a-Si_350 coating at the time points considered (2 and 24 h). For the first time, the simplified experimental setting of this study provided evidence that the hydrogen content of amorphous silicon based films may influence the early adhesion of keratinocytes. Consistently with the outcome observed for the surfaces functionalized with laminin by Gordon [44]. Cell morphology was instead substantially unchanged with a smaller area for the coated samples compared to the pristine titanium, in absence of other relevant alterations of polarity or perimeter. It is noteworthy that just a few studies are available on the adhesion behaviour of HaCaT cells on titanium and titanium alloys [44–46]. Roessler and colleagues investigated the effect of cueing peptides [46], demonstrating a positive effect on cell adhesion. Fujita et al. [45], on the other hand, were interested in elucidating the possible toxicological behaviour of ultrafine titanium nano-particles.

Notably, the pattern described for keratinocytes was not reproduced on fibroblasts: no effect could be detected as for the early adhesion dynamics of NHDF cells dependently on the amorphous silicon coatings. Fibroblasts seeded on both a-Si films were smaller and displayed a significantly lower number of focal adhesions than the cells in the control conditions. Particularly, a-Si_90 coating was

able to reduce the dimensions and to determine a rounder shape of the fibroblasts. Only one paper previously reported the adhesion of NHDF cells on titanium alloys [47,48]. Springer and colleagues [48] assessed the viability and proliferation of human dermal fibroblasts cultured on different Ti_6Al_4V surfaces, finding that cell survival was acceptable although lower than the control condition (cover glass). On these premises, it would be interesting to focus on the ability of HaCaT cells -and possibly other epithelial cells- to discriminate surfaces only on the base of their hydrogenation. Indeed, adhesion molecules including integrins could undergo different structural or functional remodeling upon surface interaction driving selective intracellular pathways triggered by substrate attachment.

5 Conclusions

The interaction of amorphous silicon (a-Si:H) coatings, grown by PECVD at 90 °C and 350 °C on titanium substrates, with human keratinocytes and fibroblasts was analyzed and compared with the behavior of bare titanium surfaces. Survey XPS analysis evidenced the presence of Si and O on the film surface, apart from the expected carbon contamination, with a higher oxygen concentration on the film grown at 350 °C. High-resolution XPS spectra revealed a pre-eminent participation of Si to the formation of amorphous Si—Si compounds, with only a small amount involved in the formation of the native oxide. Fourier-transform infrared spectrometry (FTIR) showed a higher hydrogen incorporation in the film grown at lower temperature. Optical contact angle analysis (OCA) evidenced a considerable reduction of surface energy after the film deposition, respect to bare Ti surface, only for the samples coated by the more hydrogenated film. Films with higher hydrogen incorporation showed a lower surface oxidation and a more hydrophobic behavior, compared to less hydrogenated samples. Interestingly, keratinocytes, but not fibroblasts, were sensible to the increased hydrogen content during the adhesion process. This suggests the possible role of the adhesion

molecules of epithelial cells undergoing different structural and functional remodeling upon surface interaction. Further studies are required to finely investigate the molecular mechanisms responsible for the differential behavior of the two cell types here employed.

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Figures:

Figure 1. *XPS analysis. Wide-scan XPS spectra acquired on amorphous Si coatings grown at 90°C and 350°C (A). High-resolution Si2p spectra acquired on the a-Si coatings grown at 90 °C (B) and 350 °C (C).*

Figure 2. *FTIR analysis. Results of FTIR analysis performed in transmission mode on a-Si:H thin films grown on silicon substrates at 90 °C (a-Si_90) and 350 °C (a-Si_350).*

Figure 3. *Surface roughness. Results of roughness analysis, expressed as R_a (A) and R_q (B), of Ti samples before and after the deposition of a-Si:H coatings with high and low hydrogen content.*

Figure 4. *Surface energy. H_2O (A) and CH_2I_2 (B) drops spreading on the surface of the uncoated titanium sample. H_2O (C) and CH_2I_2 (D) drops spreading on the surface of a-Si_90 sample. H_2O (E) and CH_2I_2 (F) drops spreading on the surface of the a-Si_350 sample.*

Figure 5. *Cell adhesion. HaCaT and NHDF adhesion was evaluated on all samples at 2 hours (2 h) (A, B) and 24 hours (24 h) (C, D). The level of cell adhesion was measured counting the number of adherent cells for each field. Values represent mean ± SEM; the symbol (*) indicates a statistically significant difference versus uncoated machined Ti sample (Mac), considering a p-value < 0.05.*

Figure 6. *Cell viability. Cell viability of HaCaT (A) and NHDF (B) performed through CellTiter-glo luminescent assay is shown. Data are expressed as Relative Luminescent Unit (RLU) as measured at 48 h after seeding. Values represent mean ± SEM; the symbol (*) indicates a statistically significant difference versus uncoated machined Ti sample (Mac), considering a p-value < 0.05.*

***Figure 7**. Representative pictures of cell morphology. Fluorescence photomicrographs of HaCaT (A-C) and NHDF (D-F) seeded on different samples for 24 h. The cells were stained for the nucleus (DAPI, blue), the actin (rhodamine-phalloidin, green) and the focal adhesions (paxillin, red).*

***Figure 8**. Evaluation of cell morphology. The three morphological parameters area (A, D), perimeter (B, E) and AR (Major axis/minor axis) (C, F) of both HaCaT (A-C) and NHDF (D-F) cells, are shown. Values represent mean ± SEM; the symbol (*) indicates a statistically significant difference versus uncoated machined Ti sample (Mac), considering a p-value < 0.05.*

***Figure 9**. Evaluation of focal adhesions. Focal adhesions were measured for both HaCaT (A, C) and NHDF (B, D) as number of focal adhesions/cell and focal adhesion density after 24 hours from seeding on different samples. Values represent mean ± SEM; the symbol (*) indicates a statistically significant difference versus uncoated machined Ti sample (Mac), considering a p-value < 0.05.*