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# Two-photon polymerization based large scaffolds for adhesion and proliferation studies of human primary fibroblasts

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ABSTRACT

We report on cellular adhesion of human primary fibroblasts on scaffolds fabricated by laser-induced two-photon polymerization using 515 nm femtosecond pulses. The scaffolds are large scaled with a dimension in the range of several millimeters and consist of a periodic reproducible structure. A minimum process duration of 6.3 min is achieved by an implemented nonstop single-line single-pass fabrication process and allows to write several identical specimens with different pore sizes from 10 μm up to 90 μm suitable for cell adhesion studies in a reasonable amount of time. OrmoComp®, an organic-inorganic hybrid polymer, is chosen as base material for the structures. Human dermal fibroblasts are directly seeded on scaffolds after several post-processing steps to ensure the extraction of toxic residues. Cell adhesion, proliferation, and survival are examined after three, six, and nine days of culture, respectively. Cell growth is compared depending on the different pore sizes of the scaffolds. Due to the horizontal and vertical cell growth observed on and inside the structures, we demonstrate that large scaffolds prepared from OrmoComp® qualify for three-dimensional cell adhesion and growth without support of an additional protein coating.



## 1. Introduction

Influencing the cellular behavior by its environment is an important and not yet completely understood field of research. It is known that appropriate conditions are required for the maintenance of cellular function [1]. Furthermore, additional factors cause adhesion, alignment, migration, and proliferation of the cells [2–4]. Three-dimensional scaffolds are a promising approach to develop an ideal and controlled cellular environment for tissue-engineering applications. Direct laser writing via two-photon absorption (TPA) is a favorable method to produce these scaffolds. It enables real three-dimensional writing of arbitrary predefined structures by only modifying the material around the focus position achieving a resolution down to 100 nm [5–7].

Although TPA was predicted by Maria Göppert-Mayer in 1931 [8], it is still a challenge to bridge the nanoscale resolution of this process to macroscopic structures while achieving adequate process times. Fabrication of single objects with millimeter size takes up to several hours or even days [9–12]. Multi-beam writing [13–15] is suitable for producing several devices in parallel and thus is an option for optimizing the process. Furthermore, it has been shown that the use of more than one objective [7,16] has the advantage of adapting the simultaneously polymerized volume by changing the focus and, thus, the resolution. This results in faster fabrication of larger portions of a macroscopic component with less resolution.

In this work, we introduce a nonstop single-line single-pass technique that minimizes the traveling distance during the fabrication process and enables the fabrication of porous structures. It is accompanied by the special design of the scaffolds taking advantage of real three-dimensional writing. In comparison to most of the previous work [1,9–11,17,18], we do not adhere to layer by layer fabrication or woodpile structures. The implemented method significantly reduces processing times without any additional optical components for beam splitting or shaping and enables us to realize scaffolds with an enormous surface for two-photon polymerization written structures of more than 9 $mm^2$. This exceeds the required range of 1 $mm^2$ for proof of principle studies of cellular behavior on two-photon polymerized scaffolds [1].

Cell growth and ingrowth additionally depend on the pore size and shape of the scaffold [19]. For fibroblasts ingrowth, an optimal pore size of 20 μm was found and in general, a pore size of around twice as big as the size of a single cell is recommended [20]. Here,

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we designed different pore sizes ranging from 10 μm up to 100 μm to identify suitable feature sizes in this range, which includes the range for fibroblast growth given in [4,19,20] to increase the probability that we choose an appropriate pore size range. Moreover, OrmoComp® is chosen as material for the scaffolds. This organic-inorganic hybrid polymer provides high chemical and thermal stability [21] and mechanical properties similar to those of bones [3]. Moreover, it has shown biocompatibility by several groups seeding cells onto coverslips coated with this material [3,21] and was successfully used for cell culture studies [22,23]. Further investigations of Ormocer-based components demonstrate in vitro and in vivo biocompatibility [24,25]. Thus, OrmoComp® is a promising candidate for biomedical applications. In our experiments, human dermal fibroblasts are applied to the structures and analyzed after three, six, and nine days with the aim of taking a first step toward controlled three-dimensional tissue engineering on large scaled two-photon polymerized scaffolds.

## 2. Experimental method

### 2.1. Scaffold fabrication

The scaffolds are designed with a CAD software in such a way that a whole scaffold is built up of a single line and every position is only passed once during the process excluding junction points. The process of this single-line single-pass technique is illustrated in Fig. 1. After defining the pore size, the structure is built up by cuboids with the desired pore size. Subsequently, the scaffold is designed by placing ellipses with minor and major axes equal to the edge lengths of the cuboids into the faces of the cuboids. The single ellipses are merged to generate a single line so that the whole scaffold is fabricated by a nonstop writing process. Fig. 1a shows the beginning of the traveling path during the fabrication and Fig.1b a designed scaffold with eight pores. For a specimen, several scaffolds with different pore sizes are positioned next to each other to analyze different pore sizes at the same time.

An Ytterbium doped femtosecond oscillator (Mikan, Amplitude Systems) as shown schematically in Fig. 2a is used to realize the designed structures. This laser system provides 300 fs pulses at 515 nm with a repetition rate of 55 MHz and an average output power of 550 mW. An acousto-optic modulator regulates the laser power and a reference beam is picked off for monitoring purposes. After circularly polarizing and expanding the laser beam, the light is guided through an objective to the sample. For smaller pore sizes, the ZEISS LD Plan-Neofluar 63x objective with an NA of 0.75 and for larger pore sizes the ZEISS EC Epiplan-Neofluar 20x objective with an NA of 0.5 are used. The objective is movable along the laser beam direction and the sample is movable in the plane perpendicular to the incoming beam to enable a three-dimensional writing within the photosensitive material.

A sample consists of two glass coverslips separated by spacers and a layer of OrmoComp® (micro resist technology). The structures are fixed and built up on the cover glass which is further away from the objective. One of these structures is presented in Fig. 2b and demonstrates the fabrication of large scaffolds. The

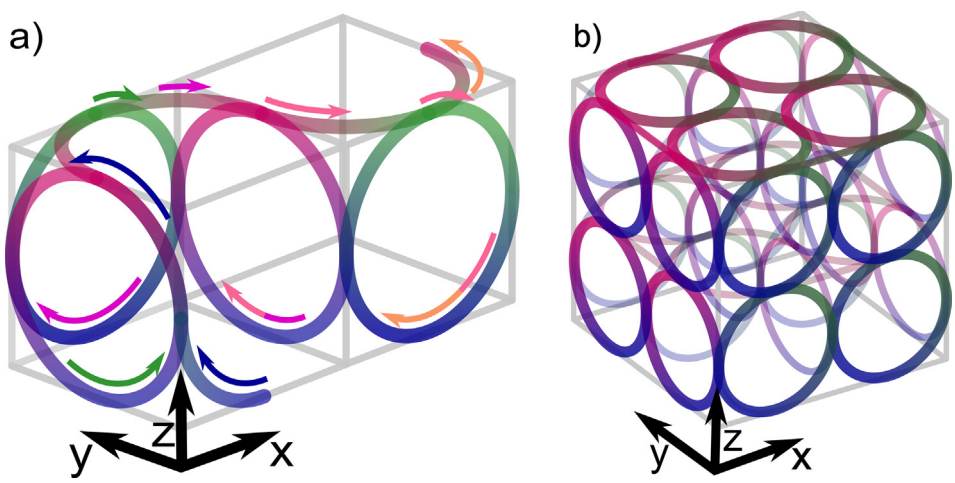


**Fig. 1.** Schematic figures showing (a) the beginning of the traveling path during the scaffold fabrication and (b) a designed scaffold with 8 pores.

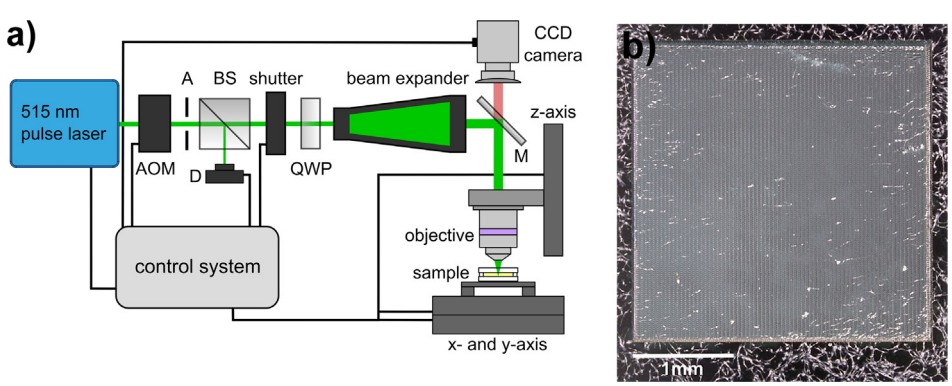


**Fig. 2.** (a) TPP setup. Laser beam power is regulated by an acousto-optic modulator (AOM), laser beam is circularly polarized with a quarter-wave plate (QWP), guided through an optical system, and coupled through an objective movable along the z-axis to a sample fixed on a movable XY stage and (b) a fabricated large scaffold.