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Proliferation of aligned mammalian cells on laser-nanostructured polystyrene

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Abstract

Biomaterial surface chemistry and nanoscale topography are important for many potential applications in medicine and biotechnology as they strongly influence cell function, adhesion and proliferation. In this work, we present periodic surface structures generated by linearly polarized KrF laser light (248 nm) on polystyrene (PS) foils. These structures have a periodicity of 200–430 nm and a depth of 30–100 nm, depending on the angle of incidence of the laser beam. The changes in surface topography and chemistry were analysed by atomic force microscopy (AFM), advancing water contact-angle measurements, Fourier-transform infrared spectroscopy using an attenuated total reflection device (ATR-FTIR) and X-ray photoelectron spectroscopy (XPS). We show that the surface laser modification results in a significantly enhanced adhesion and proliferation of human embryonic kidney cells (HEK-293) compared to the unmodified polymer foil. Furthermore, we report on the alignment of HEK-293 cells, Chinese hamster ovary (CHO-K1) cells and skeletal myoblasts along the direction of the structures. The results indicate that the presence of nanostructures on the substrates can guide cell alignment along definite directions, and more importantly, in our opinion, that this alignment is only observed when the periodicity is above a critical periodicity value that is cell-type specific.

Keywords: Cell proliferation; Cell morphology; Laser manufacturing; Nanotopography; Polystyrene

1. Introduction

Many medical and biological applications such as tissue engineering, cell culture substrates and cell bio-chips require the interaction of biological cells with the substrate. Therefore, it is relevant to investigate the influence of surface chemistry and topography on cell behavior such as cell adhesion, proliferation, and gene expression.

Numerous studies have focused on modifying the surface chemistry to improve the biocompatibility of substrates and to enhance cell attachment and proliferation [1]. This can be achieved by grafting of the polymer surface with various chemicals such as fibronectin [2–4] or RGD [5], by microcontact printing [6], monolayer self-assembly [7], ion irradiation [5,8,9], plasma treatment [10–12], electron beam [13], gamma rays [14] and UV photons [15–23]. The enhanced biocompatibility of the modified polymer surfaces is based on the incorporation of new chemical groups into or at the surface and the change of polarity and wettability.

In addition to surface chemistry, substrate topographical features have a great effect on cell adhesion and spreading, as well as on the cell shape [24–30]. Furthermore, changes in the surface roughness can significantly enhance the biocompatibility. It is known that the optimal micro-roughness is strongly cell-type dependent [25], and for many cell types, an additional nano-roughness is reported to promote cell adhesion and proliferation [31,32]. This effect may be due to

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a similarity to nanostructures which can be frequently found in the natural extra-cellular matrix (e.g., nano-stripes in collagen) [33]. A wide range of techniques, such as nano imprint lithography [6], laser holography [34,35], nano-lithography [31], nano imprinting methods [36], laser machining [17,18,20,22], electrospinning [37] and polymer demixing [38] have been employed in the recent years to structure the surface of polymers and other materials relevant for reconstructive and implantation surgery, giving rise to various kinds of nanofeatures on substrate surfaces, such as grooves [34,36], ripples [17,20,22,23], islands [38], pillars [38], particles [39], dots [31], and fibrillar networks [37].

In this study, laser irradiation has been used to generate periodic surface nanostructures on polystyrene (PS) foils. PS is widely used in cell culture applications because of its non-toxicity, high transparency, and low production cost. Previous studies have shown that the illumination of polymers by polarized UV laser beam can induce self-organized ripple structure formation within a narrow laser fluence range well below the ablation threshold [17,20,22,23,40–43]. The period of the ripples depends on the laser wavelength and on the angle of incidence of the radiation, and their direction is related to the laser beam polarization [44]. The behavior of human embryonic kidney (HEK-293) cells, Chinese hamster ovary (CHO-K1) cells and skeletal myoblasts seeded on the PS foils is investigated. We show that adhesion and proliferation of cells is enhanced for the irradiated foils in comparison to the unmodified PS. Furthermore, we report on the alignment of the cells along the direction of the ripples, which is cell-type dependent and occurs only when the periodicity of the nanostructures is above a critical value.

2. Materials and methods

2.1. UV modification of polystyrene foils

The experiments were performed on polystyrene (PS) foils with a thickness of 25 μm (Goodfellow Ltd., Cambridge, UK). Irradiation was carried out in ambient air with a linearly polarized laser beam at 248 nm wavelength. A KrF laser (Lambda Physik Compex, Germany) was employed at a repetition rate of 10 Hz. Pulses (6000) were applied in the irradiated area at a fluence in the range from 7.1 up to 8.9 mJ/cm^2. The polymer foils were irradiated at different angles of incidence, i.e. normal incidence, 15, 30 and 45°. The laser pulse energy was adjusted to achieve constant laser fluence (energy per area) at the sample surface for irradiation under all angles employed.

2.2. Characterization of polystyrene surfaces

We studied the topography and the chemistry of the polymer foils. The topography was examined via AFM imaging (diCP-II, Veeco) in non-contact mode and the chemical modifications induced by the laser irradiation were studied by contact-angle measurements, ATR-FTIR and XPS.

For contact-angle measurements, a small drop of de-ionized water (volume of about 10 μl) was placed at the flat polymer surfaces using a syringe in a way that the drop volume at the surface expands. The resulting advancing contact angle was evaluated by optical methods using a digital camera (Canon PowerShot S80). A photo of the drop was taken and analysed. All measurements were performed in air-conditioned lab at 22 °C and for each sample the measurement was repeated eight times.

ATR-FTIR spectra were recorded by a FTIR spectrometer (Equinox 55, Bruker, Karlsruhe, Germany) with a single reflection attenuated total reflectance (ATR) diamond system (Golden Gate, Specac, Orpington, UK). The spectra were recorded with a spectral resolution of 4 cm^{-1}. A spectra library (Mentor Pro, Biorad, Hercules, CA) was used to identify the peaks and the corresponding bond vibrations. The analysis depth is between 1 and 10 μm in the range of wavenumbers studied.

XPS characterization was performed employing the monochromatic Al Kα line (1486.6 eV) with a Kratos Axis165 spectrometer (Kratos Analytical, Manchester, UK). The typical analyzed depth was about 7 nm, the analyzed area 0.3 mm × 0.7 mm. The detection limit of elements is around 0.05 at% down to 0.02 at%.

2.3. Cell culture and light microscopy

HEK-293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Vienna, Austria) with 10% fetal bovine serum (FBS, Life Technologies), 2 mM L-glutamine (Life Technologies) and 100 units/mL penicillin/streptomycin (Life Technologies). The cells were incubated at 37 °C and 98% humidity in air containing 5% CO_2 and were used within 2–5 weeks. CHO-K1 cells were grown in DMEM/Ham's F12 medium (PAA, Pasching, Austria) supplemented with 5% FBS and 1% penicillin/streptomycin. A cell line derived from rat skeletal myoblasts [45] was maintained in DMEM supplemented with 10% FBS and 5 μg mL^{-1} gentamycin (EuroClone, Pero, Italy). Primary human skeletal myoblasts (a kind gift of Dr. Marksteiner, Innovacell, Innsbruck, Austria) were grown in Ham's F10 medium supplemented with 10 μg mL^{-1} insulin, 130 μg mL^{-1} creatine, 100 μg mL^{-1} Na-pyruvate, 50 μg mL^{-1} uridine (Sigma–Aldrich, Vienna, Austria) and 5 μg mL^{-1} gentamycin. Cells were harvested by trypsination and seeded onto the PS foils, typically at a density of 2600 cells/cm^2. Prior to cell seeding the foils were washed twice with 70% ethanol.

For light microscopy, the cell culture dishes were removed from the incubator and analyzed within a few minutes. Phase contrast images were acquired with two microscope setups in inverted configuration (both Zeiss, Oberkochen Germany), equipped either (a) with a PCO-sensicam (Kelheim, Germany) or (b) an AxioCam HR (Zeiss) CCD-camera.

A Pico Plus AFM (Agilent Technologies, Tempe, AZ) was used for acquiring topographical and deflection images of PS foils seeded with CHO-K1 cells using contact mode in PBS buffer at room temperature. Scan frequency was 1 Hz. The nominal spring constant of the used Silicon Nitride cantilever (Veeco, Dourdan, France) was 0.03 N/m.

2.4. Viability assay

For studying the proliferation of HEK-293 cells on unmodified and irradiated PS foils, the Cell Titer Blue (CTB) cell viability assay (Promega, Germany) was used. This assay is a fluorimetric method for determining the number of viable cells in culture. It uses an indicator dye, resazurin, to measure the metabolic capacity of cells. Viable cells retain the ability to reduce resazurin to resorufin, which is highly fluorescent (excitation 535 nm/emission 595 nm) while nonviable cells do not reduce the indicator dye, and thus do not generate a fluorescent signal. Data are presented as means ± standard error of the mean (SEM). Statistical significances were calculated by the unpaired Student's *t*-test with an error probability, p. Differences were considered as statistically significant only for $p \leq 0.05$.

For calibration of the assay, fluorescence signals obtained from various numbers of HEK-293 cells were measured. The correlation between fluorescence and the absolute number of cells was found to be linear over the range studied (5000–100,000 cells).

2.5. Cell orientation

The orientation of elongated CHO-K1 cells was evaluated by measuring the angles of individual cells by means of the image processing software package SigmaScan Pro (Jandel, San Rafael, CA). The mean angle is derived by taking the statistical average over all cell angles. The cells were then classified according to their angular deviation from the mean angle in each image. Standard deviations for the relative number of cells per class were obtained by evaluation of $n = 5$ images from each sample series.