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Functionalized ormosil scaffolds processed by direct laser polymerization for application in tissue engineering

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ABSTRACT

Synthesized N,N′-(methacryloyloxyethyl triehtoxy silyl propyl carbamoyl-oxyhexyl)-urea hybrid methacrylate was polymerized by direct laser polymerization using femtosecond laser pulses with the aim of using it for subsequent applications in tissue engineering. The as-obtained scaffolds were modified either by low pressure argon plasma treatment or by covering the structures with two different proteins (lysozyme, fibrinogen). For improved adhesion, the proteins were deposited by matrix assisted pulsed laser evaporation technique. The functionalized structures were tested in mouse fibroblasts culture and the cells morphology, proliferation, and attachment were analyzed.

1. Introduction

In recent years, the synthesis of hybrid composites has proved to be an attractive class of materials with widespread applications. Among them, organically modified silicates (ormosils) are considered as a new emerging class of organic–inorganic materials. Ormosils find multiple applications as dental materials [1], in microelectronics and optical components [2], as protective coatings with tailored properties [3], etc. They combine the properties of organic polymers (the possibility to be functionalized, low processing temperatures) with silicate type material properties (hardness, chemical and thermal stability, transparency) generating new, complementary properties, unmatched by conventional composites [4]. Moreover, their properties can be modified by varying the composition from inorganic to organic, choosing the desired amount of structural elements. All these characteristics make ormosil materials suitable for applications in medical devices and also as scaffolds for tissue engineering [5].

In order to be considered a suitable scaffold for tissue engineering, a given structure should exhibit a series of physical, chemical and mechanical properties. It should be biocompatible, easily processable, and highly porous [6]. The method by which the scaffold is fabricated must allow the realization of 3D structures with controlled mesh architecture, compatible with specific cell cultures [8 and the references therein]. Various methods for producing 2D and 3D scaffolds have been used over the years: gas foaming [9], electrospinning [10], melt molding [11], temperature induced phase separation (TIPS) [12], fiber bonding [13], sintering [14], solvent casting/particulate leaching [15], etc. For some of the conventional techniques, several disadvantages may appear, for example the use of improper solvent, uncontrollable cells behavior, etc. In the last decades, laser-based methods such as laser machining [7], selective laser sintering/melting [16], stereolithography [17], MAPLE DW (matrix assisted pulsed laser evaporation direct write) [18] or direct laser writing with ultrashort pulses [19] have been intensively studied as alternatives for scaffolds production.

Direct laser writing using infrared femtosecond laser radiation via multiphoton absorption and avalanche absorption processes has the advantage of allowing the realization of any complex 2D and 3D structure of photosensitive materials in a single step procedure [20]. Very high spatial resolution, below Abbe's diffraction limit, has been reported by Sakellari et al. [21]. The technique has various applications in photonics, medical implants, and tissue engineering.

In this work, ormosil scaffolds were produced by direct femtosecond laser writing. In a recent study we have demonstrated the non-cytotoxicity behavior of the photo structured polymer [22]. We have also analyzed the cell viability, proliferation and senescence for substrate-adherent or free-standing polymeric scaffolds, leading to the conclusion that the hybrid methacrylate can be successfully used in bio applications [23].

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Herein we analyze two functionalization techniques: (i) proteins (lysozyme or fibrinogen) immobilization on the obtained polymeric structures and (ii) low pressure plasma treatment at room temperature.

Lysozyme and fibrinogen were deliberately chosen, as they are well known and characterized in the literature. Lysozyme is an antibacterial enzyme found in egg white, tears and other human secretions. The lysozyme molecule is about 4.5 nm × 3.0 nm × 3.0 nm and has a molecular weight of around 14 kDa. It is soluble in water, but it cannot be dissolved in ethanol or acetone. Fibrinogen is a blood protein that is involved in clotting and in producing fibrin, a natural biodegradable scaffold [24]. Fibrinogen has a molecular weight of around 340 kDa and is soluble in water as well. Both proteins were deposited onto the polymeric structures by matrix assisted pulsed laser evaporation (MAPLE), a technique which offers some important advantages: very good adhesion of the deposited protein layer to the substrate and controlled average thickness of the deposited film. Previously, we have proved that thin lysozyme films with intact and active molecules, as tested by mass spectrometry methods and standard kits, can be obtained by MAPLE [25]. Successful deposition of fibrinogen thin films by MAPLE has also been reported [26].

Different plasma treatment methods have been applied to polymers over the last decade in order to improve the cell attachment and proliferation [27]. In this work, we have applied low pressure plasma treatment to the ormosil polymeric structures, with the aim of improving material biocompatibility.

Preliminary studies were performed on simple bidimensional structures functionalized both with proteins and by plasma treatment. The structures were used as scaffolds for testing the in vitro culture and proliferation of L929 mouse fibroblasts.

2. Experimental

2.1. Materials

The synthesized hybrid monomer used for direct laser polymerization by femtosecond laser pulses experiments and subsequent biological tests is a methacrylate with triethoxysilane group: N,N′-(methacryloyloxyethyl triethoxy silyl propyl carbamoyloxyhexyl)-urea (SIM-3). The chemical structure and the material properties have been presented in Refs. [22,23].

2.2. Scaffold preparation

The monomer and the Irgacure 369 photo initiator dissolved in tetrahydrofuran were drop casted onto glass substrates. An amplified Ti: Sapphire femtosecond laser (Clark CPA-2010) emitting at a wavelength of 775 nm, with a repetition rate of 2 kHz and 200 fs pulse duration, was used to irradiate the monomeric sample. The laser beam was focused on the sample by a 100 mm focal distance f-theta lens resulting in a spot with a diameter of 35 μm at the focus. The scanning of the laser spot across the sample surface in a raster pattern was achieved using a laser scanning head provisioned with galvanic mirrors. The laser fluence was in the range 0.2–0.5 J/cm^2 and the scanning velocity was 1–3 mm/s, as previously used in our experiments [23].

2.3. Scaffold surface functionalization

Two approaches were used for the functionalization of the polymeric surface: protein immobilization and plasma treatment.

2.3.1. Protein deposition by laser technique (MAPLE)

The MAPLE system, at DTU Fotonik, used for lysozyme and fibrinogen thin films deposition has been described in [28]. A Nd:YAG laser emitting at 532 nm and having a pulse length of 6 ns and 2 Hz repetition rate was used to irradiate the target at an angle of 45°. The glass substrates containing the polymerized structures were placed parallel to the target at a distance of 6 cm. The number of pulses used for the depositions was 7200 for all the experiments; the spot size was 0.008 cm^2 and the fluence 1.7 J/cm^2. The targets were frozen solutions of 1 wt% chicken egg white lysozyme (Aldrich, molecular weight: 14,307 Da) and 1 wt% fibrinogen from bovine plasma (Sigma, approximate molecular weight of 340 kDa) in Milli-Q water. Aqueous solutions of 1:1 lysozyme: fibrinogen (1 wt%) were also used as targets.

2.3.2. Plasma treatment

The plasma treatment experiments were performed in a cylindrical glass chamber of 12 cm diameter and 18 cm height. The chamber was initially pumped down to 3.4×10^{-2} mbar. A capacitively coupled RF discharge (13.56 MHz) was generated at 20 W in a parallel-plane configuration. The samples were mounted on the grounded electrode at a distance of 6 cm from the RF powered electrode. A working pressure of 4.9×10^{-1} mbar was kept during plasma treatment by flowing Ar at a constant flow rate of 20 sccm. The plasma treatment time was set to 5 min for all the samples.

2.4. Analysis

Optical microscopy, scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to investigate the surface morphology and the topography of the polymerized structures. The protein film thickness was measured by quartz crystal microbalance (QCM), usually as a result of 3600 pulses.

As mentioned, sets of polymerized structures (untreated) and sets of functionalized structures were used as scaffolds for the in vitro culture of L929 mouse fibroblasts. The grids on the glass substrates were sterilized for 30 min using a UV lamp, being placed on a 70% alcohol wet paper. After drying, the samples were transferred in a 12-well plate. The cells were grown in MEM (minimal essential medium), supplemented with L-glutamine and 10% inactivated fetal bovine serum. An inverted Olympus microscope (CKX31) was used for cell observation. The pictures were taken using a CCD camera attached to the microscope. "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 grids, 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 was uniformly placed onto the grid surface. The plate was incubated at 37 °C, 5% CO_2 in humid atmosphere and then 1.5 ml of medium was added in each well and incubated for 24 h. After that, the grids on glass were moved in other wells and 400 μl of viability solution was added (20 μl MTS at 100 μl MEM solution). The plates were incubated for 3 h at 37 °C, 5% CO_2 in humid atmosphere, movements of the plates leading to homogeneous MTS solution and reduction of the active element. After the incubation, the MTS solution was transferred into a 96 well plate and absorption at 490 nm was read by a TECAN Sunrise Basic plate-reader. For cells observation, they were colored with acridine orange stain.

3. Results and discussion

Areas of 25 mm^2 were polymerized by femtosecond laser pulses and grid-like structures with different spacing between lines have been obtained. Fig. 1 presents SEM images of three structures containing 1, 2 and 3 layers of polymer. Different magnifications

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