

Direct Laser Fabrication of Polymeric Implants for Cardiovascular Surgery

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In this work we present Multi-Photon Polymerization fabrication technique for biomedical applications. Optimal structuring parameters were defined in different polymeric materials using various lasers with different pulse durations (8 ps, 300 fs, 80 fs) and excitation wavelengths (532 nm, 515 nm and 800 nm), respectively. The applied photopolymers were: acrylate based AKRE, hybrid organic-inorganicOrmocore b59 and SZ2080, biodegradable PEG-DA-258 mixed with radical polymerization photoinitiators optimized for specific exposure conditions. It was defined that photoinitiators' molecules did not affect materials cytotoxicity. Biocompatibilities of the used materials were investigated and showed positive results *in vitro* and *in vivo*. Furthermore, various in size and form artificial scaffolds were designed and fabricated as sample prototype implant structures for further experiments for tissue engineering applications in cardiovascular surgery.

Keywords: laser non-linear lithography, 3D microstructures, photopolymerization, biocompatible polymers, biodegradable polymers, stem cells, tissue engineering.

1. INTRODUCTION

Direct Laser Writing (DLW) is an attractive fabrication technology, which has evolved rapidly during past decades. Multi-Photon Polymerization (MPP) is a branch of DLW, which allows to modify polymeric materials in nano-scale. Due to non-linear nature, MPP can be easily employed for the fabrication of three-dimensional (3D) structures for number of applications, such as microfluidics [1], microoptics [2], photonics [3] as well as biomedicine and tissue engineering [4].

MPP has been first demonstrated in 1997 by S. Kawata group [5] and since then has expanded as a flexible technique that allows creation of microscopic objects with nano-scale resolution. Varieties of micro-structures have been fabricated since, ranging from complex micromechanical components [1] to conductive metamaterials [6]. Lately it has been demonstrated that biocompatible polymeric, gelatin or even protein structures can be used for biomedical applications as well [7-9]. Polymers are attractive for the possibility to dope them and in this way to add desired functionality, for example: fluorescent dyes can help imaging of scaffold cell interaction [10]. One of the most promising biomedical applications of MPP is engineering of artificial tailor-made tissues, which could be transplanted into patients to cure diseases or traumas [11]. MPP has been employed for the fabrication of artificial scaffolds, which could serve as an Extra Cellular Matrix (ECM) and sustain stem cell growth *in vitro*.

Controllable biomimetic and geometrical properties of the scaffolds can affect cell viability, adhesion and direct their differentiation and this can be used for constructing artificial tissues of desirable form and functionality [12, 13].

Cardiovascular disease is an important issue in nowadays medicine. Artificial blood vessel implants fabricated via DLW out of acrylate based materials can be a competent replacement for currently used synthetic grafts (i.e. polytetrafluoroethylene or polyethylene terephthalate), because last-mentioned are usually suitable as large diameter (> 6 mm) blood vessel prostheses due to poor mechanical properties [14]. Controllable elastic modulus combined with high strain at break and high tear resistance are important features of biocompatible materials which are suitable for polymerization structuring. These properties can be applied for producing small size blood vessel implants [15]. Physical design of the scaffold also plays an important role as it significantly affects cell organization, proliferation and differentiation [16]. MPP satisfies the requirement to precisely control surface geometry, scaffold configuration and pore structure in micro-scale. In this case, it is a more reliable structuring technique than the traditional ones used for scaffold fabrication, such as fiber bonding [17], gas foaming [18], solvent casting/particulate leaching [19], phase separation [20]. However, to date there is a lack of knowledge of cell-matrix and cell-cell interactions at micro-scale. Therefore, the current research should be aimed to the production of synthetic ECM with suitable biological and chemical properties, which could mimic the native tissues and help to investigate cell behavior on 3D scaffolds.

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The aim of this study is to apply MPP technique for fabrication of artificial scaffolds as cardiovascular implants. The scaffolds with precisely controllable pore sizes have been prepared. Overall dimension of the implants was large enough for the surgical practice (> mm). The photostructurable materials used in this work proved to be biocompatible for the rabbit's stem cells *in vitro* and for the laboratory rat's *in vivo*. Different materials and light sources have been used for fabrication to demonstrate technological flexibility. Overall interdisciplinary study showed that proposed rapid structuring technique can be used for tissue engineering applications.

2. EXPERIMENTAL

2.1. Materials

In our experiment we used four different photopolymers: custom made acrylate based material AKRE [21], hybrid organic-inorganic SZ2080 (ORganically MODified SILica ORMOSIL, FORTH) [22] and Ormoclear (ORganically MODified CERamics ORMOCER, Micro Resist Technology GmbH) [11], biodegradable PEG-DA-258 (Poly Ethylen Glycol Di-Acrylate of M.w. = 258, Sigma-Aldrich GmbH) [23]. The monomers were photosensitized adding 1 wt.%–2 wt.% of 2-Benzyl-2-dimethylamino-1- (4-morpholinophenyl) buta-none-1 photoinitiator (Sigma-Aldrich GmbH) or 4,4'-Bis (diethylamino) benzophenone (Sigma-Aldrich GmbH) (depending on the laser wavelength used). PDMS thermo elastomer (Poly(DiMethyl Siloxane), Dow Corning Corp.) was used producing transparent mask for UV micromolding, it was mixed with matched curing agent (Dow Corning Corp.) using weight ratio 10 : 1.

Samples for fabrication were prepared by drop-casting the photopolymer on a cover glass substrate. After laser processing samples were treated with the appropriate organic solvent in order to wash out unexposed material. Photopolymer exposed to light underwent polymerization and became insoluble in the developer. Polymerized structures sustained during the development process. In this way, the free-standing structures were fabricated on a glass substrate. Scanning Electron Microscope (SEM) and optical profilometer were applied to evaluate the microstructured scaffolds.

2.2. Laser Fabrication Setup

The MPP system used in this work is depicted in Fig. 1. Three different lasers as irradiation sources were used: a) diode-pumped picosecond Nd:YVO laser oscillator with cavity dumping (Ekspla Ltd.), b) a high peak power femtosecond Yb:KGW laser amplifier (Pharos, Light Conversion Co. Ltd.), c) femtosecond Ti:Sapphire laser oscillator (Super Spitfire, Spectra Physics). Their parameters are compared in Table 1. Also average output power and peak pulse intensity ranges used in experiment are given. Light intensity was calculated using following equation:

$$I = \frac{E_p}{\tau_p \pi w^2}, \quad (1)$$

where $E_p = P/f$ is pulse energy, P – average laser output power, f – repetition rate, τ_p – pulse duration, $w = 0.61\lambda/NA$ is the waist of the beam, λ – wavelength, NA – numerical aperture of the objective. Values are calculated assuming that 40×0.65 NA objective was used, which was the most practical for fabrication of the scaffolds.

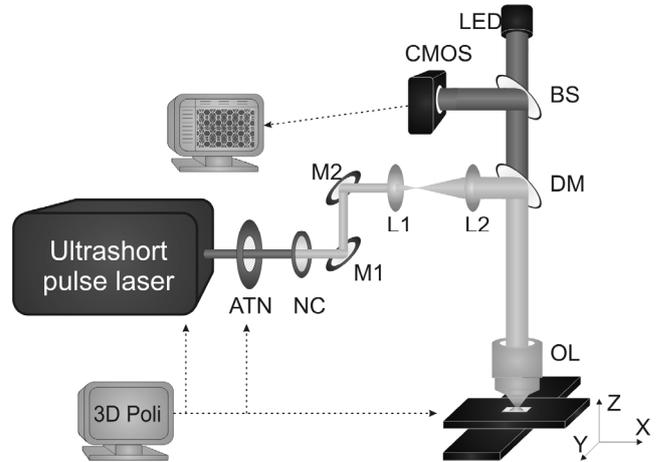


Fig. 1. MPP fabrication setup. Ultrashort pulsed laser beam is guided through a shutter to nonlinear crystal (NC), reflected by dichroic mirror (DM) and coupled to objective lens (OL). The sample is fixed on XYZ stages which are computer controlled. LED provides illumination needed for CMOS camera to monitor the fabrication process online

Table 1. Parameters of the lasers used in experiment

Laser source	Pulse duration	Repetition rate	Wave-length	Average output power	Peak intensity
Nd:YVO	8 ps	1 MHz	532 nm	1–5 mW	0.02–0.08 TW/cm ²
Yb:KGW	300 fs	200 kHz	515 nm	1–10 mW	2.3–23 TW/cm ²
Ti:Sapphire	80 fs	80 MHz	800 nm	15–24 mW	13–21 TW/cm ²

The laser beam was guided through an optical system to a high numerical aperture objective and focused to a volume of photopolymer. The sample was mounted on a high speed and wide working area positioning system which consisted of linear motor driven stages (Aerotech, Inc.): XY-ALS130-100, Z-ALS130-50. These stages ensure an overall travelling range of 100 mm in X and Y directions and 50 mm in Z direction and support the scanning speed up to 300 mm/s. Upon irradiation the monomers underwent transition from liquid to solid (or from gel to solid), which resulted in the change of the refractive index. It enabled wide-field transmission microscopy to be used for monitoring the manufacturing process in real time. A microscope was built by adding its main components to the system: a source of red light provided by LED, a CMOS camera (mvBlueFOX-M102G, Matrix Vision GmbH) and a video screen. The ability to image photostructuring while performing MPP is an

important feature for successful fabrication process. Control of all equipment was automated via custom-made software “3DPoli” specially designed for MPP applications. By moving the sample three-dimensionally the position of laser focus was being changed inside the resin and this enabled writing complex 3D structures (Fig. 2).

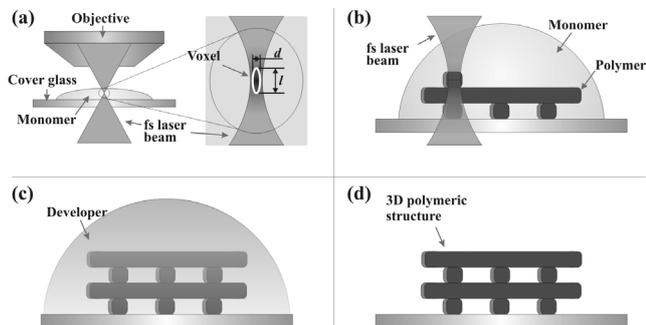


Fig. 2. Fabrication steps: a – direct laser writing; b – 3D structuring; c – development; d – 3D micro/nanostructure

Structures can be imported from Computer Aided Design (CAD) files or programmed directly. This MPP system was tested for structuring in various photosensitive materials at large scale. The ability to scale up and speed up the fabrication was ensured by changing laser beam focusing objectives in the range from $100 \times \text{NA} = 1.4$ to $10 \times \text{NA} = 0.25$, thus at the sacrifice of the resolution from 200 nm to 4 μm [21].

3. RESULTS

3.1. Fabrication of scaffolds

One of the most important advantages of MPP compared to the alternative above mentioned technologies is the precise control of structuring resolution, which allows fabricating precise objects with almost no geometrical restraints. Spatial resolution can be flexibly tuned by varying laser output power and translation speed of the sample, by replacing focusing objectives or altering sensitivity of the material itself. In our previous work we have shown the resolution dependence on these factors using three different laser sources, and we have demonstrated the possibility to accurately reproduce spatial resolution of 200 nm [12, 21, 24, 25].

The use of different lasers has revealed some aspects of fabrication flexibility, such as different Fabrication Windows (FW – ratio between threshold intensity of optical damage and photopolymerization, I_d/I_{th}). This parameter characterizes fabrication throughput and flexibility: a higher FW means a possibility to change structuring resolution and throughput in a wider range. Picosecond laser exhibits a lower FW compared to femtosecond lasers, due to long pulse duration. However, the use of it gives an opportunity for low-cost practical applications. The accessed maximum output power from Ti:Sapphire laser oscillator in our case was not enough to increase FW to its maximum capabilities, because a part of laser beam was split to the other experiments. As a consequence, femtosecond Yb:KGW laser amplifier was the most convenient light source for a rapid, high

throughput and flexible fabrication. Additionally, excess of laser output in carried experiments could be employed for interference lithography technique, enabling much higher fabrication throughput of the periodic microstructures [26].

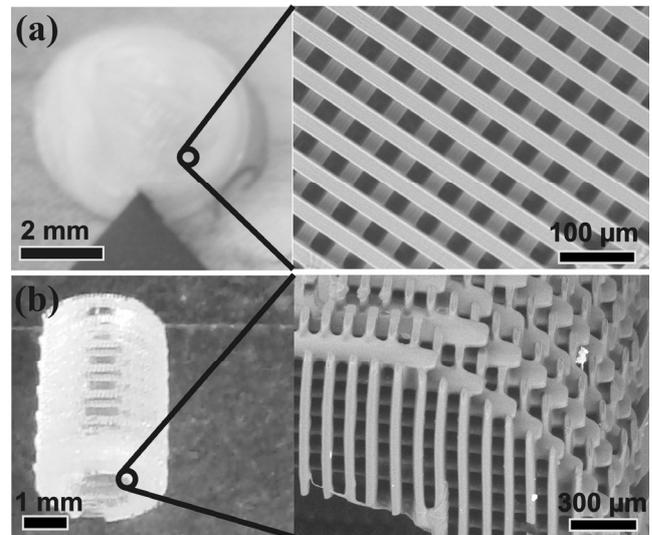


Fig. 3. 3D artificial scaffolds fabricated via MPP. (a) $5 \times 5 \times 0.3 \text{ mm}^3$ disc shape scaffold out of SZ2080 polymer. Pore size is $\sim 40 \times 40 \times 40 \mu\text{m}^3$, and general porosity is $\sim 40\%$. 10 mm/s sample translation velocity and $40 \times 0.65 \text{ NA}$ objective were used. Fabrication took 8 hs. (b) 3 mm in outer diameter with 1.5 mm internal diameter of a hollow tube and 6 mm long artificial blood vessel scaffold fabricated out of Ormocer polymer. Pore size is $\sim 50 \times 50 \times 100 \mu\text{m}^3$, and general porosity is $\sim 60\%$. 2 mm/s sample translation velocity and $10 \times 0.3 \text{ NA}$ objective lens were used. Fabrication took 12 h

Using optimized fabrication parameters we prepared micro-porous scaffolds over large area in order to have objects of acceptable size for later implantation *in vivo*. Scaffolds were up to few millimeters in size, with 30 μm –100 μm pores and $\sim 40\%$ –60% of general porosity (Fig. 3). The pore size of the scaffolds should be around twice as large as a single cell, and for mammals it corresponds to tens of micrometers [27]. Precise control of the pore size, their homogeneity and interconnection is believed to be beneficial for cell proliferation [16].

3.2. Replication of scaffolds

For repeated cell growth experiments there is a demand for many equal scaffolds. In such case fabrication with MPP system is a rather expensive and time consuming process. As an alternative method, UV micromolding can be used for rapid production of series of equal samples [28]. Fig. 4 shows micromolding steps: PDMS elastomer is placed on the original structure and cured via thermal reaction, then transparent PDMS mold is placed on the new monomer and the latter is cross-linked by exposing to UV light.

By using this technique, we have successfully replicated large area 2D scaffolds for stem-cell growth. Fabrication time was reduced up to twenty times for $(15 \times 15) \text{ mm}^2$ 2D scaffolds. We have shown that via UV

micromolding technique we can reproduce surface roughness with 2 % inaccuracy (Fig. 5).

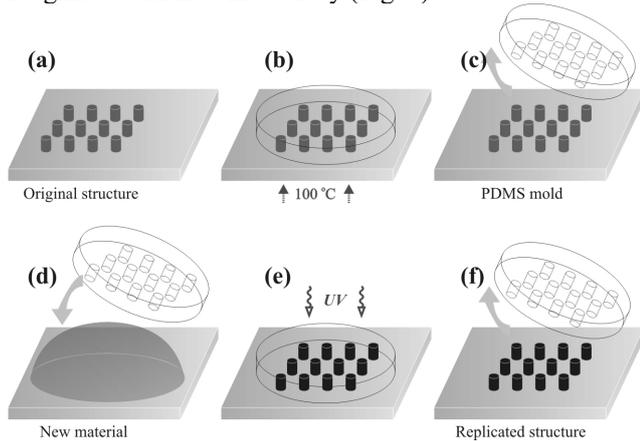


Fig. 4. Replication steps: a – original structure; b – PDMS elastomer is poured onto original structure and cured thermally; c – PDMS mold is removed from the substrate; d – mold is placed on new monomer material; e – monomer is polymerized with UV radiation; f – mold is removed revealing replicated structure

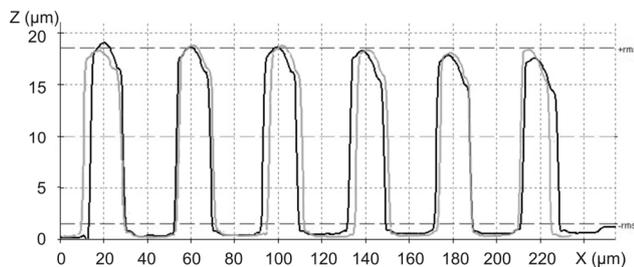


Fig. 5. Profilometer image comparing original and molded structures. It shows high quality reproduction possibilities of micromolding technique

3.3. Biocompatibility of materials

Our experiments *in vitro* and *in vivo* showed that all four of the used polymers (Ormoclear, SZ2080, PEG-DA-258 and AKRE) are biocompatible. Adult myogenic stem cells derived from rabbit muscle were seeded on non-structured polymeric films *in vitro* for 48 h and their viability was registered by staining with dye-mix solution (Fig. 6, a). The results demonstrated that polymers were as biocompatible as control polystyrene and glass surfaces.

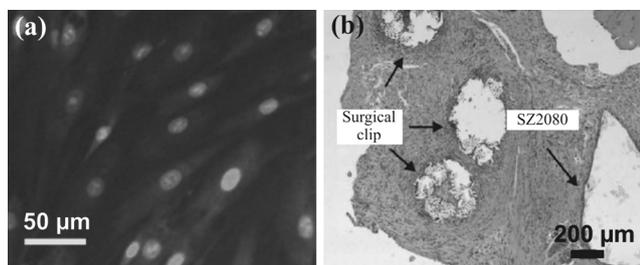


Fig. 6. Alive rabbit stem cells growing *in vitro* on the non-structured polymer SZ2080 surface (a). Section of biocompatible polymer SZ2080 and surgical clip implanted in rat's muscle *in vivo* (b)

Furthermore, biocompatibility of polymeric samples manufactured as shapeless granules were tested *in vivo*. For comparison of tissue response, surgical suture was

taken as a control sample (Fig. 6, b). After three weeks of implantation in rat's muscle all tested materials were found non-cytotoxic and as biocompatible as surgical suture, showing them to be suitable for biomedical practice.

4. CONCLUSIONS

In conclusion, in present research biocompatibility of four different photopolymers is stated by experiments *in vitro* and *in vivo*. Three-dimensional scaffolds of scale suitable for surgical practice were fabricated from these materials having desired pore sizes and general porosity. Additionally, replication technology of two-dimensional scaffolds was applied and fabrication throughput of the structures for stem cell growth is increased. Finally, multi-photon polymerization of scaffolds is demonstrated with three different laser sources, including low cost picosecond laser, lowering technological costs comparing to traditionally used femtosecond laser sources and opening opportunities for practical applications in tissue engineering. Future work is targeted to create and test *in vivo* three-dimensional scaffolds for applications in tissue engineering for cardiovascular surgery. Such biomedical constructs could serve as biodegradable stents or vein replacement implants with stem cells grown on them.

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