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Submicron Laser-Induced Dot, Ripple and Wrinkle Structures and Their Applications

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***Abstract*—**Polymers exposed to laser or plasma treatment or modified with different wet methods which enable the introduction of nanoparticles or biologically active species, such as amino-acids, may find many applications both as biocompatible or anti-bacterial materials or on the contrary, can be applied for a decrease in the number of cells on the treated surface which opens application in single cell units. For the experiments, two types of materials were chosen, a representative of non-biodegradable polymers, polyethersulphone (PES) and polyhydroxybutyrate (PHB) as biodegradable material. Exposure of solid substrate to laser well below the ablation threshold can lead to formation of various surface structures. The ripples have a period roughly comparable to the wavelength of the incident laser radiation, and their dimensions depend on many factors, such as chemical composition of the polymer substrate, laser wavelength and the angle of incidence. On the contrary, biopolymers may significantly change their surface roughness and thus influence cell compatibility. The focus was on the surface treatment of PES and PHB by pulse excimer KrF laser with wavelength of 248 nm. The changes of physicochemical properties, surface morphology, surface chemistry and ablation of exposed polymers were studied both for PES and PHB. Several analytical methods involving atomic force microscopy, gravimetry, scanning electron microscopy and others were used for the analysis of the treated surface. It was found that the combination of certain input parameters leads not only to the formation of optimal narrow pattern, but to the combination of a ripple and a wrinkle-like structure, which could be an optimal candidate for cell attachment. The interaction of different types of cells and their interactions with the laser exposed surface were studied. It was found that laser treatment contributes as a major factor for wettability/contact angle change. The combination of optimal laser energy and pulse number was used for the construction of a surface with an anti-cellular response. Due to the simple laser treatment, we were able to prepare a biopolymer surface with higher roughness and thus significantly influence the area of growth of different types of cells (U-2 OS cells).

***Keywords*—**Polymer treatment, laser, periodic pattern, cell response.

I. Introduction

IN the last few decades, an interest has grown in the usage of products made from renewable resources and products which are able to decompose into environment-friendly constituents. Biodegradable polymers are of great importance in biomedical applications, mostly as substrates that can temporarily support different types of cell replacements or in the field of delivering therapeutics [1], [2]. The surface of a solid material is important for various applications, since the surface nanostructures or microstructures and the properties of surfaces are usually rather different from the bulk material. The few surface layers of the solid surface are the most exposed to both physical and chemical interactions with its environment [3]. Thus, the knowledge of surface parameters can be further used for the construction of scaffold materials to support tissue growth in vitro and in vivo and can be altered by different types of surface treatments [4].

It is desirable to alter the surface by introducing reactive functional groups whereas the bulk of the polymer material remains intact. This can be achieved by several treatment methods; there are various ways of modifying the surface of polymers, such as chemical treatment or treatment based on interaction with particles or a beam - plasma treatment or laser treatment [5]. Since treatment by excimer laser is able to modify only small a depth which ranges from fractions of a micrometer to several tens of micrometers due to the wavelength of the laser, the chemical and physical changes occur only in the top few surface layers, and the bulk remains unaltered [6]. Excimer laser treatment may be applied in the field of biochemistry and tissue engineering, as the laser-treated surface can facilitate the adhesion of living cells [7]. The rapid development of different methods used for the pattern construction on polymer materials based on processes involving the preparation of surface structures was described in several papers recently [8], [9]. These methods can be divided into two main categories: "top-down" and "bottom-up". Top-down methods are based on the creation of microstructures, nanostructures and devices mainly of large-sized materials, where the spatial resolution and minimum size of structure depends on the precise removal of material [10], which is influenced by a several variables.

If the laser beam hits the surface of a polymer, several phenomena may occur. The energy can be either reflected or can be scattered or absorbed by the bulk, depending on the chemical structure of the material. Presence of double bonds between two carbon atoms and/or carbon atom and a heteroatom shifts the absorption peak to higher wavelengths compared to single bonds. Furthermore, crystallites scatter incident light and thus increase its path through the bulk of the material [11]. Chemical modification of the surface layer and its surface morphology depends on laser fluence and the number of laser pulses, a UV excimer pulse laser treatment of polymer foils can lead to ablation of the material, granted the laser fluence is high enough. Different surface structures were observed and described on the basis of their periodicity and orientation which is influenced by the polarization of laser

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beam. Periodic surface structures with the periodicity corresponding to the wavelength of the incoming laser beam are known as low spatial frequency LIPSS, while the second group where lower period than wavelength is achieved are called high spatial frequency LIPSS [12], [13]. The period of LSFL structures depends on wavelength of incoming laser light and the angle of incidence [13].

Formation of surface structures with a high degree of periodicity can provide better control or enhancement of material properties. Periodic gratings can be constructed by UV radiation caused by excimer lamps, plasma discharge, laser beam radiation, or by ion-beam sputtering [14]. The most common periodic nanostructures obtained by self-organization are ripples and dots. Their application can be found in production of biomedical surfaces [15] or they can be easily metalized, since the pattern has a positive influence on adhesion [16]. The potential application of such patterns is also in electronics for the manufacturing of microchips and memory devices [17] or they can be useful in construction of biosensors. The dimensions (width and height) of the surface pattern can be modified by radiation under a different angle [18]. The strong absorption coefficient allows us to prepare the periodic pattern also on other types of polymers, such as polyethyleneterephthalate (PET) [19], polyethylenenapthalate (PEN) [13], [20], polystyrene (PS) [21] or PES [22], which also allows us to prepare metal nanowires or other structures on treated surfaces. As was discussed, the periodic pattern is constructed on the basis of the laser beam interference at the surface and the subsequent surface response.

Construction of a periodic pattern on a biopolymer surface is more complicated, since the common biopolymers lack the benzene ring in their chain, therefore their absorbance of excimer laser wavelengths is much smaller, which can be bypassed by addition of special chemical substances able to absorb the desired wavelengths [23]. In recent years, new materials in tissue engineering, regenerative medicine or controlled drug delivery have promoted the need of new properties of biomaterials with biodegradability [24]. Polymer research led to development of new polymer-based materials which can provide interesting possibilities in areas of surface engineering, electronics, biocompatible systems or devices friendly to the environment [25].

We have focused on surface treatment of two types of polymers in this paper. As representative of non-biodegradable polymers, PES was chosen, and as a biodegradable polymer, PHB was chosen. These polymer substrates were exposed to laser fluence below and above their ablation threshold. This exposure may lead to the formation of different surface structures. The most frequently observed types on aromatic polymers were LIPSS and surface dots. It was found that the ripple pattern has a period roughly comparable to the wavelength of the incident laser radiation, and the pattern dimensions depend on many factors, such as chemical composition of the polymer substrate or laser wavelength. We have found that the laser treatment can be used both for the construction of polymers with enhanced compatibility or, on the contrary, for polymers with significant decrease of surface adhesion and proliferation, which can be consequently used for single-cell assays application.

II. Experimental

A. Materials and Modification

As a substrate, we used 50 μm thick polymer foils: PES (density 1.37 g cm^{-3}) and PHB (with 8% polyhydroxyvalerate, density 1.25 g cm^{-3}). The polymers were obtained from Goodfellow Ltd., Cambridge, Great Britain. For the modification, we used a KrF excimer laser (Coherent Compex Pro 50 wavelength of 248 nm, pulse duration of 20-40 ns, repetition rate 10 Hz). The beam of KrF laser was polarized linearly with cube of UV grade fused silica 25x25x25 mm^3 with active polarization layer. For homogeneous illumination of the samples, we used only the central part of the beam by means of an aperture (0.5x1.0 cm^2). The samples were mounted onto a translation stage at three different positions of the sample and laser beam: Perpendicular (0°) and under the angles of 22.5 and 45° with respect to the surface normal. We used 100-6000 pulses with laser fluences in interval 4-40 mJ cm^{-2}.

B. Measurement Techniques

Surface morphology and roughness of the pristine and modified polymer samples were examined by the atomic force microscopy (AFM) technique using a VEECO CP II device in tapping mode. A Si probe RTESPA-CP with the spring constant 20-80 N m^{-1} was used. The mean roughness values (Ra) represent the arithmetic average of the deviations from the centre plane of the sample.

Contact angle was determined by goniometry with static water drop method. The measurements of water contact angles were performed using distilled water (6 different positions) using the Surface Energy Evaluation System (SEE System, Advex Instruments, Czech Republic).

C. Modification SEM Analysis of U-2 OS Cells Adhesion

Detailed morphology of human U-2 OS (derived from osteosarcoma) cells adhered to and growing on pristine and laser modified PHB was examined by scanning electron microscopy (SEM) 48 h after seeding. Microscopic glass slides served as controls. First, the tested samples were sterilized in 70% ethanol for 1 h, inserted in 12-well plates (Ø 2.14 cm), washed by PBS and weighted by hollow plastic cylinders from poly(methyl methacrylate). U-2 OS cells were seeded on the samples in the density of 20,000 cells·cm^{-2} in 1 ml of high glucose Dulbecco´s Modified Eagle Minimum Essential Medium (DMEM; Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific, USA) and 2 mM L-Glutamine (Sigma, USA). The cells were cultivated for 24 and 72 h at 37 °C, humidity 95% and 5 % CO_2. Then, the cells were washed three times with PBS (pH = 7.4), fixed by Karnovsky solution in cacodylate buffer and dehydrated with ethanol (50, 70, 80, 90 and two times 99.9% for 10 min.). As a next step, hexamethyldisilazane (HDMS) was used to further dehydrate the samples (two times 10 min.). The cells intended for analysis by SEM were coated with a thin layer of gold

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(20 nm) and analyzed by TESCAN LYRA3 GMU (Tescan, CZ) in a secondary-electron mode.

III. Results and Discussion

A. Surface Morphology of PES

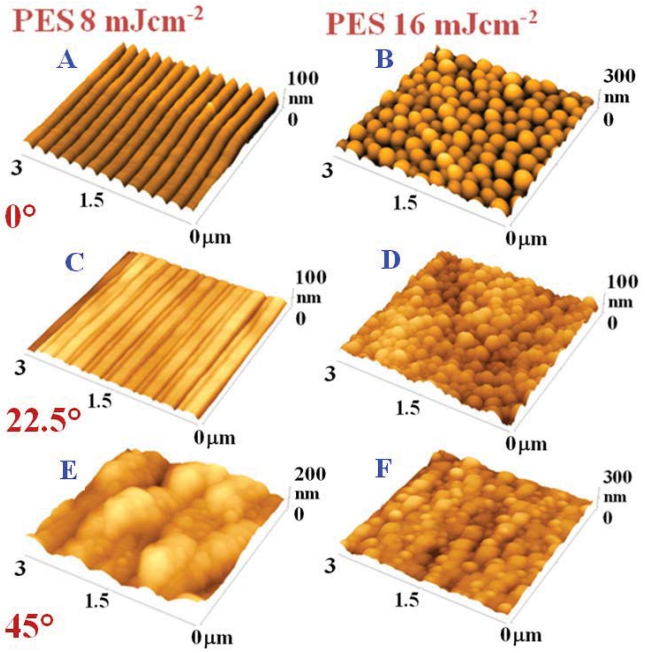


Fig. 1 3D AFM images (3.0×3.0 µm^2) of PES: surface morphology of samples treated by laser beam at the fluence of 8 mJ cm^{-2} and 6,000 pulses at different angles of laser beam incidence (0°- A, 22.5° - C and 45° - E) and samples treated by laser beam at the fluence of 16 mJ cm^{-2} and 6,000 pulses at different angles of laser beam incidence (0°- B, 22.5° - D and 45° - F)

The main aim of our study was to investigate the influence of laser treatment on different polymers, mainly one representative of biopolymers, PHB, and one representative of aromatic polymers, which is also frequently used in the field of tissue engineering, PES. We have determined that the most regular structures have formed after laser treatment by 6,000 pulses at two characteristic laser fluencies of 8 and 16 mJ cm^{-2}. Non-modified (pristine) samples are naturally very flat, with surface roughness not exceeding 1 nm. Using laser fluence of 8 mJ cm^{-2} and 6,000 pulses led to the formation of regular ripple pattern (Fig. 1). We have also observed the formation of a dot patterning if higher laser energy was applied. The width and height of PES pattern can be also determined, e.g. the width of the pattern prepared under 8 mJ cm^{-2} and 0° was approx. 240 nm and the height was approx. 55.0 nm. Generally, the most regular structures were obtained by laser treatment under perpendicular angle of incidence. The tilt of 22.5° caused a decrease in regularity and the extension of the dimension of the surface structures. This effect was even more pronounced when an angle of 45° was applied, where the regular surface arrangement was fully lost for PES samples. Similar behavior was observed after treatment by laser fluence of 16 mJ cm^{-2}, for which regular dots were formed. In the same manner as for the ripples, the dot pattern regularity was lost during the tilting of the stage to higher angle values (Fig. 1). The modified surface morphology is connected with surface area and roughness of the treated material. Because of that we have introduced the results of surface area analysis into Fig. 2, more importantly the values of surface roughness for samples treated with laser fluence 8 mJ.cm^{-2} (A) and 16 mJ.cm^{-2} (B) are introduced on the right of the figure. It is obvious that surface area increases with higher laser fluence. As for surface roughness, we have observed that the lowest roughness was created by the tilt of 22.5° for all modifications. On the other hand, the highest increase in roughness was observed after tilting to 45°.

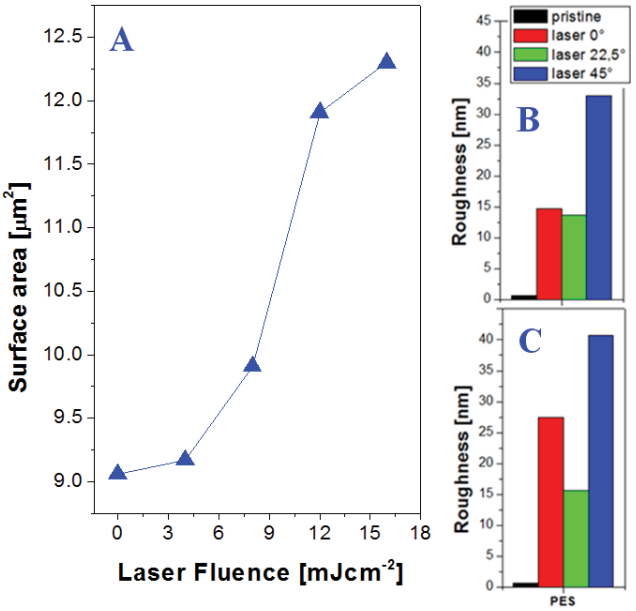


Fig. 2 The dependence of the surface area of laser-treated PES (6,000 pulses) on laser fluence (A), the dependence of PES roughness on the angle of the stage tilt at the laser fluence of 8 mJ cm^{-2} (B) and (B) 16 mJ cm^{-2} (C) is introduced

B. Wettability of PES

The changes of contact angle depending on the laser fluence are introduced in Fig. 3. The errors of measurement for all samples were in the range of ± 3.0°. The contact angle value of pristine PES was 66.1°. A treatment even by a smaller laser fluence caused a rapid decrease in the contact angle (laser fluence 4 mJ cm^{-2} induced a drop in the contact angle down to approx. 10°). We have determined that PES became more wettable after the excimer laser treatment; this effect is connected with changes in surface oxidation and also roughness [13], [22], [27], [28]. Surprisingly, with increasing laser fluence, the contact angle also increased. The values for PES treated by higher laser fluence (25 mJ cm^{-2}) even exceeded the value of a pristine polymer. The impact of the changes of the incidence of the laser beam with respect to the surface normal (tilting) on surface wettability was also studied (Fig. 3, right part). It was determined that tilting of a sample to the angles of 22.5° (A) and 45° (B) induced only small changes of contact angle in comparison to the major change

induced by changing the laser fluence. For most of the treatments, we determined the highest value of the contact angle to be achieved under the tilt of 22.5°; nevertheless, the difference was in the range of the measurement error (± 3.0°). We can conclude that changes in contact angle are induced mostly by chemical changes on the polymer surface, but for higher values of roughness of polymer substrates, the roughness also noticeably influences the surface wettability [29].

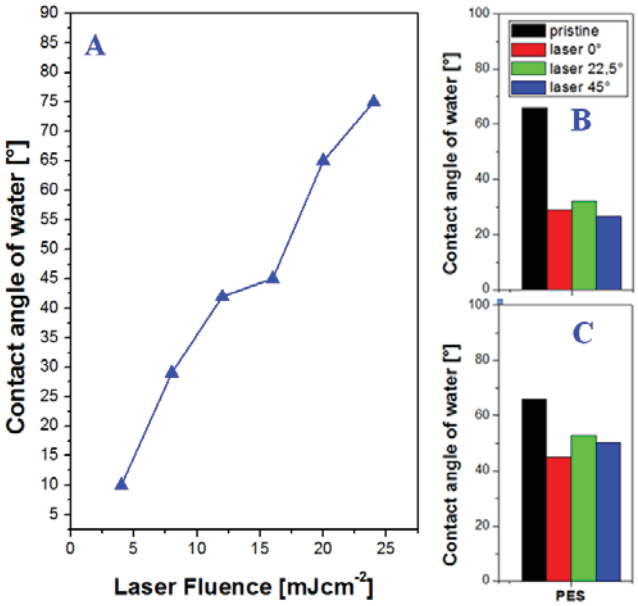


Fig. 3 The dependence of the water contact angle of laser-treated PES (6,000 pulses) on laser fluence (A), the dependence of the water contact angle of PES on the angle of the stage tilts for the laser fluence of (B) 8 mJ cm^{-2} and of (C) 16 mJ cm^{-2}

C. Surface Morphology of PHB

For consequent study, the biopolymer PHB, which has no aromatic ring in its chain, was chosen. Our aim was to study the wettability and morphology of a polymer without benzene ring and its comparison with a polymer in which the aromatic ring is present (PES). The pristine PHB foils consist, as is the case for most biopolymers, of larger segments, which can be positively detected on AFM images as grain of granular structure [29]. A significant change in surface roughness induced by simple excimer laser processing causes significant change in surface roughness, which are visible in Fig. 4. Due to the increase in laser fluence, a significant increase in surface roughness is induced on the PHB surface (31.9 → 47.1 → 270.0 nm). The higher laser fluence is a source of heat accumulation induced by higher laser dose. This effect supports the distortion of the lamellar structure. This phenomenon (the roughness change) may as a consequence guide the growth of cells and it may very well exhibit a natural barrier for cell growth based on altered surface roughness and/or chemistry.

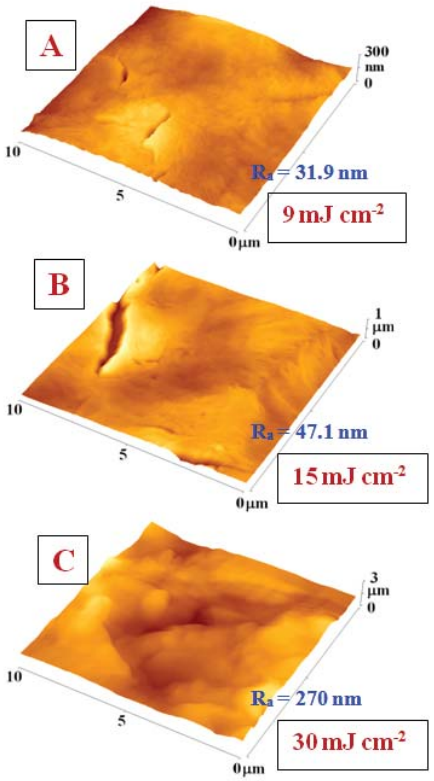


Fig. 4 3D AFM scans of PHB laser treated by 6000 pulses and different laser fluence – 9 (A), 15 (B) and 30 (C) mJ cm^{-2}. Ra represents arithmetic mean roughness

D. Surface Wettability of PHB

Biopolymer PHB was treated by an excimer laser and different number of laser pulses (from 1000 to 6000). We have observed that that the laser treatment contributes as a major factor to the contact angle/wettability change. The contact angle value for pristine PHB is approx. 64° (Fig. 5). As it is apparent from this figure, both laser fluence and number of laser pulses contribute significantly to changes in surface wettability. It is obvious that the lowest values of contact angle were observed for samples treated by the highest number of laser pulses, 6000. It is obvious from the measured values that for a constant number of laser pulses, an increase in laser fluence leads to the decrease of the contact angle. This effect was observed for all three studied numbers of laser pulses, therefore the lowest value of contact angle and thus the "highest" wettability was detected for samples which were treated by 40 mJ.cm^{-2} and 6000 pulses, the contact angle was determined to be 42.9°, which is significantly lower value than that which was measured on a pristine PHB. On the contrary, on samples treated only by 1000 pulses and 5 or 10 mJ.cm^{-2}, the values of contact angle were even higher than for pristine PHB, and with increasing laser fluence the contact angle decreases further, corresponding to the number of applied laser pulses. As we have observed earlier, the contact angle is strongly influenced by the surface chemistry and ablation (modified surface layer is removed), which is the main reason for wettability alteration [26].

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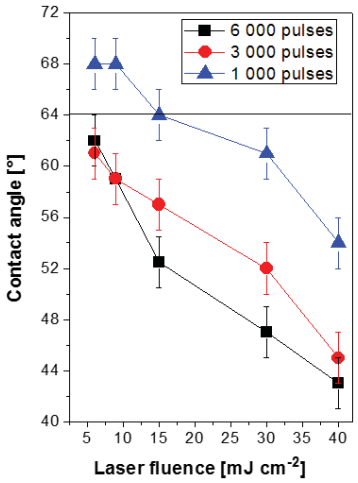


Fig. 5 Dependence of the contact angle on laser fluence for excimer laser treated PHB. Different numbers of laser pulses (1000, 3000 and 6000) were applied. Black line represents contact angle for pristine PHB

E. Cell Testing on PHB

We have focused on the study of adhesion and proliferation of human osteosarcoma bone cells (U-2 OS). Only the basic properties were studied, the surface morphology of cells which were seeded was analyzed. The differences in cell morphology of pristine PHB and PHB treated with 9 mJ.cm^{-2} and 6000 pulses were tested. Laser treated polymer matrices were prepared by varying combinations of laser fluence and number of pulses. The cell response of surface treated PHB samples was followed in an interval of 6 - 72 hours. Selected samples 24 hours after seeding were visually evaluated (Fig. 6). It has been proven previously that several types of laser treatment may improve the cell response and guide the cell growth [30]-[32], therefore we have focused on the biopolymer opposite cell response, with the aim of preparing an "anti-cellular" surface, thus applicable in the field of single-cell assays. It was confirmed that the biopolymer itself acts as a biocompatible substrate and after 24 hours after seeding the cell response on pristine PHB can be successfully compared to TCPS. For several applications, it is necessary to precisely determine the area of cell growth (rectangular or circular shapes) and thus to construct single cell assays. As it is obvious from Fig. 6, different combinations of laser fluence and number of laser pulses (e.g. lower laser fluence and higher number of pulses and vice versa) may lead to the construction of a surface with a negative cell response, the shape of the preserved cells is globular, the response of the cell can be characterized as an apoptosis stage of cell growth, thus the cell count on treated surface is significantly decreased. The results were confirmed by SEM analysis of cell morphology, where it is apparent that shape of U2-OS cells on pristine PHB is rather different from that on laser treated PHB (Fig. 7). The cells on pristine PHB are well spread with a number of cell filopodia on the surface. The laser treated PHB has higher surface roughness with cells randomly spread across the surface with almost no filopodia attached to the surface.

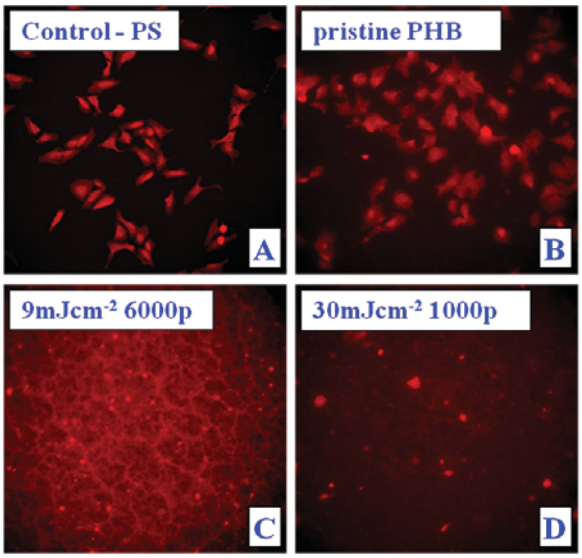


Fig. 6 Photographs of adhered and proliferated U-2 OS cells 24 hours after seeding on tissue polystyrene (A), pristine PHB (B), and PHB treated by 9 mJ.cm^{-2} 6000 pulses (C) and 30 mJ.cm^{-2} and 1000 pulses (D)

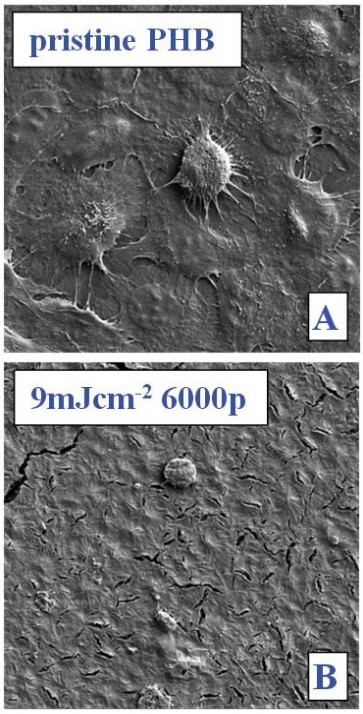


Fig. 7 SEM images of U-2 OS cells cultivated for 48 h on pristine PHB (A) and PHB treated with 9 mJ.cm^{-2} and 6000 pulses (100 µm scan) (B) photographs

IV. Conclusion

An excimer laser was used for the treatment of a polymer containing a benzene ring (PES) and a biopolymer PHB. Excimer laser treatment of PES films by excimer laser light was successfully applied for foil nanostructuring with the aim of nanopattern construction. We have determined the optimal

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laser parameters for the construction of a regular ripple pattern and of a nanodot pattern. We have observed increasing trend of surface wettability measured immediately after laser treatment. For PES, the optimal conditions for the construction of the two patterns are 8 $mJ.cm^{-2}$ for 6000 pulses for the ripple one and 16.mJ cm^{-2} for 6000 pulses for the dot one. The relationship between the treatment and the changes of the contact angle (wettability), surface morphology and surface area was characterized. The contact angle of treated PES increased with laser fluence, which corresponded with an increase in roughness and surface area.

Pristine PHB was confirmed to be an excellent substrate for U-2 OS cell growth. It was determined that the excimer laser treatment plays an important role in PHB wettability changes. An increase in laser fluence leads to a significant increase in both the surface roughness and the surface area of PHB surface (of one order). Certain combinations of laser fluence and number of pulses were used for construction of a specific surface with an anti-cell response. By a simple laser treatment, we can thus construct a biopolymer surface with an U-2 OS cells growth decrease and thus limit the area of cell growth precisely. This modified surface may be used for cell growth guidance with a natural barrier for cell growth and can therefore be applicable is single-cell assays.

ACKNOWLEDGMENT

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