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Ultra-fast laser microprocessing of medical polymers for cell engineering applications

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ABSTRACT

Picosecond laser micromachining technology (PLM) has been employed as a tool for the fabrication of 3D structured substrates. These substrates have been used as supports in the *in vitro* study of the effect of substrate topography on cell behavior. Different micropatterns were PLM-generated on polystyrene (PS) and poly-L-lactide (PLLA) and employed to study cellular proliferation and morphology of breast cancer cells. The laser-induced microstructures included parallel lines of comparable width to that of a single cell (which in this case is roughly 20 μm), and the fabrication of square-like compartments of a much larger area than a single cell (250,000 μm^2). The results obtained from this in vitro study showed that though the laser treatment altered substrate roughness, it did not noticeably affect the adhesion and proliferation of the breast cancer cells. However, pattern direction directly affected cell proliferation, leading to a guided growth of cell clusters along the pattern direction. When cultured in square-like compartments, cells remained confined inside these for eleven incubation days. According to these results, laser micromachining with ultra-short laser pulses is a suitable method to directly modify the cell microenvironment in order to induce a predefined cellular behavior and to study the effect of the physical microenvironment on cell proliferation.

1. Introduction

During the last few years applications of biocompatible and biodegradable polymers have experienced a big expansion. This increase is, to a large extent, linked to their potential in biomedical applications like tissue engineering [1] and clinical diagnostics [2]. Most animal cells need to attach to a surface for surviving, growing and proliferating; in other words, they are anchorage-dependent [3]. In this context, specially designed biomaterials can act like temporal scaffolds inside the human body, mimicking extracellular matrix structures and promoting cell growth and organization into a specific architecture. Recent reports have shown that cell behavior is strongly dependent on the surface properties of the substrate material, such as the topology, the charge, the degree of hydrophobicity or hydrophilicity and other mechanical or chemical properties [4–9]. Thus, by changing the physicochemical properties of the substrate, cell–material interactions can be modified and hence influence cell adhesion, migration and proliferation. Among the biomaterial properties that affect cell behavior, the substrate mechanics has a particular strong effect upon cell fate [10–12]. Cells in vivo are subjected to mechanical forces (i.e. tensile or compressive) through the extracellular matrix (ECM). Under these conditions, the cellular cytoskeleton suffers stress changes that affect cellular shape. These morphological changes may affect the direction and accumulation of cells at a proper site or even influence cell phenotype [13–15]. Stem cells can be propagated in suspension, as floating spherical colonies [16]. The influence on cell fate is especially important in the case of stem cells. Several reports have shown that the above mentioned surface properties that affect cell proliferation, adhesion, and morphology, also affect stem cell differentiation [17–20]. Thus, with different substrates that transmit different external forces to the cell it may be possible to induce or favor a certain cell fate and therefore, the development of a certain tissue. Some of the cellular processes that can be controlled by physical interactions between cells and their ECM or microenvironment (growth, differentiation, motility, apoptosis) are critical for cancer development [21–23]. Several researchers have observed that cancer

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epithelial cells revert to normal behavior (healthy cells) when they are in contact with the ECM of an embryonic tissue [24–26]. In this regard, the design of biomaterials with architectures that mimic natural cell microenvironments may be a powerful tool to better understand and manipulate cell function as a strategy for future cell-based therapeutics. In this context, surface microstructuring and micromanufacturing techniques can play an important role in the field of three-dimensional scaffold fabrication, which can make it possible for cells cultured *in vitro* to exhibit some important characteristics of *in vivo* cells, such as the cellular tridimensional networks and the structural organization of human tissues [27,28].

Surface micro- and nanopatterning of polymers can be achieved through different techniques by chemical or physical methods. The most common techniques used for surface micropatterning are lithography-based fabrication technologies and microcontact printing (μCP) [29]. Soft lithography is frequently used to create a surface pattern on a silicon wafer that can be easily replicated onto a polymeric material surface to form regular topographies like ridges, grooves, pillars and dots, but the average height of these features usually does not exceed 10 microns. Microcontact printing is a technique very extended in surface chemistry and cell patterning that enables to transfer topographic surface patterns onto chemical surface patterns with high precision by means of an elastomeric stamp. However, the transfer efficiency depends on the chemical affinity between the stamp and the substrate and therefore, only few materials can be presently patterned by this technology. μCP also shows some limitations when producing complex geometries and 3D microstructures. Moreover, these techniques are generally restricted to flat substrates and small areas, and the pattern remains in the substrate only for one or two weeks. Another novel photolithographic method with potential applications in tissue engineering is the two-photon polymerization technique (2PP) [30], which uses femtosecond laser pulses to create 3D microstructures with high spatial resolution (nearly 100 nm) by solidification of liquid photo resins due to high intensities focused on small volumes. Although it is possible to obtain almost any type of topography by means of a computer model, not all polymers can be processed by means of this technique since the solidification process depends on the solubility of a suitable photoinitiator present in the polymer solution and the density of polymerizable vinyl groups. Another drawback is the extended processing time that involves the microstructuring of large areas. Furthermore, a promising technique to fabricate topographical cues on different materials is the Direct Laser Interference Patterning technology (DLIP) [31]. This technique produces patterns in a one step process based on the interference of two or more laser beams, but only periodic structures can be fabricated.

Pulsed laser ablation is a well-established universal tool for direct surface modification of almost all type of materials. Ultra-short laser pulses (picosecond and femtosecond) enable to obtain 3D microstructures with high precision, taking advantage of the "cold" ablation process. In this process the high energy laser pulse is initially absorbed by the electrons in the material and due to the short pulse duration compared to the electron–phonon relaxation time, the energy transfer from the electrons to the lattice is not immediate. Under these conditions, the thermal effects are minimized and the melt depth produced approaches a minimum value. Thus, the laser radiation can directly etch the material by turning solid into plasma, increasing the accuracy of the topographical features generated on the substrate surface. This technology represents a very versatile method for 3D microstructuring, allowing a direct and fast processing of a wide variety of substrates and geometries; including the micromachining of complex structures on flat and non-flat surfaces.

As we mentioned before, surface modification by laser technologies is a promising technique for scaffold microstructuring. Liu et al. [32] applied for the first time the ultra-short pulsed laser technique for this purpose. They used a femtosecond pulsed laser to obtain microstructured collagen substrates (holes, grooves and grids), and analyzed the growth, adhesion and viability of human fibroblast and mesenchymal stem cells from rat bone marrow on the patterns. Only five subsequent reports have applied laser ablation by femtosecond laser for the 3D microstructuring of biocompatible materials for cell seeding [33–37]. The purpose of the research reported in this paper is to evaluate the picosecond pulsed laser ablation technology (much faster than femtosecond pulsed laser ablation technology) applied to the fabrication of three-dimensional scaffolds aimed for cell engineering. To the best of our knowledge, no other reports exist on the application of picoseconds pulsed laser ablation in the study of the effect of topographical cues on cell proliferation. We examined the effect of the laser-created topography on cell behavior by observing proliferation and adhesion of breast cancer cells on different laser structures on poly-L-lactide (PLLA, a biocompatible and biodegradable polymer used in scaffold fabrication) [38,39] and polystyrene (PS, a biocompatible polymer frequently used in the fabrication of devices for cell culture applications) [40]. It is worth noting that PLLA has been investigated for many years as scaffold for tissue engineering [41–46] showing morphological, mechanical and degradation properties that make this material very suitable for such purpose. Likewise we examined the influence of the topography in cell morphology when cells were cultured in confined environments.

2. Materials and methods

2.1. Materials

Poly-L-lactide (PLLA) was supplied by Purac Biochem (The Netherlands) [47]. PLLA pellets were dried at 40 °C for 24 h and dissolved in chloroform at a concentration of 2 wt.%. PLLA films were prepared by casting the PLLA solution on glass Petri dishes. After preparation, the films were dried at least 24 h at 60 °C to ensure complete evaporation of the solvent. Under these conditions, films of 50 μm thickness were obtained. The degree of crystallinity of these films was 4% as measured by differential scanning calorimetry. Polystyrene (PS) Petri dishes for cell culture were manufactured by SARSTEDT (Germany).

2.2. 3D micro-structuring technique

Surface microstructuring of polymer samples was carried out by means of a picosecond pulse Nd:YVO_4 laser (RAPID: Lumera Laser [48]), which is integrated in a micromachining workstation by 3D-Micromac. The laser source delivers 10 ps pulses at 1064 nm wavelength with energy of 12 μJ operating at a maximum repetition rate of 1 MHz. In addition to the fundamental mode, the laser emits at second and third harmonic wavelengths of 532 and 355 nm, with maximum energies of approximately 2.5 and 1.5 μJ, respectively, at the same repetition rate (1 MHz). The laser beam was focused over the sample by a focusing lens placed in air that has a focal length of 100 mm for light of wavelengths 532 and 1064 nm, and of 103 mm for light of 355 nm wavelength. Spot sizes (beam radius at $1/e^2$) of 30 μm and 20.5 μm were obtained at energy of 0.2 μJ for wavelengths of 532 nm and 355 nm, respectively, by selecting optional fixed beam expanders. Sample position can be selected by an XY stage with lateral resolution in the μm-range and a Z positioning system with a vertical resolution of roughly 10 μm.

By means of pulse overlapping different trenches can be fabricated. Trench width and depth were controlled by selecting an appropriate energy (E), frequency (f) and overlapping distance between pulses (d). By using a galvanometric scanner and appropriate control strategies, any desired topography and geometry can be generated on the workpiece. The optimization of the microstructuring process for PLLA was described in a previous report [49]. Flat PLLA substrates (Ra = 240 nm) and PS Petri dishes (Ra = 20 nm) were laser-irradiated applying an energy of 0.9 μJ at a frequency of 100 kHz, and 5 μm of overlapping distance. After this treatment, the average surface roughness (Ra) increased, leading to values of 700 nm and 500 nm for PLLA and PS Petri dishes, respectively (Fig. 1). Microgrooves of different width and depth were obtained on PS Petri dishes by applying pulse energies