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Direct laser writing of nanorough cell microbarriers on anatase/Si and graphite/Si

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ABSTRACT

The formation of hierarchical structures consisting of microstripe barriers decorated with nanorough ablated materials prepared by direct laser writing is described. Linear features of circa 25 μm width and 12 μm height are achieved on amorphous and crystalline titania and graphitic carbon films deposited on silicon. Ablated protrusions build up barriers decorated by nanoscale Si-film reconstructions, as indicated by EDX maps and micro-Raman spectroscopy. Wettability tests show a dramatic change in water contact angle, which leads to almost full wetting after irradiation, irrespective of the original film composition. Fluorescence microscopy images of human mesenchymal stem cells cultured on 1D and 2D structures demonstrate the short term biocompatibility of the ablated surfaces. It is shown that cells adhere, extend and polarize on feature edges, independently of the type of surface, thus suggesting that the created nanoroughness is at the origin of the antifouling behavior. In particular, irradiated anatase and graphite surfaces demonstrate an increased performance of crystalline films for the creation of cell guiding and trapping devices. The results suggest that such laser processing of films may serve as a time-and-cost-efficient method for the design of few-cells analytical surfaces.

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1. Introduction

The surface modification of functional materials by laser irradiation opens their applicability to, among other fields, biomedicine and biotechnology [1]. Laser techniques are increasingly used in view of the progress reached in tunability, pulsing and power control. The modification can activate a precise chemistry or modify the topography of a biomaterial to adapt to cell/tissue restrictions. In fact, cells in osteochondral tissues are well organized [2,3]. Thus, the formation of 1–3D patterns on complex substrates is expected to improve the biocompatibility of the chosen material. In particular, the anisotropic adhesiveness of cultured cells can be improved, as required for many biomaterials [4]. These patterns are designed to mimic the structures that cells find in tissues and it is a way to control their mechanotransduction, that is, determining the mechanical constraints that allow the multi-potent cells to generate the biomolecular signals to induce a change in function (e.g. proliferation, differentiation or apoptosis).

Additionally, this laser micromachining route has been selected for implantation of microfluidic/bioelectronic devices as biosensors in the living body [5]. By controlling locally surface properties, one can potentially construct other hybrid bio-microfluidic devices for single cell analysis, stimulation or cellular sorting [6]. For instance, this kind of structures can be used to evaluate collective and individual migrations, following the epithelial-mesenchymal transition [7], and could be used to evaluate the efficiency of new antitumoral drugs. Depending on the effect induced to cell mobility (selective migration, anchoring) such devices can be categorized as cell guides or cell traps.

The conventional methods for micromachining have relied on indirect processes assisted by photolithography [8]. However, these techniques are time consuming, making them non-ideal for cost-efficient production of lab-on-a-chip platforms. The laser-enabled micromachining for biotechnology is an emerging and ongoing research topic [9]. When envisaging micropatterns with laser technology one is faced to two main alternatives: direct laser writing (DLW) versus diffraction generated patterns. The former, also known as laser scanning photolithography [10], is advantageous when free forms are to be designed, large areas are required and ablation threshold of the material

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is high. It can be further developed to deposit biomaterials using an absorbing matrix (i.e. matrix assisted pulsed laser deposition or MAPLE) [11]. In the second family of processes, the use of diffraction gratings (or phase masks) can very efficiently produce periodic motives. Laser beam energy distribution reduces the patternable areas, which shall be created on soft organic/inorganic materials [12] or photosensitive resins (UV lasers are seldom used). From this description, it stems that DLW appears as the most promising option for the processing of inorganic biomaterial scaffolds.

This study focusses on the photoablation of TiO_2/Si and graphite/Si interfaces using an infrared laser and their primary use for the creation of platforms for few cell cultures. In fact, Martins et al. [13] measured the area of material removed by calibrated ablations and opened the path to the formation of micropatterns from composite/layered materials. TiO_2 is recurrently targeted as an appropriate biocompatible material, since it is known to act as a protective oxide on Ti implants [14] and it can be advisable as cell carrier material in view of its limited induction of fibrous tissue [15]. More specifically, sol-gel derived TiO_2 has demonstrated promising biocompatible properties for prosthetic and surgical applications [16,17]. On the other hand, it has been also shown that the deposition of thin graphitic layers improves the biocompatibility of C composites [18] and stems as a model surface for further chemical modification. Preliminary forms processed herein by DLW are assayed using human mesenchymal stem cells (hMSCs). As precursor cells of osteochondral tissues, adequate material adhesiveness and anisotropy remain key to mimic real tissue microenvironments, which is determinant for both, in-vivo biocompatibility and in-vitro stimulation of differentiation routes [19].

2. Experimental procedures

2.1. Preparation of thin-films

Si wafers ((100) orientation) were cut in 1.5×1.5 cm^2 cleaned with ethanol and used with the native oxide. Polished Si was used for TiO_2 deposition and microstructured Si (KOH homogenized pyramids with no polishing) selected for graphite deposition. The TiO_2 precursor sol contained 0.4 M TIPT (Tetraisopropyl Orthotitanate, Ti(OCH(CH3)2)4) in ethanol with total water molar ratio 0.82 and a pH of 1.27 adjusted with 0.1 M HCl as previously reported [16]. TiO_2 Spin-coating was performed by dispersing 50 μl of the precursor solution at 2000 RPM for 45 s. The thin-films were differentiated in terms of their structure by applying different thermal annealing (500 °C for the crystalline and 100 °C for the amorphous) for 2 h. This process was repeated to increase film thickness below the mechanical stress limit (3 layers for amorphous, 6 layers for crystalline coatings). Carbon (graphite) was adsorbed on microstructured Si wafers by direct scrapping of a high purity spectroscopy grade graphite bar (Le Carbone, Lorraine). Derived samples were labeled as CSiM. The films were observed to be water stable at room temperature with and without ultrasound agitation with no need for chemical grafting.

2.2. Direct laser writing

The photoablation process was carried out with an infrared laser (Nd:YVO_4, $\lambda_e = 1064$ nm, Spectra-Physics) to underline the absence of any relationship of specific absorption of the target materials (i.e. TiO_2 is a strong UV absorber) and the general applicability to any other coating/substrate systems. The ablation threshold/power calibration for DLW was controlled by the laser pulse frequency (ν) and emitted power (P) making arrays on the materials by scanning these two parameters and directly observing by SEM the produced features. Laser ablation thresholds of Wp ~0.75 and Wp ~1 W/mm^2 for amorphous and crystalline films were identified, respectively. The PC controlled X–Y stage for sample scanning below the laser allows a resolution of 1.25 μm.

2.3. Materials characterization

TiO_2 film thickness was derived by Ellipsometry (Gaertner L116B) with a 632.8 nm wavelength and 70° beam incidence, where the phase and polarization measurements of light reflected off the samples are used to fit against theoretical models of Titania on Silicon substrate. The thickness of the films can also be later confirmed by Scanning Electron Microscopy (SEM, Philips XL-40FEG and Hitachi S-3000N) images where the Energy-Dispersive X-ray spectroscopy (EDX, INCAx-sight, Oxford Instruments) is also available to perform chemical composition maps. Furthermore, X-ray diffraction (XRD) is carried out on the crystalline films in an X'Pert PRO-Panalytical system equipped with a graphite secondary monochromator. Cu Kα radiation was used in grazing incidence of 0.5° obtaining diffractograms in the 20°-60° range with 6 s integration every 0.04°. Raman spectra were taken at room temperature with a Renishaw Ramascope 2000 microspectrometer. The excitation light source was a HeNe laser with a 632.8 nm emission wavelength. The light was focused on the sample surface with a 100× microscope objective. Laser power on the sample was below 3 mW. The spatial resolution was around 1 μm. The spectral resolution and precision were about 3 and 1 cm^{-1}, respectively. Static Water Contact Angle (WCA) measurements (five droplets for each condition) were carried out in static mode in a KSW 100 with droplet volumes of 3 μl.

2.4. Cell culture and fluorescence microscopy

Pluripotential hMSCs were chosen in this work because their adhesion is known to be especially sensitive to the materials properties and their controlled transformation from progenitor to differentiated osteochondral tissues is a motivation for the processing of microstructured materials. hMSCs were isolated in a Percoll gradient from 1 or 2 ml of human bone marrow samples from anonymous healthy donors and provided by hematology services of Hospital La Princesa, Jiménez-Díaz Foundation and the Biobank of University of Málaga. Cells were plated and incubated using Dulbecco's Modified Eagle's Medium — Low Glucose (DMEM-LG) plus 10% fetal bovine serum (FBS) of selected batches. Cells were collected by treatment with 0.25% trypsin-ethylenediaminetetraacetic acid. Cell culture mediums were prepared by the research services of Molecular Biology Center "Severo Ochoa" (CSIC-UAM). Laser-processed samples were exposed to UV–light during 10 min, thoroughly washed with phosphate buffer saline (PBS), individually placed on a 24-multiwell plate (Falcon) and seeded with 5×10^3 hMSC. Then cells were incubated in DMEM-LG plus 10%FBS at 37 °C in 5% CO_2 95% O_2. After 72 h cells were rinsed with ice-cold PBS and fixed in 3.7% formaldehyde in PBS during 30 min at RT and equilibrated in PBS. For immunofluorescence, cells were permeated and all cell soluble proteins removed by incubation with 0.5% Triton in cystoskeleton buffer containing 10 mM pipes, pH 6.8, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 0.3 M sucrose for 30 min on ice. After the treatment, samples were cleaned and fixed with 3.7% formaldehyde and equilibrated in PBS. Cytoskeleton morphology samples were blocked incubating with PBS-5% bovine serum albumin (BSA) during an hour and then equilibrated with PBS-0.5% BSA and incubated successively with α-Tubulin (1:2000, Sigma), a mouse-derived secondary antibody labeled with Alexa-488 (1:500, Invitrogen) and 4′,6-diamidino-2-phenylindole (1:5000, Calbiochem)) for nuclei. Finally samples were dehydrated with ethanol (Merck) mounted with Mowiol/Dafco and visualized in fluorescence microscope Olympus IX81 coupled to a charge coupled device camera.

3. Results and discussion

3.1. Optical and structural properties

All the obtained TiO_2 films, whether amorphous or crystalline, showed good optical homogeneity, which allowed the determination

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