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Temperature responsive functional polymeric thin films obtained by matrix assisted pulsed laser evaporation for cells attachment–detachment study

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ABSTRACT

Multifunctional thin films used as thermoresponsive substrate for engineering cell sheets represent an important area in tissue engineering. As the morphology and the chemical characteristics of the thin films directly control their interaction with cells, it is important to correlate these characteristics with the biological answer. In this study, thermally sensitive poly(*N*-isopropylacrylamide), (pNIPAAm) thin films were prepared by matrix assisted pulsed laser evaporation and utilized in L929 cell adhesion and detachment studies. Fourier transform infrared spectroscopy (FTIR) and atomic force microscopy (AFM) were used to determine the pNIPAAm thin films chemical and morphological characteristics. The FTIR data demonstrated that the functional groups in the MAPLE-deposited films remained intact for fluences in the range of 200–600 mJ cm^{-2}. Within this fluence range, the AFM topographical studies showed that the roughness of the coatings was dependent on laser fluence and the obtained surfaces were characterized by a granular aspect. L929 cell viability studies onto the pNIPAAm coatings showed little or no toxic effect for fluences below 600 mJ cm^{-2}, while for higher fluences, viability was decreased with more than 50%. The adhesion and detachment of the cell was found to be mainly dependent on the film surface morphology.

1. Introduction

In the field of bioengineering applications, there is an increased interest in the use of various surfaces that can respond to different stimulus (e.g. temperature, pH, light, magnetic field) [1–4]. The stimuli control on bio-adhesion and manipulation of microorganisms or mammalian cell; or on bio-adsorption of proteins is given by the chemistry of the surface, morphology and stability in the aqueous media. The chemistry and the morphology of the surface are given by the nature of the materials used, but are also related to the fabrication method.

Among so called smart materials, poly(*N*-isopropyl acrylamide) (pNIPAAm) is a thermoresponsive polymer that present a special interest because of the phase change that undergoes in a physiologically relevant temperature range, which leads to cell/protein release [5,6]. At values above lower critical solution temperature (LCST), pNIPAAm becomes more hydrophobic and interacts more readily with proteins; whereas at lower temperatures numerous hydrophilic groups are exposed to prevent protein interaction. This phase change constitutes the main advantage of pNIPAAm, as it can be used for cell sheet harvesting in a noninvasive way [6], as trypsinisation method, which can cleave important surface molecules, can be avoided. In this way, the detachment of the cell sheet occurs from the surface and it allows that the structure of the basal lamina and cell surface receptors to be retained. Its applicability varies from obtaining single type of cell constructs, spheroids to multiple types of cells patterning or shaping for cell-based research areas [7–9]. It is very important to underline the fact that cell interaction and answer to pNIPAAm surfaces is dictated not only by the chemical characteristic, but also by its thickness, surface morphology (porous, uniform, low roughness). There are studies indicating that pNIPAAm surfaces with thicknesses higher than several tens of nm were inhibiting the cell adhesion, or that highly porous surfaces inhibit formation of cells monolayer interfering in the attachment detachment process [7–11].

There were many approaches for obtaining pNIPAAm surfaces, in the form of films, nanoparticles, membranes or any other kinds of supports. Some of the most common/usual methods for the modification of large surface area with thin polymer films include dip-, spin-, or spray-coating. However, for these techniques, problems such as dewetting or delaminating often remain. Other techniques for the immobilization of pNIPAAm layers on

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surfaces are plasma polymerization, radiation-induced grafting of *N*-isopropylacrylamide, atom-transferred radical polymerization (ATPR), reversible additional fragmentation transfer polymerization (RAFT), nitroxide mediated radical polymerization (NMP), polymerizations on self-assembled monolayer (SAM) modified surfaces, covalent grafting by ultraviolet and electron beam irradiation and plasma methods [12–17]. Besides the advantages of each technique, the main disadvantages are related to high cost, but also on the lack of flexibility in controlling the surface density, morphology or thickness of the films [17].

Our approach is based on the use of laser based method, namely matrix assisted pulsed laser evaporation (MAPLE), for obtaining functional and stimuli responsive polymer surfaces. MAPLE is already an established method for obtaining monolayer or multilayers thin films from various sensitive materials, from nanoparticles to proteins, enzymes or other types of sensitive materials [18–24]. The method is simple, and involves directing a pulsed laser beam onto a cryogenic target made of the material to be deposited dissolved in a solvent matrix. By the laser beam interaction with the surface layers of the target, these are evaporated and the solvent and solute molecules are released into the deposition chamber. As the volatile solvent is pumped away, the material molecules are deposited onto a substrate and form a thin film [24].

The advantage of using this approach in this work is given by the possibility of tailoring not only the thickness of the pNIPAAm layer, which is an important parameter in the cell attachment, but also on tailoring the morphology of the deposited thin films and its increased stability in the fluid medium. Obtaining pNIPAAm thin films with controlled thickness, roughness and surface chemistry enabled the correlation between the physical and chemical characteristics of the coatings with L929 fibroblast cell line response.

2. Materials and methods

2.1. Materials and target preparation

pNIPAAm and chloroform were obtained from Sigma–Aldrich. For target preparation, solutions consisting of pNIPAAm polymer dissolved in chloroform (1 wt%), were homogenized for several minutes and rapidly frozen drop by drop in a liquid nitrogen cooled copper container. For avoiding that the target melts during the deposition, the copper container with the target was mounted on a cryogenic holder inside the deposition chamber. The target was rotated with a motion feed through driven by a motor to avoid local overheating and drilling following multiple pulses of laser irradiation.

2.2. MAPLE deposition system

For the MAPLE experiments, a "Surelite II" pulsed Nd: YAG laser system (Continuum Company) working at a wavelength of 266 nm, 6 ns pulse duration and 10 Hz repetition rate was used. The laser spot size measured on the surface of the frozen targets was 0.02 cm^2. The laser fluence range used was 200–800 mJ cm^{-2}. Two thermocouples were placed at two different positions of the target holder for checking the temperature. Pfeiffer-Balzers TPU 170 turbomolecular pump was used for adjusting the background pressure ($1–4 \times 10^{-3}$ Pa).

2.3. Substrates preparation

1.3 cm^2 round glass coverslips were used as substrates for the MAPLE experiments for the in vitro assays.

For FTIR post-characterization, 1 cm^2 square double polished Si (1 0 0) transparent in the IR was used. The substrates (glass, Si) were carefully cleaned in an ultrasonic bath in acetone, ethyl alcohol and deionized water and blow-dried with N_2 gas before use.

During the deposition the substrates were kept at ambient temperature and placed at a distance of 4 cm from the frozen target.

2.4. Structural and morphological characterization of the deposited polymer thin films

For all the FTIR measurements, a Jasco FT/IR-6300 spectrometer was used in transmission mode by accumulation of 1024 scans in the spectral range from 400 to 4000 cm^{-1}, with a resolution of 4 cm^{-1}.

Atomic force microscopy (XE 100 AFM setup from Park Systems) was used for surface morphology characterizations. AFM measurements in non-contact mode were performed to analyze the films surface roughness. The images were acquired with an Axiovert 200 Microscope coupled to a Carl Zeiss AxioCam MRm camera.

KSV CAM101 microscope with a video camera with FireWire interface (640×480 pixels resolution) was used for the contact angle measurements in static mode. The sessile drop method was applied at two temperatures: 37 °C and 23 °C using a syringe with double distilled water, which ensured droplets with a volume of 0.5–1 μl. The reported values for the contact angle were obtained upon averaging 5 measurements performed on different areas of the sample.

2.5. Cell culture experiments

2.5.1. Sterilization

Prior starting the biological assays, the samples were placed on a 70% alcohol wet paper and placed for 60 min under a UV lamp. Then, the samples were rinsed gently twice with sterile serum-free Hank's balanced salt solution (SF-HBSS) and placed in Petri dishes.

2.5.2. Cell adhesion and detachment assay

The experiments were performed with fibroblast line L929 cells. 2 ml of L929 cells suspension in Dulbecco's Modified Eagle Medium DMEM (with phenol red, 10% FCS and 0.1% penicillin/streptomycin) were dropped onto the sterilized samples surface. Cells were seeded onto triplicate samples at 2×10^6 cells/ml in 24-well plates and cultured in a CO_2 incubator at a temperature of 37 °C up to 48 h for viability testing. An inverted Olympus microscope (CKX31) was used for cell detachment observation. For the fluorescence microscopy studies, the cells were stained with acridine orange for 20 min, at room temperature, and then washed twice with sterile SF-HBSS medium. Based on fluorescent staining, cells morphological characteristics were analyzed. The images were taken by a CCD camera (ANDOR iXon DU897 E-CSO-UVB) and Olympus IX71 microscope.

For cell morphology analysis, respectively for the cell detachment quantification, up to 10 fields were captured for each of the 10× and 20× objectives, for each investigated sample. Image analysis and counting was performed by using the grayscale representations of each field and the ImageJ software (National Institute of Health, USA, http://rsb.info.nih.gov/ij/).

2.5.3. MTS assay

"Cell Titer 96Aquous One Solution Cell Proliferation Assay" Promega kit was used for the viability and proliferation testing of the cells. This kit uses the mitochondrial reduction of a tetrazolium salt (MTS) into a formazan product as metabolic marker, soluble in the culture medium. Such reaction is induced only by the viable cells.

Prior to seeding on the samples, the cells were detached with trypsin, resuspended in medium and afterwards brought to a concentration of 2×10^6 cells/ml. A volume of 15 μl of this suspension