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Micro and nano-structured surfaces

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The study of cell reaction to micro and nanotopography is dependent on the method of manufacture available. Several methods of manufacture have been developed: polymer demixing, embossing and photolithography. Surfaces obtained with these different techniques, having micro and/or nanodomains, have been studied toward the same type of cells, i.e. human endothelial cells (HGTFN) and mouse fibroblasts (3T3). Polymer demixing of polystyrene (PS) and poly(4-bromostyrene) (PBrS) producing nanometrically islands of 18, 45 and 100 nm height, polycarbonate (PC) and polycaprolactone (PCL) grooved with grooves 450 nm wide and 190 high, the natural polysaccharide hyaluronic acid (Hyal) and its sulfated derivative (HyalS) photoimmobilized on silanized glass as grooves 250 nm high and 100, 50, 25 or 10 μm wide have been obtained. The morphology and polarization of the cells has been studied by optical microscopy and scanning electron microscopy. Cells respond in different way to the topography of the materials, but the surface chemistry is dominant in inducing different cell behavior.

Introduction

The idea of nanofabrication materials containing few geometrically ordered atoms is very charming, by scientific and practical point of view. In the last decades several techniques have been developed to realize nanostructures, but just in the last years the study of their properties and their possible applications has been extended. While the application of nanotechnology in the electronic and optical engineering field in which smaller, faster and cheaper dispositives are required, is well established, the use of nanostructured materials in other fields such as medicine and biology is really innovative. Up to now, microscopic biosensors able to record cellular activity [1] and the realization of nanopatterned surfaces able to work as cell guidance have been realized. The realization of micro or nanostructured materials mean to obtain ''intelligent'' biomaterials capable to modulate two important signals due to the cell-substrate and cell–cell interactions in such a way to build a new tissue or organ. The surface chemical composition and topography have strong effects on cell cytoskeleton and shape, and cells may react according to the topography type [2, 3]. In order to create the nano or micro-features it is useful to consider that the cell dimensions are about 10–20 μ, but on the cell membrane some functional domain present dimensions in the order of the nano scale [4, 5].

In this paper different techniques such as polymer demixing, injection molding and photoimmobilization have been used in order to fabricate different micro and nanofeatures and to study cell behavior on them. The polymer demixing is a quite new method. Blends of polymers spontaneously undergo phase separation during spin casting onto glass substrate. By controlling the concentration and the proportions of the polymers, different topography can be produced: pits, islands, ribbon of varying height or depth [6]. Polystyrene (PS) and poly(4-bromostyrene) (PBrS) nanohills were

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obtained with mean heights from 95 to 18 nm. The injection molding concern the pouring of a polymer solution on to a specific stamp and let the polymer take shape of the pattern [7]. Polycarbonate (PC) and polycaprolactone (PCL) grooved surfaces with grooves 450 nm wide and 190 nm deep have been obtained using this procedure. The photoimmobilization is based on creating the pattern on the surface by UV irradiation through a photomask with the desired pattern after casting a polymer solution [8].

The reaction of an established human endothelial cell line (HGTFN) and a cancer mouse fibroblast line (3T3) to the micro and nanostructured surfaces have been investigated.

Materials and methods

Fabrication of the nano and microstructured surfaces

Three different types of materials have been obtained.

- The polymer demixing procedure using a mixture of polystyrene (PS) and poly(4-bromopolystyrene) (PBrS) to fabricate nano scale hills of reproducible height has been already described [7, 9]
- Polycarbonate (PC) and polycaprolactone (PCL) linear grooves were manufactured by injection molding. The obtained grooves were 450 nm wide and 190 nm deep separated by a 1000 nm wide ridge
- Micropatterned surfaces were prepared by photo-immobilizing the hyaluronane (Hyal) or its sulfated derivative (HyalS) adequately functionalized with a photoreactive moiety, on aminosilanized glass substrate in the presence of a chromium photomask. Four different patterns having stripes of 100, 50, 25 and 10 wide and about 250 nm high were obtained as previously described [10, 11]. The same pattern was obtained via laser ablation. The glass surfaces having a continuous thin layer of hyaluronic acid or sulfated hyaluronic acid obtained by UV irradiation, were irradiated by focused UV-laser pulses which went through a photomask with a stripes pattern. The treatment allows the controlled polysaccharide's removal thereby printing the printing.

The dimensions of the different type of features were evaluated by atomic force microscopy (AFM ThermoMicroscope, Veeco Instruments).

Cell behavior analysis

In order to study the influence of the nano and micropatterned surfaces on mouse fibroblasts (3T3 line), and an established human endothelial cells (HGTFN line) behavior adhesion and proliferation tests were performed.

All the patterned samples were sterilized by dipping into 70% ethanol for 10 min and left to dry at room temperature. Fibroblasts 3T3, endothelia HGTFN were seeded at a density of 1×10^4 on the materials and added of 1 ml of complete medium. The cell behavior as well as the cell number was evaluated time by time with a phase contrast and optical microscope.

Cell morphology was also analyzed by scanning electron microscopy (SEM) using the procedure already described [10, 11]. Briefly, each sample was rinsed with sodium cloride (NaCl 0.9%) and incubated in 1.5% (v/v) glutaraldehyde in 100 mM sodium cacodylate for 30 min at room temperature. The samples were then washed with NaCl 0.9% twice and left standing in primary dehydration solution [50% (v/v) ethanol in H_2O] for 15 min. They were then transferred to a secondary dehydration solution [70% (v/v) ethanol in H_2O] for 15 min and subsequently to absolute ethanol for 15 min for total cells dehydration. Finally, the samples were desiccated overnight and gold sputtered with an automatic sputter coater, and analyzed by SEM (XL20 Philips, The Netherlands) at 15 kV accelerating voltage.

Results

Polymer demixing materials

The surface chemistry of the nanohills was essentially PS, because the materials subjected an annealing process. Topographical structures of the samples showed a smooth profile of the bumps, as evidenced by AFM analysis. The hills height (ranging from 95, 40 to 18 nm for the different samples) was a mean value and there was a random distribution of hill center to center distances. A micrograph of PS/PBrS having 95 nm average high bumps is reported in Fig. 1.

The fibroblast (3T3 line) and endothelial cells (HGTFN line) behavior show a similar trend. On the PS and on PBrS flat materials, used as control, cells spread well, showing a good affinity for both the materials. No significant difference in the number of adhered fibroblasts appears on the native PS or PBrS surfaces, while the number of spread HGTFNs is significantly larger on the flat PBrS one. On the nanobumped materials cell density was higher than on flat ones, and among the different nanofeatures the highest number of adhered cells was present on the smallest features (18 nm high) either with fibroblasts or endothelial cells. The trend of cellular counts is reported in Fig. 2. On flat materials SEM analysis showed cells with a normal morphology with flattened appearance, sometimes following the surface irregularities with pseudopodia which stuck on the holes of the surface. On the nanohill materials their morphology was

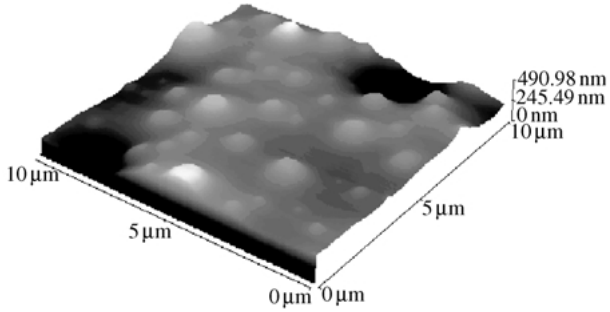

Figure 1 AFM micrograph of PS/PBrS nanobumped material. The hills average height is 95 nm.

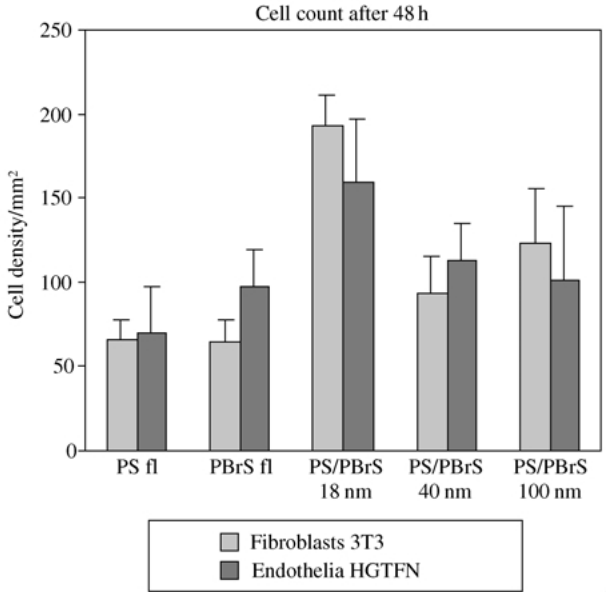

Figure 2 Cell counts graph showing fibroblasts (3T3) and endothelia cell density/mm^2 on polymer demixing materials having hills of different average height.

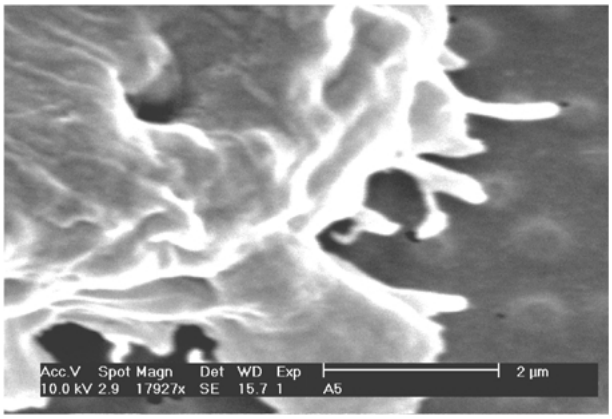

Figure 3 SEM images of an endothelial cell (HGTFN line) spreading on PS/PBrS 18 nm high nanohills: the pseudopodia adhered on the top of the hills.

generally spread; in some non-adhering regions cells emitted many pseudopodia which adhered on the top of the bumps. This type of behavior was strongly remarked on 18 nm nanohill materials; a SEM image showing HGTFN behavior on PS/PBrS 18 nm nanohills is reported in Fig. 3.

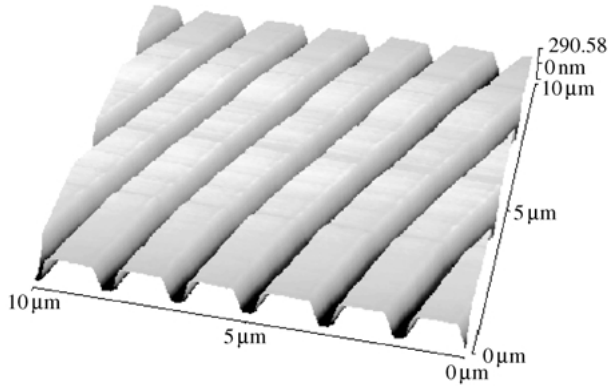

Figure 4 AFM micrograph of PCL grooved material. The grooves are 450 nm wide and 190 deep separated from a space of 1 µm.

Analyzing another cellular line, i.e. HUVEC primary endothelial cells, a similar behavior to the previous ones was observed allowing us to exclude any influence of the cellular line. Different cell lines react in the same way following the surface topography. The smallest features seemed the most suitable for cell proliferation and morphology control.

PC and PCL grooved materials

The surface topography consists in continuous linear grooves 450 nm wide and 190 nm deep having a very sharp edge and no irregularities on the features. An AFM micrograph of PCL grooved material is reported in Fig. 4. Fibroblasts (3T3 line) and human endothelia (HGTFN) cell density was quite high on PC and PCL flat materials. Fibroblasts and endothelial cells spread assuming a round shape demonstrating a pretty good affinity for the native materials. Many cells in some regions expressed pseudopodia going down and adhering on the substrate.

On PC and PCL grooved materials, cells lined up along the grooves assuming an elongated shape. Some of them emitted pseudopodia that stuck on the top part of the grooves. The large dimensions of endothelia and fibroblasts induce the cells to occupy a space containing more grooves although the elongated morphology follows the groove's direction. Fig. 5 shows a fibroblast 3T3 aligning along the grooves with pseudopodia adhering on the ridge of the grooves. The different chemistry of PC and PCL materials do not absolutely

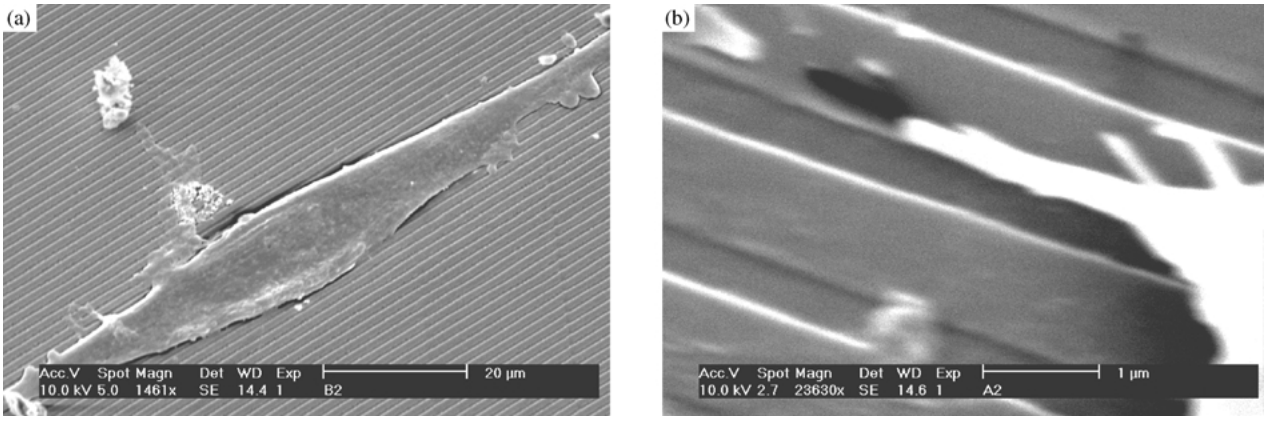

Figure 5 SEM micrographs of fibroblasts (3T3 line) lining up along PCL grooved materials (a), a detailed SEM image showing a cell emitting pseudopodia which adhere and align along the top of grooves (b).

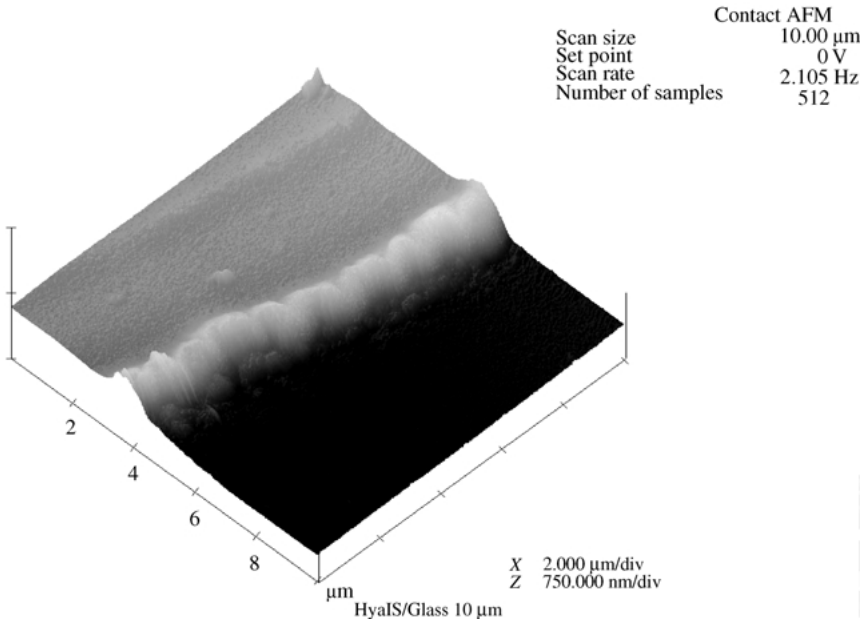

Figure 6 AFM image of a micropatterned HyalS surface on glass substrate having stripes 10 µm wide. The average height is 1 µm.

induce any change in the behavior of the cells. Only the topographical cues are responsible of the morphology of the cells, irrespective of their line.

Polysaccharides microstructures on aminosilanized glass substrate

The microstructured Hyal surfaces analyzed by AFM did not present a very sharp profile, perhaps due to the softness characteristic of the polysaccharide. The thickness of the stripe's step was about 250 nm in all the samples fabricated, and the Hyal surfaces exhibited small defects at nanometer level.

Regarding the micropatterned HyalS samples, the topographical structures showed a smooth morphology of the HyalS domains and a roughness of few nanometer on glass domains. The area around the edges were sometimes irregular at nanometer level, but sharp, as reported in Fig. 6. The thickness of the stripes was about 500 nm for the different samples fabricated.

On all HyalS samples HGTFN cells spread lining up along the polysaccharides stripes [10]. The behavior and shape was slightly different according to the stripe dimensions. On stripes 100 µm wide, cells spread on HyalS assuming a flat shape and occupying the largest available area. On stripes 50 µm and 25 µm wide they lined up along the edges, while on the microfeatures 10 µm wide cells lined up along the HyalS stripes, assuming an elongated morphology. As an example SEM images showing endothelia behavior on 100 µm and 10 µm microdomains is reported in Fig. 7(a) and (b). On the contrary, on Hyal micropatterned surfaces endothelial cells spread lining up along the surface's glass grooves independent of the stripes dimension. The typical cell response on the widest microfeatures (100 µm) was an adhesion on the Hyal stripes edges in the first 24 h of culture and a later placing in the middle part of the glass stripes. On the thinner stripes (50, 25, 10 µm) cells lined up along glass stripes in the first 24 h of culture. The fibroblasts 3T3 showed the same type of reaction of the endothelial cells on micropatterned Hyal surfaces.

Also a primary endothelial cell line (BAEC) was studied on Hyal and HyalS micropatterned surfaces. On Hyal micropatterned surfaces cells spread lining up along the glass stripes in the first 24 h of culture independent of the microdomain wideness and maintained the alignment even after a week. On HyalS micropatterned materials BAECs do not spread easy, but after two days they were

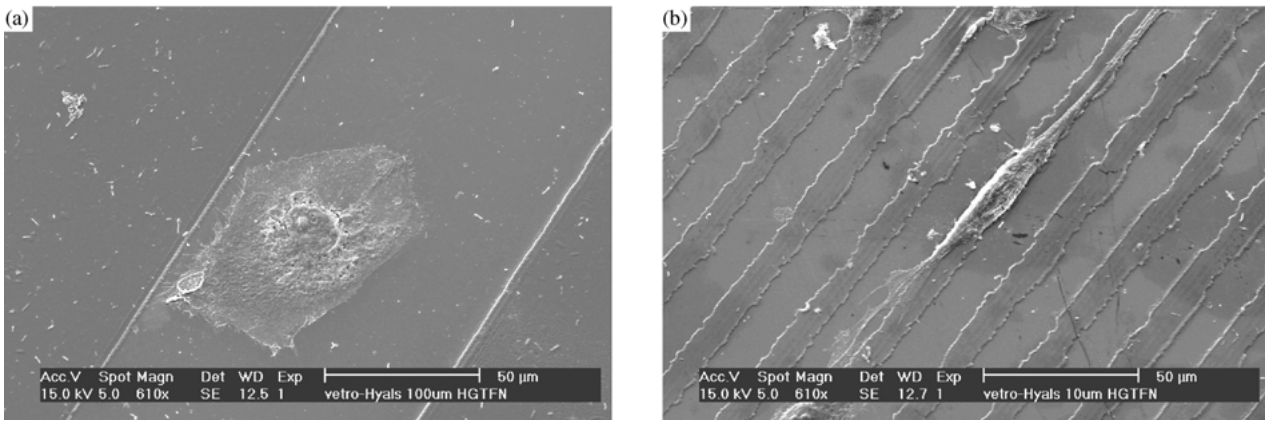

Figure 7 SEM micrographs of endothelial cells (HGTFN line) spreading on HyalS stripes 100 µm wide (a) and on HyalS stripes 10 µm wide (b). Cell assumes a very flatten shape on the bigger stripes, while an elongated morphology on the smaller ones.

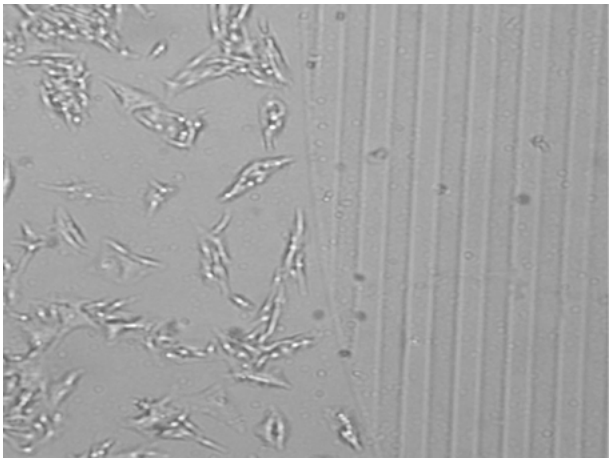

Figure 8 Optical microscope micrograph showing fibroblasts (3T3 line) adhering on micropatterned Hyal on Hyal substrate. Cells adhered on silanized glass outside the micropatterned area.

able to line up along the glass stripes performing the same behavior as Hyal micropatterned materials. According to these results it is evidence that the cell behavior depends on the type of cells, but it is strongly influenced by the surface chemistry. Micropatterned Hyal surfaces were tested also with cancer melanocities (B16F1). The behavior was completely different: cells spread on silanized glass out of the micropatterned area with a random distribution [11]. That behavior was ascribed to the presence of a thin layer of Hyal also between the polysaccharide stripes, masking the presence of the aminosylanized glass. A similar behavior was found with the micropatterned Hyal photoimmobilized on a continuous layer of Hyal substrate. A step of Hyal 200 nm high was obtained. The influence of topography on cell behavior was zero, because cells moved from the micropatterned area to the outside and spread on glass surface as reported in Fig. 8, showing the scarce affinity of Hyal to allow cell adhesion.

Conclusions

Using three different techniques, three different type of patterned surfaces have been obtained:

- Polystyrene-poly(4-bromopolystyrene) nanohills of different mean height ranging from 18 to 100 nm
- Polycarbonate and polycaprolactone with grooves 450 nm wide and 190 deep
- Hyaluronane and sulfated hyaluronane stripes on glass substrate (about 250 nm high and ranging from 10 to 100 μm wideness

Both the human endothelial and mouse fibroblast cells were investigated, obtaining similar results with the first two specimens, i.e. a poor discrimination in the behavior of the cells either related to the different cellular lines or to different nano dimensions of the material or to a different chemistry (PL and PCL).

On the contrary significant distinctions were obtained with the third specimen. The behavior of the cells was different in relation to the cellular line, chemistry of the surfaces and stripes dimensions. The micrometer dimension being of the order of that of cell is the most adapt to influence and to stimulate cell response.

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