Protein adsorption and fibroblast adhesion on irradiated polysiloxane surfaces

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A very peculiar case of differential cell response towards polysiloxane surfaces of very similar composition is investigated. Poly(hydroxymethylsiloxane) (PHMS) surfaces treated either by O_2-plasma or 6 keV Ar^+-beams have been used to test the adhesion, proliferation and spreading of human fibroblasts. The surface chemical structure and nanomorphology were investigated by means of X-ray photoelectron spectroscopy (XPS), surface free energy measurements and atomic force microscopy (AFM). In spite of the close compositional and morphological similarity of the modified surfaces, the viability of the adhered cells, evaluated by means of optical microscopy and epifluorescence microscopy, was found to be very different in the two cases. The study of the features of the adsorbed protein adlayer on the two types of surfaces was performed by XPS and AFM and indicated that the overall cell behavior is connected to a quite different protein aggregation process, occurring respectively on the plasma- and Ar^+-modified polysiloxane surfaces. It is suggested that the specific biological response of the modified surfaces is determined by the chemical structure at the nanometric level.

1. Introduction

The problem of the understanding and control of the mechanisms of cell interaction with organic and inorganic solid surfaces is one of the most important, in view of a number of relevant applications in the field of tissue engineering, new prosthetic devices, microsystems aimed to manipulate cells, etc ... [1, 2]. These applications demand a very careful control of cell–surface interaction processes, including, as one of the critical issues, the directionally guided cell growth [3, 4].

Recently, it has been shown that several types of cells exhibit a preferential adhesion to ion-irradiated surfaces [5]. The improvement of the cell response on the irradiated surfaces has been discussed in terms of the change of contact angle of proper liquids (i.e., the surface free energy), of the chemical structure, of the electronic properties of the irradiated surfaces, of the effect of the long-living radicals or other metastable species among many others ... [6, 7].

In this scheme, the analysis of the way the modified surfaces may drive the modified cell response is still lacking. It is well known that the first phenomenon occurring during the cell–surface interaction basically consists in the surface adsorption of some of the proteins present in the medium [8], with a manifold of possible conformation according to the nature (chemistry, morphology, physical properties ...) of the surface. The further evolution of the cell adhesion process is then determined by the structure, conformation and composition of the protein adsorbed layer. Accordingly, in our laboratory, we have undertaken a systematic study of the adsorption process of different proteins, including human serum albumin, collagen IV, vitronectin and fibronectin on polymeric irradiated surfaces [9]. However, these studies, basically addressing the identification of the differences in the adsorption behavior of a single protein onto modified surfaces, have not yet involved the study of the competitive adsorption processes observed for complex solutions of many proteins.

In a previous paper we suggested that the striking differences observed in cell adhesion, their morphology and viability may be related to the formation of different phases in plasma- and ion-irradiated surfaces [10]. In particular, it was suggested that the biological response was probably specific of the peculiar electronic structure of plasma- and ion-irradiated layers, possibly connected to a different nanometric scale organization. In any case, we suggested that the response should involve a complex modification of the mediating adlayer of proteins.

The present paper is aimed at studying the features of the protein adlayer formed following the exposure of irradiated polymer surfaces to fetal bovine serum (FBS), one of the most important components in the cell culture

media. In particular, these effects have been studied for the case of PHMS films treated respectively by using O_2-plasma and 6 keV Ar^+ beams, which in a previous related paper have been shown to exhibit highly specific cell response, depending on the surface treatment [10]. Accordingly, XPS, surface free energy and AFM measurements have been used to characterize the irradiated and FBS-exposed surfaces and to obtain some picture of the organization of the protein films for the various surfaces. The results are discussed in connection with the cell adhesion behavior, involving respectively proliferation and spreading processes.

2. Materials and methods

2.1. Materials

PHMS thin films (thickness of 500 ± 15 nm) were spin coated onto the monopolished side of silicon (1 0 0) wafers from a commercial solution (Accuglass512B, Allied Signal, USA). The wafers were then cut in (1×0.5) cm^2 pieces and surface modified by using either O_2 plasma treatment or 6 keV Ar^+ ion beams.

Oxygen plasma modification was carried out by a March Instrument solid-state Plasmod® unit (Concord, CA, USA) powered by RF generator at 13.56 MHz. The plasma treatment was operated according to the following experimental parameters: 1 min of exposure time, an applied power of 100 W and a residual pressure of 67 Pa of pure oxygen (99.95% minimum purity). After the plasma treatment the samples were kept in ultrapure MilliQ-water for 1 week. This specific aging procedure has been chosen according to the previous results on the effect of various aging process, respectively in air and water, extensively reported in Satriano *et al*. [10]. In particular, the water-aged surfaces resulted in a higher wettability with respect to the ones aged in atmosphere [10]. The depth of the modified PHMS layer was estimated higher than about 9 nm by measuring the disappearance of the C 1s signal in angular resolved XPS measurements, corresponding to the conversion of the plasma-treated polymer layer into an inorganic-like amorphous SiO_x phase [10].

Ion irradiation treatments were performed with 6 keV Ar^+ at a fluence of 1×10^{15} ions/cm^2 by using a VG EX05 ion gun. The irradiations were carried out at room temperature with less than 10^{-5} Pa of pressure in the chamber and an ion current of 1.5 μA/cm^2, in order to avoid heating effects in the samples. By using TRIM code [11], the projected range (i.e., the average penetration depth) of 6 keV Ar^+ ions in PHMS was estimated to be 8.5 ± 3.0 nm, with a total deposited energy of about 700 eV/nm. The ion-irradiated samples were aged for two weeks in atmosphere before the next measurements.

2.2. Characterization techniques

2.2.1. XPS measurements

XPS analysis was carried out with a PHI 5600 Multi Technique Spectrometer equipped with a dual Al/Mg anode, a spherical capacitor analyzer and an electrostatic lens system (Omni Focus III). The spectra were acquired at a photoelectron take-off angle of 45°, in fixed analyzer transmission mode by using a standard $MgK\alpha_{1,2}$ (1253.6 eV) source and pass energies of 187.85 and 11.75 eV for survey and detailed scans, respectively. The estimated sampling depth is about 9 nm, according to an attenuation length of 3.0 nm for Si 2p peak in organic materials [12]. Such value is actually comparable to the estimated thickness of the ion- and plasma modified layers. The spectra were analyzed by using an iterative least squares fitting routine based on Gaussian peaks and the Shirley background subtraction. Binding energies (BE's) of all the spectra were referenced to the intrinsic (before irradiation) hydrocarbon-like C1s peak of PHMS set at 284.6 ± 0.2 eV or to the adventitious one set at 285.0 ± 0.2 eV (after irradiation).

2.2.2. Atomic force microscopy

A Multimode/Nanoscope IIIA AFM (Digital Instruments) was used for the visualization of surface micro- and nano-topography and for the analysis of serum proteins adsorbed on the modified PHMS surfaces. The AFM images were measured in tapping mode with a standard silicon tip, in order to minimize the interaction between tip and proteins. The relative room humidity was 30% and the room temperature was 23 °C. Images were recorded using both height and phase-shift channels, with 512×512 measurement points (pixels). Measurements were made twice or three times on different zones of each sample. Representative images are shown. Image processing was performed by using the Nanoscope III software. Quantitative evaluation of protein coverage from AFM images was performed using the Scion Image software (Windows version of NIH Image software), in terms of integrated density (I.D. $= N \times [M - B]$, where N is the number of pixels in the selection, M is the average gray value of the pixels and B is the most common pixel value).

2.3. Cell culture and cytocompatibility tests

Normal human dermal fibroblasts-adult (NHDF, Clonetics, Bio-Whittaker) cell line was used to test the cell adhesion on the various modified surfaces. The cells were routinely maintained at 70–80% confluence in 25-cm^2 flasks (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle medium (DMEM, Euroclone) supplemented with 10% (v/v) FBS, 0.1 μg/ml streptomycin, 2 mM L-glutamine, and 100 million units/ml penicillin (all Euroclone), at 37 °C in a humidified 5% CO_2 atmosphere.

Fibroblasts were released from culture flasks by incubation with 0.25% (w/v) trypsin and 0.02% ethylenediaminetetracetic acid (EDTA) in phosphate-buffered saline (PBS) for 5 min, then centrifuged and resuspended in the fresh culture medium. The PHMS treated and untreated samples were placed in the bottom of six-well tissue culture plate (Nunc), and a 3-ml volume of cell suspension (1.5×10^2 cell/ml) was seeded into each well.

The cell adhesion as well as proliferation and tendency to spread were evaluated respectively after 5 and 48 h of incubation.

Following incubation, the samples were rinsed with

PBS, then fixed with 4% *p*-formaldehyde (30 min at room temperature), and treated with Triton X-100 (0.1% in PBS). Finally, the cells were stained (30 min at 37 °C) with Hoechst (50 µg/ml) and Blue Evans (10 µg/ml) in order both to visualize adhered cells on polymer surfaces without optical interferences, and to evaluate cell vitality and spreading by differential staining of nucleus and cytoplasm.

The samples were washed twice in magnesium and calcium-deprived PBS, dried in air and mounted with mounting medium (SIGMA) for observation by epifluorescence microscopy. At least 10 microscopic fields per sample were randomly acquired with × 20 magnification by a COHU High Performance CCD Camera and Leica Qwin software.

Quantitative evaluation of adhered cells was performed using the Scion Image software (Windows version of NIH Image software). Results of the image analysis are expressed as mean-standard deviation for each group of treated samples. Differences among groups were established by *t*-student test analysis by a two population comparison. Statistical significance was considered at a probability $P < 0.05$.

2.4. Protein adsorption studies

The incubations with serum proteins were carried out by soaking the PHMS substrates in Petri dishes containing 2 ml solution of FBS (Sigma) for 30 min at room temperature. After that the samples were gently washed by using ultra pure Millipore water with a micropipette performing a number of sucking–rinsing steps, in order to wash out the non-adsorbed proteins. The samples were then dried in atmosphere before the measurements.

3. Results and discussion

3.1. Cell–surface interaction

NHDF cells were seeded on untreated and irradiated surfaces. Cell adhesion, morphology and spreading were determined after 5 and 48 h, respectively. These incubation times, which are significantly shorter than the typical time (6–9 days) needed for complete spreading, are already sufficient to demonstrate dramatic differences in the number, morphology and viability of the adhered cells for the untreated, plasma- and ion-irradiated PHMS surfaces.

Indeed, the quantitative estimation of the cell coverage reported in Fig. 1 shows that while no significant cell adhesion was found on the untreated PHMS, both the ion and plasma irradiation induced a dramatic and comparable increase of cell adhesion. Furthermore, for both the incubation times, the number of adhered cells is actually almost the same, indicating that already after the shorter incubation the attachment is stable.

Fig. 2 shows the epifluorescence microscopy analysis of NHDF cells incubated for 48 h. It can be seen that both cell morphology and endocellular structure strongly depend on the type of surface treatment. In fact, only a few cells adhered on untreated PHMS surfaces (Fig. 2(a)), showing clear evidence of poor viability, i.e., a globular shape, with thickened chromatin. On the contrary, on the plasma-treated samples (Fig. 2(b)) the

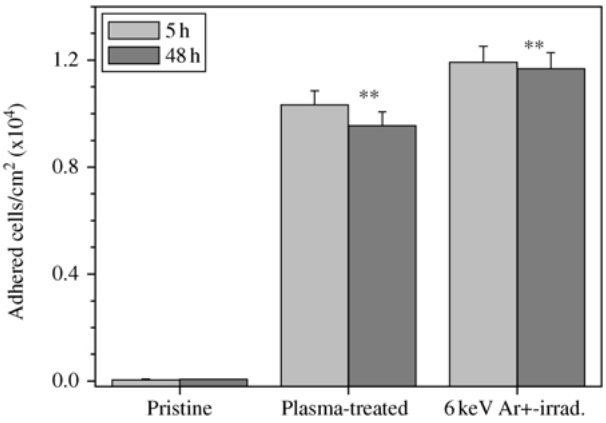

Figure 1 NHDF cell coverage on the various PHMS surfaces 5 and 48 h of incubation (** = statistical significance $P < 0.05$).

cells look viables in general, with a characteristic elongated and well-shaped morphology, without major clustering effects. It is to note, however, that about 15% of the cellular population still show a globular shape,

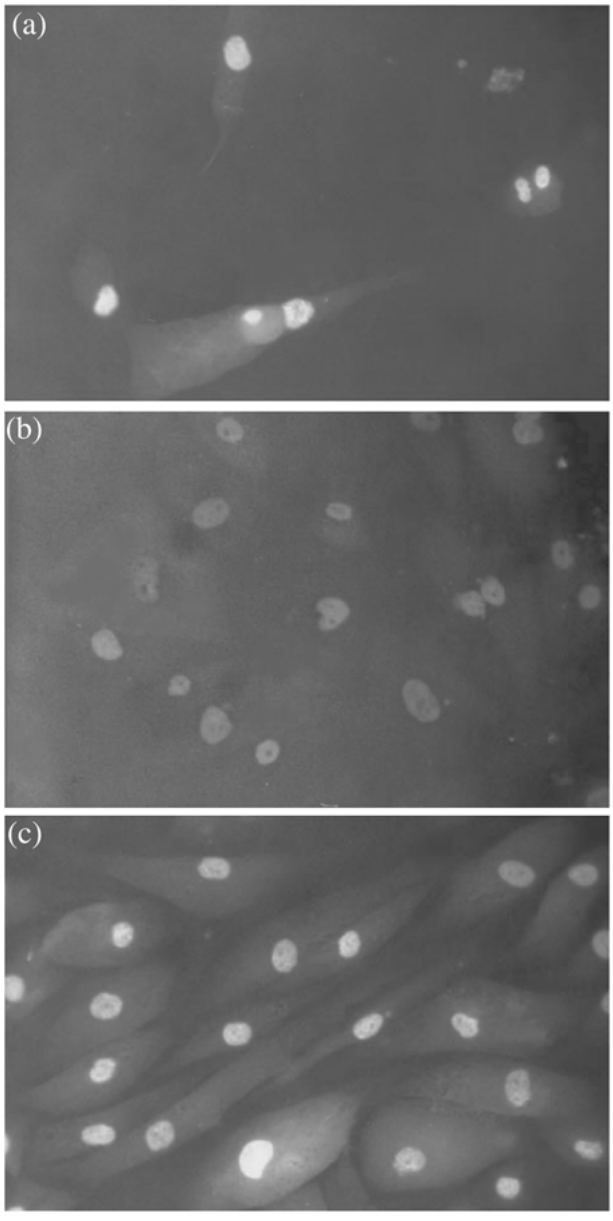

Figure 2 Light micrographs of NHDF cells adhered and spread on PHMS: (a) untreated, (b) plasma-treated and (c) ion-irradiated.

suggesting that the plasma-treated surfaces are only partially cytocompatible. Finally, the cell adhered onto the 6 keV Ar^+ irradiated surfaces (Fig. 2(c)) looked tapered and evenly distributed throughout the irradiated surfaces with a complete spreading and confluence, and a regular mitotic activity.

3.2. Protein–surface interactions

The adsorption of proteins from FBS onto the untreated and plasma- and ion-irradiated PHMS surfaces has been studied at short incubation time, 30 min, in order to identify the first stage events, which are responsible for the immediate cell attachment process.

The analysis of the chemical and structural modification induced in polysiloxane surfaces has been reported in great detail elsewhere [10, 13]. Let us summarize the relevant results for the present work. Both plasma and 6 keV Ar^+ irradiation induced a marked decrease of the carbon content from the original value of $\sim 23\%$ to respectively 6% for plasma- and 12% for 6 keV Ar^+-irradiated samples. This corresponds respectively to the formation of an a-SiO_x phase, for O_2-plasma-treated samples, and a peculiar amorphous a-$SiC_xO_y(H_z)$ phase, still containing Si–C–O bonds and other carbon-containing species, for 6 keV Ar^+ irradiation.

In agreement with the observed differences in chemical structure, the wettability behavior of plasma-modified surfaces is quite different with respect to that of Ar^+-irradiated PHMS. In fact, the static water contact angle value of $\theta_s \sim 90°$ for the untreated PHMS surfaces (quite high hydrophobic character), becomes $\theta_s \sim 50°$ for 6 keV Ar^+-irradiated surfaces (moderately hydrophilic character) and $\theta_s \sim 10°$ for the oxygen plasma-treated ones (highly hydrophilic character).

The described surfaces have been incubated with FBS and, after rinsing and drying steps, characterized by using XPS and AFM measurements.

The XPS study of the FBS-exposed surfaces shows that an almost complete coverage is achieved for untreated as well as irradiated PHMS surfaces. In fact, assuming the nitrogen content as a XPS marker of the firmly adsorbed proteins (i.e., those resisting the washing steps), a 12–14% of N atomic concentration is measured for all the samples, in fair agreement with previous results on surfaces having adsorbed protein layers (e.g. HSA adsorbed on PHMS show about 16% of nitrogen content [9]). This fact, together with the disappearance of the substrate-characteristic XPS (Si 2p and Si 2s) peaks confirms the almost complete surface coverage.

A further evidence of the formation of a relatively thick protein adlayer, similar for all the investigated surfaces, was obtained by a close analysis of the C1s photoelectron peaks for bare surfaces (Fig. 3(a)–(c)) and protein-covered surfaces (Fig. 3(d)–(f)). The peak fitting analysis revealed the following components for the bare substrates: C_{C-Si}, at 284.6 ± 0.2 eV of BE, due to $>$C–Si bond; C_{C-O}, at 286.6 ± 0.2 eV of BE, assigned to $>$C–OH and $>$C–OC groups (present also in the untreated PHMS samples due to polymer chain terminations or to solvent residuals). These peaks are modified in plasma-treated samples by the addition of a new component at 288.5 ± 0.2 eV of BE due to $>$C(=O)O bonds (component C_{COO^-} in Fig. 3(b)). In ion-irradiated samples the C 1s peakshape is modified by the addition of two new components, respectively at $\sim 286.3 \pm 0.2$ eV, assigned to $>$C–O–Si moieties and at 287.2 eV of BE, due to $>$C=O [10].

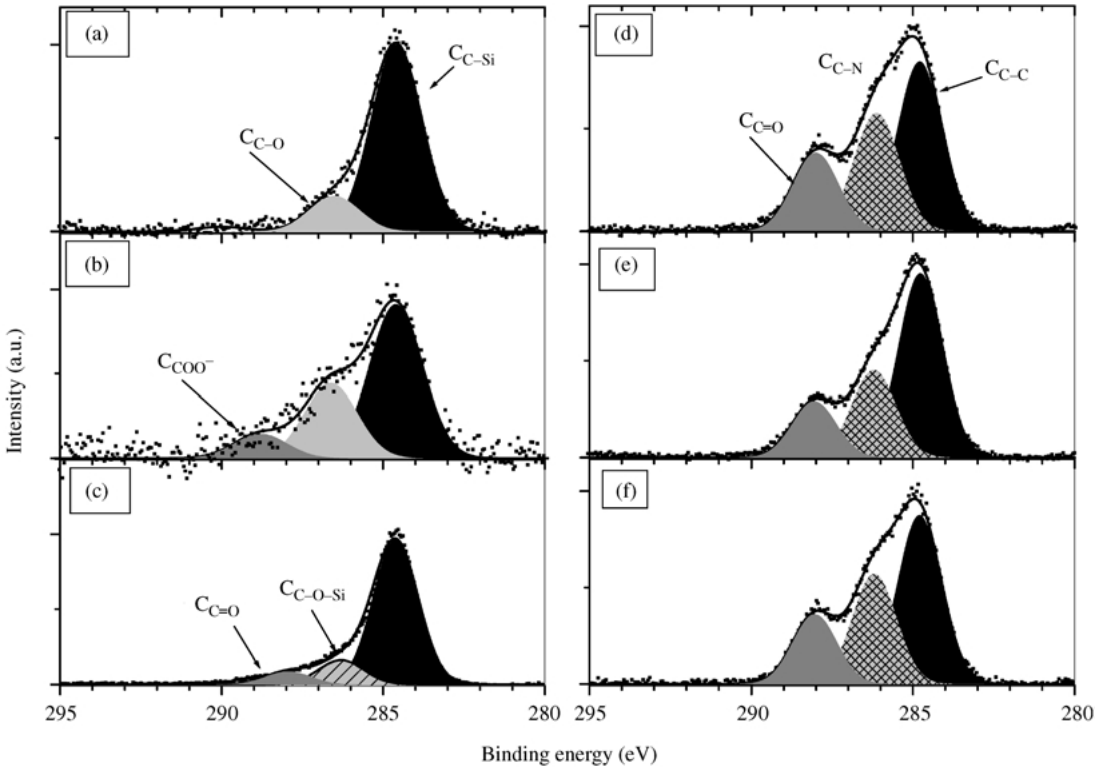

Figure 3 C1s photoelectron peaks for bare (a)–(c) and FBS-incubated (d)–(f) PHMS: untreated (a), (d) plasma-treated (b), (e); 6 keV Ar^+-irradiated (c), (f).

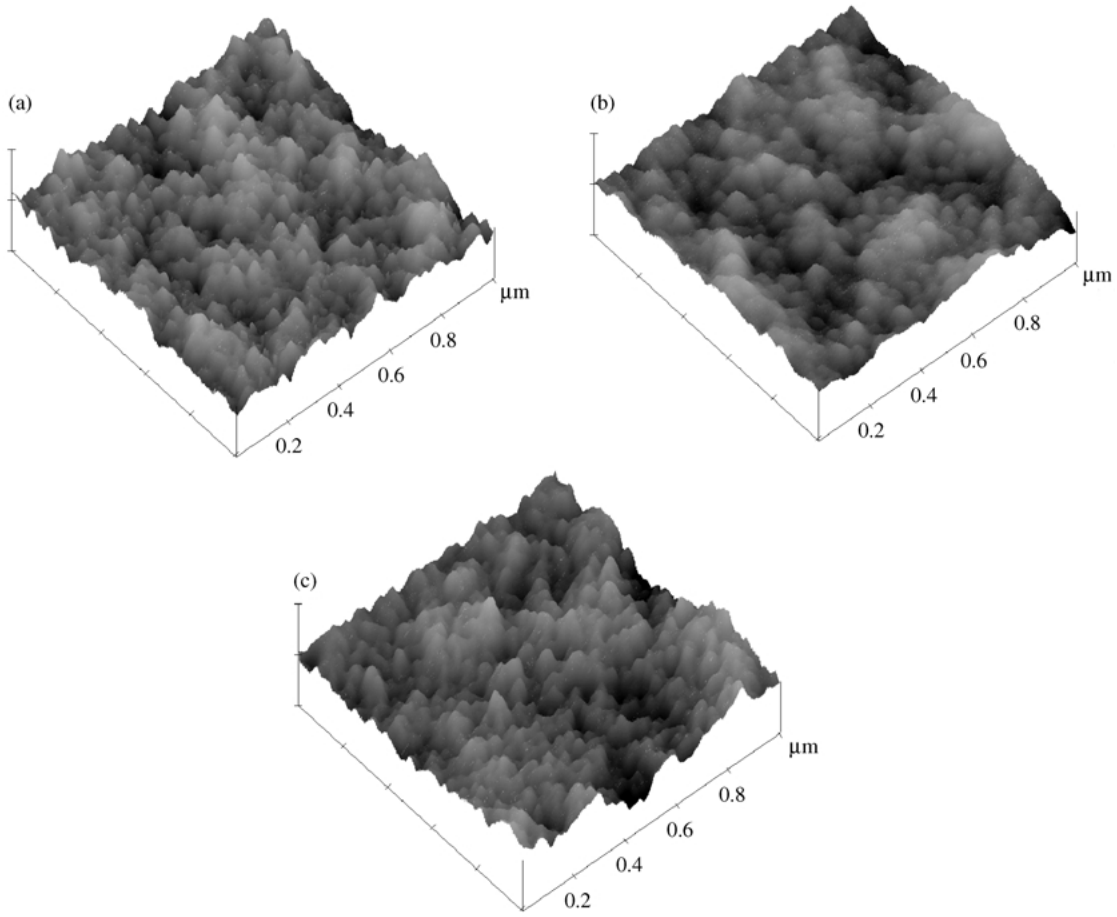

Figure 4 AFM height images of PHMS: (a) untreated, (b) plasma-treated and (c) ion-irradiated. z range = 4 nm/div.

As to the FBS-incubated samples, Fig. 3(d)–(f) show the appearance of the characteristic components of peptide-related groups, i.e., $C_{C=O}$ and C_{C-NH}. In particular, two basic facts appeared clear: the first is that the adsorption step produces layers of very similar chemical structures. The second one is that the relative intensity of the C_{C-C}, C_{C-NH} and $C_{C=O}$ components is quite different, the ratio $C_{C-C}/C_{C-NH}/C_{C=O}$ being respectively of 2.1/1.5/1.0 for untreated PHMS, 2.4/1.6/1.0 for ion- and 3.3/1.5/1.0 for plasma-irradiated surfaces. In other words, untreated and ion-irradiated samples show a very similar structure of the adlayer, different from the one obtained onto the very hydrophilic plasma-treated PHMS surfaces. In particular, the polar groups, corresponding to C_{C-NH} and $C_{C=O}$ components in the XPS spectrum, seem mostly oriented towards the surface in the case of the more hydrophilic surfaces, while such a domains would be oriented mostly towards the solution in the case of the mildly hydrophilic and hydrophobic ones.

It should be made clear that the present observation should not be taken as an indication of the real behavior in biological fluids, as far as the measurements obviously refer to dried surfaces. However, the result is still representative of how much the surface response with respect to adsorption processes can differ for the plasma- and ion-treated samples.

AFM measurements were carried out (1) to determine whether the different surface modification treatments create different topographical features; (2) to ''see'' if the organization of the protein layer ''senses'' the modified PHMS surfaces.

Fig. 4 displays the AFM images of untreated, plasma- and ion-modified PHMS surfaces. No visible differences in morphology and roughness (see also Table I) with respect to the untreated PHMS are observed for both kinds of modification treatments.

In Fig. 5 are shown the same surfaces after the protein adsorption step from FBS solution. The morphological differences among the three samples are evident and can be related to different behavior of adsorbed proteins on the different-treated surfaces. In fact, on untreated PHMS we have found that the adsorbed layer is characterized by the formation of few aggregates with average dimensions of about 70×120 nm. In the case of plasma-treated surfaces, the adsorption process produces a more dense population of smaller protein aggregates, characterized by a relatively uniform distribution throughout the surfaces (average size: 90×40 nm). Finally, for ion-irradiated surfaces, relatively large aggregates are unevenly distributed through the surfaces, roughly showing two different size populations respectively consisting of very large (having the long axis of ~ 150–200 nm and the short one of ~ 90 nm) and smaller aggregates (with ~ 100–130 nm and ~ 40 nm respectively for long and small axis), these last seeming the growing nuclei for the larger ones. Table I reports the AFM quantitative data for the various protein-covered

TABLE I Root mean square (RMS) roughness and integrated density (ID) values of surface coverage by protein aggregates from AFM analysis

PHMS	RMS (nm)	ID
Untreated	0.541	—
Untreated + FBS	0.903	686.2
Plasma-treated	0.429	—
Plasma-treated + FBS	3.632	2955.52
6 keV Ar^+	0.589	
6 keV Ar^+ + FBS	2.911	1384.98

samples, both in terms of change of the surface roughness and the amount of surface covered by the protein aggregates.

It is to note that the XPS data clearly indicated that an almost complete coverage is found for all the investigated surfaces, i.e., the aggregates seen in AFM lay on a smooth and probably continuous protein film.

AFM phase images (see Fig. 6) suggest that the observed topography effect has also a chemical nature, as far as the phase imaging is determined by the modulation of the tip-surface interactions depending on the chemical groups on the surfaces.

Finally, it has to be stressed that the images were acquired in air, and they actually represent the dried protein adlayers on the surfaces. Accordingly, a quite different structure with respect to the one in real medium has to be expected. However, the reported data are still representative when examined in comparison for the plasma- and ion-treated samples.

4. Conclusion

The reported results, dealing with the different chemical structure of the PHMS surfaces by using either plasma- or ion-irradiation treatments, can be phenomenologically related to cell adhesion, spreading and viability behavior. In fact, fibroblasts were found to adhere with comparable effectiveness on plasma- and ion-irradiated surfaces, while the spreading and viability effects are slightly higher for the ion-irradiated surfaces. It is to note that we cannot exclude that the observed difference in the spreading behavior reflects different spreading velocities on the two types of surfaces.

In this paper, we tried to correlate the cell behavior to the protein adsorption phenomena occurring in medium and to the structure of the irradiated surfaces. In this experimental framework, we have selected only one treatment condition respectively for the plasma and low energy ions, taking advantage of the previously reported results [9, 10, 13].

In particular, we have found that also if an almost complete surface coverage with protein adlayer is obtained for all the treated and untreated PHMS surfaces, a clear evidence of the dependence upon the surface treatment of the protein layer orientation and aggregation was found.

However, as to the protein orientation, we must conclude that it is not able to explain the different cell response obtained for the various surfaces.

Thus, the attention should be focused on the aggregation phenomena with respect to cell spreading and viability, whose extent and features are drastically

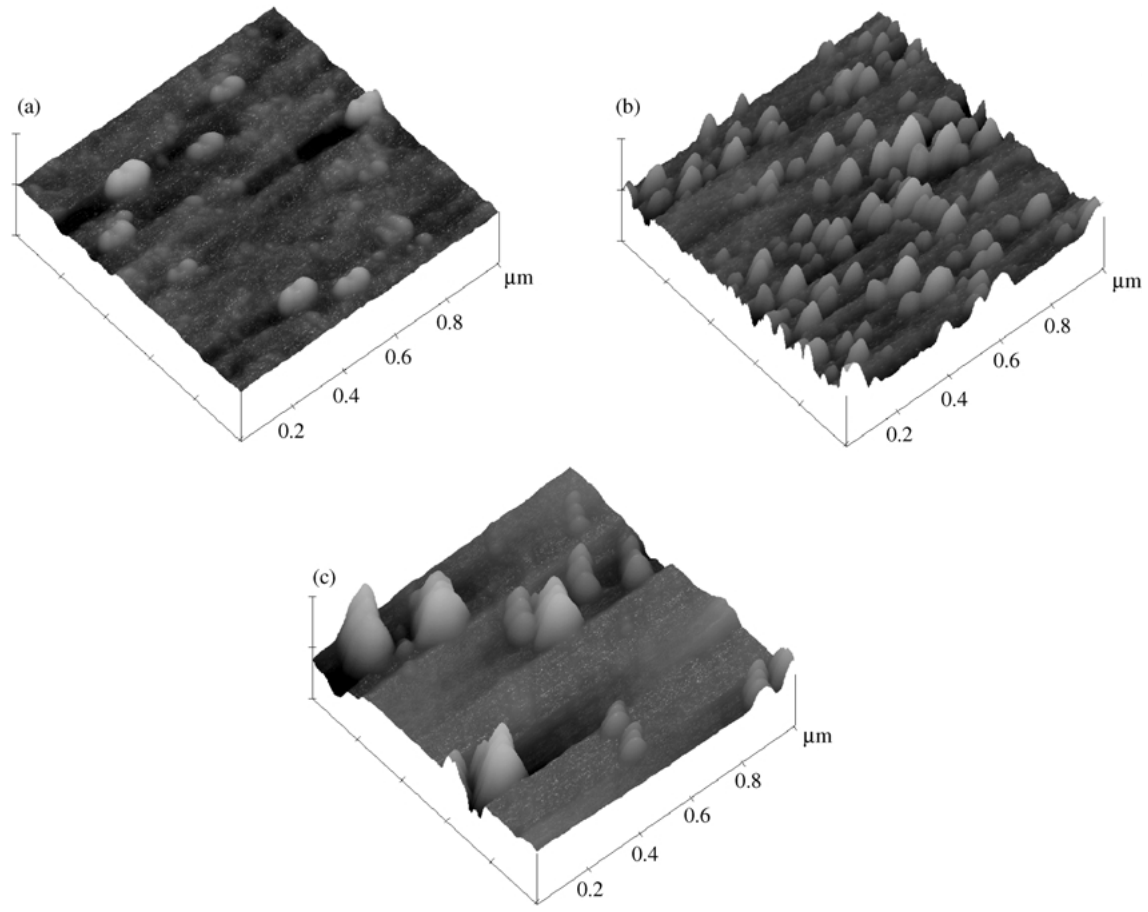

Figure 5 AFM height images after incubation with FBS of PHMS: (a) untreated, (b) plasma-treated and (c) ion-irradiated. z range = 30 nm/div.

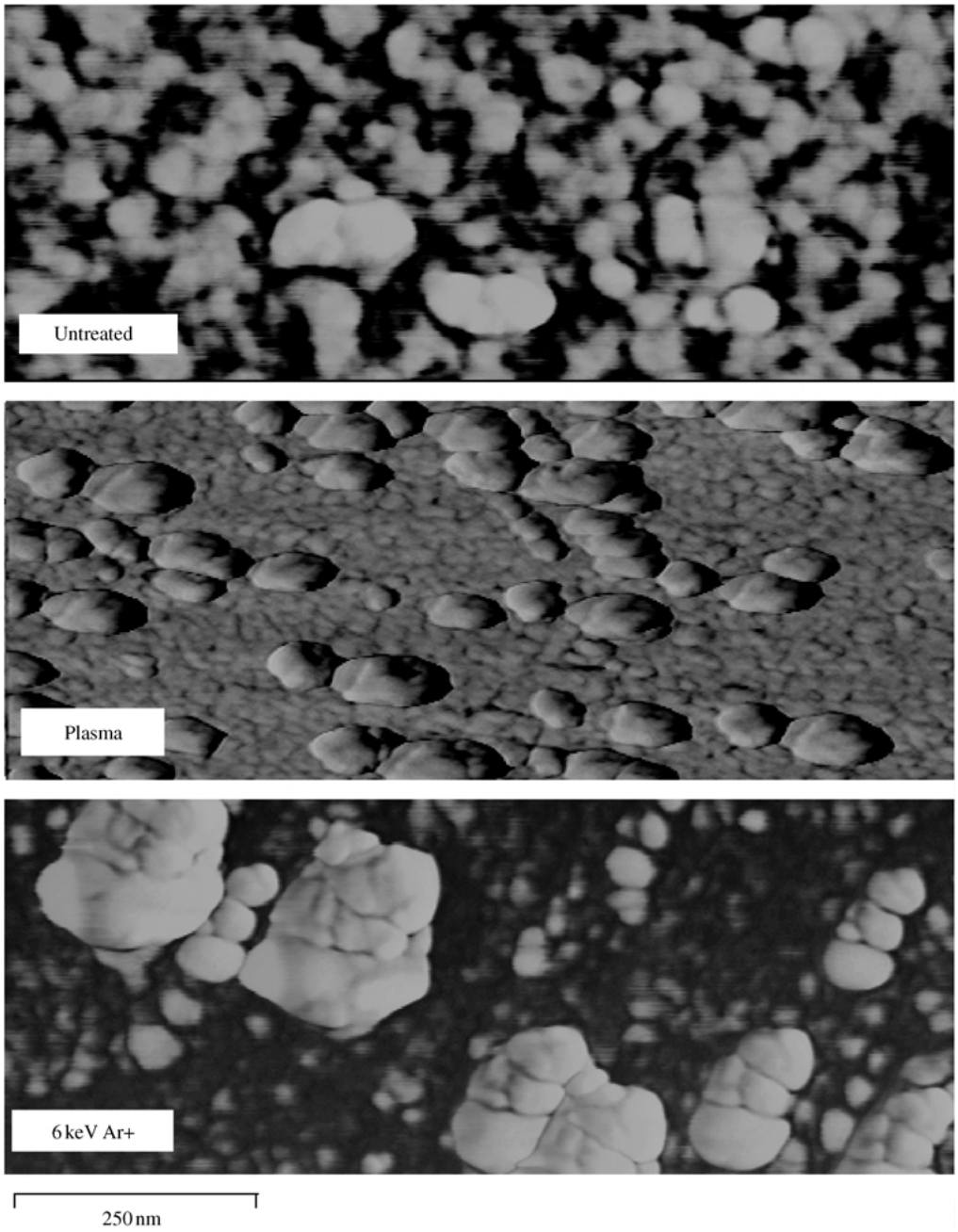

Figure 6 AFM phase images after incubation with FBS of untreated PHMS, plasma-treated and ion-irradiated PHMS surfaces. z range = 120 deg/div.

different for the various surfaces. In particular, in the case of ion-irradiated surfaces, the catastrophic aggregation process of serum proteins can be correlated to an optimal NHDF cell adhesion and spreading behavior. On the other hand, the scarce formation of protein aggregates for O_2-plasma and untreated surfaces can be related to less viable character of the NHDF cells on these surfaces. The reported effects deserve as much interest as it is known that highly hydrophilic surfaces, also prompting a good protein adsorption, are not particularly effective in promoting in itself a significant cell adhesion [10].

Finally, it should be stressed that in order to understand the real connection between surface structure and protein aggregation effects, one should extend the investigation to the fine physical and chemical structure of the growing domains.

In fact, it is to expect that also factors like the different electrical properties (as for instance, electrical conductivity or the presence of electrically charged domains) or the density of specific chemical groups on the plasma- and ion-irradiated surface layers can be important to determine the protein aggregation effects.

Acknowledgments

The authors acknowledge partial support from PRIN 2001 (MIUR-Rome). Profs G. Strazzulla and G.A. Baratta (Osservatorio Astrofisico of Catania) are acknowledged for access to the ion-beam irradiation facility.

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Received 1 October 2002
and accepted 15 January 2003