Two-photon polymerization technique for microfabrication of CAD-designed 3D scaffolds from commercially available photosensitive materials

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Abstract

We report on recent advances in the fabrication of three-dimensional (3D) scaffolds for tissue engineering and regenerative medicine constructs using a two-photon polymerization technique (2PP). 2PP is a novel CAD/CAM technology allowing the fabrication of any computer-designed 3D structure from a photosensitive polymeric material. The flexibility of this technology and the ability to precisely define 3D construct geometry allows issues associated with vascularization and patient-specific tissue fabrication to be directly addressed. The fabrication of reproducible scaffold structures by 2PP is important for systematic studies of cellular processes and better understanding of in vitro tissue formation. In this study, 2PP was applied for the generation of 3D scaffold-like structures, using the photosensitive organic–inorganic hybrid polymer ORMOCER® (ORGanically MOdified CERamics) and epoxy-based SU8 materials. By comparing the proliferation rates of cells grown on flat material surfaces and under control conditions, it was demonstrated that ORMOCER® and SU8 are not cytotoxic. Additional tests show that the DNA strand breaking of GFSHR-17 granulosa cells was not affected by the presence of ORMOCER®. Furthermore, gap junction conductance measurements revealed that ORMOCER® did not alter the formation of cell–cell junctions, critical for functional tissue growth. The possibilities of seeding 3D structures with cells were analysed. These studies demonstrate the great potential of 2PP technique for the manufacturing of scaffolds with controlled topology and properties. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords 3D scaffolds; two-photon polymerization; microstructuring; hybrid polymer; gap junction; DNA strand breaks

1. Introduction

One of the most popular approaches for tissue engineering relies on the application of scaffolds. Scaffolds help to guide cell growth and create certain milieux required for cells to form a functional three-dimensional (3D) tissue. Generally, scaffolds have to fulfil a few basic requirements; they should promote cell attachment and migration, enable diffusion of vital cell nutrients and expressed products, and have appropriate mechanical properties (Hutmacher, 2001). There are several established techniques for scaffold fabrication, including CAD/CAM technologies, nanofibre self-assembly, textile technologies, gas foaming, liquid–liquid phase separation, and direct ink-jet printing. Among all these techniques, only CAD/CAM technologies allow the fabrication of predefined structures. Unfortunately, structural resolution of the currently applied CAD/CAM fabrication technologies is limited to 10–100 µm. In this study, the two-photon polymerization (2PP) technique was applied
for the microfabrication of CAD-designed 3D polymeric scaffolds. This technology allows structural resolution down to 100 nm to be achieved (see Ovsianikov et al., 2006, and references therein).

Parallel to the development of scaffolds, an understanding of cellular processes, responsible for the formation of 3D tissue constructs, is of great importance. Systematic studies imply the ability to precisely reproduce the scaffold structure and its properties. The 2PP technique fulfills all these requirements and was applied in this study for the fabrication of 3D structures using the photosensitive materials Ormocer® and SU8. The biocompatibility of both Ormocer® and SU8 was tested by comparing the proliferation rate profiles of various cells grown on flat surfaces of these polymers.

Taking its origins from multiphoton microscopy, 2PP relies on the ability of high localization of the light–material interaction region, due to the non-linear absorption of laser radiation. In the case of Ormocer®, this interaction leads to polymerization, e.g. solidification of the material in the laser focus region. By moving the laser focus within this photosensitive material, one is able to write any desired 3D structure. In the case of SU8, which is initially solid, illumination by laser pulses results in the generation of a 3D photoacid pattern and polymerization is achieved later, during the subsequent post-bake processing step. The resolution of 2PP can be as high as 100 nm (and even better); it is controlled by adjusting the illumination dose, i.e. the average laser power and the scanning velocity (Ovsianikov et al., 2006). 2PP technology is advantageous for the fabrication of patientspecific implants and scaffolds, as well as for performing systematic tissue formation studies. Such common issue as in vitro-grown tissue vascularization is also addressed by this method. Using 2PP it is possible to precisely control both external shape and internal porosity of the fabricated scaffold. To the best of our knowledge, this study presents a first attempt towards the application of 2PP technique for the fabrication of 3D scaffolds for tissue engineering.

Parallel to the development of scaffolds, there is a demand for cell culture techniques allowing cell growth on the vertical/steep surfaces, in order to create true 3D tissue. Specific difficulties related to cell growth on vertical surfaces are related to the fact that many cells, especially adherent cells, sink down to the bottom of the culture container (Hammond et al., 2000). As shown in this paper, placing the dish with cells on a shaking table is sufficient to keep the cells in suspension and gives them time to adhere to the steep facets of the 3D structures. Additionally, it is demonstrated that Ormocer® does not alter cell viability and the capability of cells to form junctions, such as gap junctions. The fabrication of 3D scaffolds from these materials is discussed.

2. Materials and methods

2.1. Polymers and polymer processing

For the fabrication of 3D structures, near-infrared Ti:sapphire femtosecond (fs) laser pulses are applied. The laser wavelength, pulse duration and repetition rate are, respectively, 780 nm, 80 fs, and 94 MHz. The detailed description of the experimental set-up can be found in our previous publications (Ovsianikov A, 2006; Serbin J, 2004). In these experiments, the laser beam is tightly focused into a volume of photosensitive polymers in order to initiate the 2PP process in a small focal region. By moving the laser focus in 3D, one leaves a modified material trace, creating in this way 3D patterns and objects.

Ormocers® are hybrid organic–inorganic polymeric materials produced from liquid precursors using the sol–gel process. They include urethane- and thioether-(meth)-acrylate alkoxysilanes and contain strong covalent bonds between the organic and inorganic components. The cross-linking of inorganic and organic moieties leads to the formation of a 3D network, which provides Ormocers® with significant chemical and thermal stability (Obi et al., 2004; Hass and Wolter, 1999). By changing the ratio of organic/inorganic network density, it is possible to design Ormocer® materials with the desired mechanical, optical, chemical and surface properties. In this study, Ormocomp® (Microresist Technology GmbH, Germany), a member of the Ormocer® family containing 1.8% photo-initiator Irgacure 369 (Ciba Specialty Chemicals, Switzerland), was used. This is a liquid photosensitive material that can be locally transferred into the solid phase through a free-radical polymerization reaction taking place immediately after laser exposure. After irradiation, the non-solidified material is removed by a 1:1 solution of 4-methyl-2-penthanone and 2-propanol to reveal the generated structure. Manufactured structures were seeded with various cell types in order to determine the possibilities for growing tissue in 3D. Flat Ormocer® surfaces for cell viability control experiments were obtained by UV illumination of the Ormocer®, spin-coated onto glass coverslips. Afterwards these samples were rinsed in 4-methyl-2-penthanone and 2-propanol.

SU8 is an epoxy-based polymer, widely used in lithography. From the fabrication point of view, its main difference to ORMOCER® is that SU8 is initially solid. Therefore, the fabricated structure was immobilized throughout the whole fabrication time (Serbin et al., 2004). SU8 is polymerized via a cationic reaction, which takes place during the post-bake processing step. The refractive index of this material changes only slightly during the illumination step, therefore there are no disturbances of the laser beam focus by the structures written beforehand. This allows very flexible patterning strategies. A standard formulation of SU8-2050 (Microresist Technology GmbH, Germany) is used to fabricate the structure shown in Figure 1b.
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Figure 1. CAD model (a) and SEM image (b) of a 3D vascular microcapillary structure fabricated by two-photon polymerization technique of SU8. (c, d) SEM images of porous structures fabricated from Ormocomp®

The material was spin-coated onto the 170 µm thick glass coverslips. Processing required pre- and post-bake steps, which were performed in accordance with the protocol provided by the manufacturer (Microchem, http://www.microchem.com/products/su_eight.htm). In order to remove the non-illuminated material, standard SU8 developer was applied. Flat SU8 samples were obtained by exposing the spin-coated and pre-baked SU8 surfaces to UV light. After post-baking, the samples were rinsed in SU8 developer and 2-propanol.

2.2. Cell proliferation on Ormocomp® and SU8 surfaces

To test the biocompatibility of Ormocomp® and SU8, the proliferation profiles of cells grown on coverslips covered with these materials, in comparison to those grown on uncoated glass coverslips, were studied. Samples were placed in Petri dishes (diameter 35 mm) filled with 2 ml DMEM/F12 medium (Sigma, Taufkirchen, Germany) with the addition of penicillin/streptomycin (100 U/ml and 10 mg/ml, respectively), patricin (0.5 µg/ml) and 5–10% fetal calf serum. The Petri dishes were then placed in a cell culture incubator (Heraeus, Hanau, Germany), in which a 95%:5% air:CO₂ atmosphere and 80% humidity were maintained. In order to determine the growth parameters, the cells were trypsinized and counted after different periods of cultivation.

2.3. Analysis of the genotoxicity of Ormocomp®

In order to determine whether Ormocomp® affected the DNA of the cells, DNA strand breaking for cells grown on Ormocomp® and those grown under control conditions was analysed using the Comet assay; a detailed description of the Comet assay procedure can be found in previous publications (Ivancsits et al., 2002; Ngezahayo et al., 2005). Cells grown on control coverslips and on the coverslips coated with Ormocomp® were trypsinized, collected and centrifuged for 10 min at 800 × g (Biofuge; Heraeus, Osterode, Germany). The pellets were resuspended in PBS to 2 × 10⁶ cells/ml, then 20 µl cell suspension was mixed with 100 µl low-melting agarose (0.6%); 100 µl of this mixture was spread onto agarose-coated glass slides and covered with a glass plate. The slides were left for 10 min at 4 °C for solidification of the coated material, then the glass plate was removed and a further 100 µl agarose added. After solidification at 4 °C, the slides were incubated for 90 min in a lysis buffer containing 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 1% lauryl sarcosin, 1% Triton X-100 and 10% DMSO, pH 10. Subsequently, the coverslips were placed in a horizontal gel electrophoresis chamber, filled with electrophoresis buffer for alkaline Comet assay (1 mM Na₂EDTA, 300 mM NaOH, pH > 13). After 40 min adaptation to the buffer, electrophoresis was performed (25 V, 300 mA, 4 °C for 20 min). For neutralization, the slides were washed three times with Tris buffer (400 mM...
Tris, pH 7.5) and dried at room temperature. Comets were visualized by ethidium bromide staining (20 µg/ml) and examined with a fluorescence microscope (Zeiss, Oberkochen, Germany) at ×200 magnification, using a xenon lamp and an ethidium bromide filter set (excitation at λ = 520 nm). The images were recorded with a CCD Camera (software ‘Xaw TV’). For a quantitative analysis of the DNA breaking, the tailmoment (amount of DNA damage) was evaluated using Comet scoring software (Comet, http://www.autocomet.com/home.php).

2.4. Cell culture on Ormocomp® 3D structures

The schematic representation in Figure 5 shows how the cells are grown on the 3D structures. The Ormocomp® structures were fabricated on coverslips which were inserted into a Plexiglas holder. The whole construction was placed inside plastic Petri dishes (diameter 60 mm) containing 5 ml cell culture medium (DMEM/F12), as described above. The holders with the structures mounted on them were placed so that the structures were pointing towards the bottom of the Petri dish and remained covered by the culture medium. To avoid sedimentation of the cells on the bottom of the Petri dish and to promote cell adhesion on the 3D structures, the Petri dishes with cells were placed on a shaking machine in the cell culture incubator. The culture medium was changed every 3–4 days. To observe the cells grown on the lateral surfaces of the 3D structures, a Nikon stereo microscope (Nikon, Düsseldorf, Germany) was used.

3. Results and discussions

3.1. Application of 2PP for the fabrication of 3D scaffolds

In recent years, a few technologies have been proposed and investigated for the fabrication of 3D scaffolds for tissue engineering. Nevertheless, cellular processes responsible for the formation of 3D tissue constructs are not yet fully understood. Systematic studies of cellular responses require the availability of identical 3D scaffolds and the ability to precisely reproduce the scaffold structure and its properties on request. Our studies show that 2PP technology is capable of microfabrication of CAD-designed three-dimensional (3D) polymeric scaffolds with structural resolution much higher than available by conventionally applied CAD/CAM techniques (Ovsianikov et al., 2006). Virtually any photosensitive material can be structured by means of the 2PP technique. In this study, the 2PP technique was applied for the microstructuring of commercially available Ormocer® and SU8 materials. An example of microstructure having a shape of microcapillaries, fabricated by 2PP of SU8 material, is shown in the scanning electron microscope (SEM) image in Figure 1b. The corresponding CAD design of this microstructure is shown in Figure 1a. Sustenance of cellular survival and proliferation by both Ormocer® and SU8 was tested by comparing the growth rate of various cells grown on flat unstructured surfaces of these polymers. Cell growth on vertical Ormocer® surfaces has been demonstrated. Application of the 2PP technique provides the means for relatively simple modification of the external shape and internal porosity of the fabricated scaffold (Figure 1c, d). The capability to control the individual pore size independently addresses such common issues as in vitro grown tissue vascularization. Our future studies will include influence of 3D architecture and material composition on the cellular processes.

3.2. Sustained cell proliferation by Ormocomp® and SU8

For adherent cells, proliferation is only possible if the cells attach to the cultivation surface. Different properties of the culture material affect the adherence of the cells to the surface. It is known that a favourable distribution of charges influences the interaction between cellular adhesion molecules, such as integrins, and the material (Seyfert et al., 1995). Glass is known to present a favourable charge distribution on its surface, therefore cells grow efficiently on glass (Cukierman et al., 2001). In contrast, plastic materials are known to inhibit cell adhesion and, thereby, the cell growth. To improve the adhesion on plastic, proteins of the extracellular matrix (ECM), such as fibronectin or collagen, are generally applied to the plastic surface (Seyfert et al., 1995). Due to the charges of ECM proteins, as well as surface structures, the cells are stabilized.

In order to test the biocompatibility of Ormocomp® and SU8, various cells were seeded onto flat coverslips coated with these materials. GFSHR-17 granulosa cells, GM-7373 endothelial cells and SH-SY5Y neuroblastsoma cells were used with Ormocomp®. In the case of SU8, only the GFSHR-17 granulosa cells were applied. The cells were counted after different periods of cultivation and their proliferation rates were compared to those of cells cultivated on regular coverslips, using Student’s t-test (p < 0.05, p < 0.01 and p < 0.001).

The growth behaviour of the cells is given in a bulk diagram (Figure 2). At least four experiments with cells grown on polymeric surfaces and four experiments with cells grown under control conditions were carried out (5.76 × 10⁶ cells/ml were seeded). The error bars indicate the standard deviation (SD).

It was observed that the presence of Ormocomp® or SU8 did not significantly influence cell growth. The cells proliferate at the same rate as those cultivated under control conditions (Figure 2). The doubling times for different cells cultivated on Ormocomp® and under control conditions, are presented separately in Table 1. In order to complete the biocompatibility tests, analysis of inflammation and immunological effects of the materials should be performed. However, such tests involve animal
Figure 2. Comparison of growth profiles for granulosa cells on flat SU8, ORMOCER® and glass samples (5.76 x 10^6 cells/ml) were seeded. The results are presented as average ± SD for n = 4 experiments for each material.

studies, and are therefore beyond the scope of the present paper, which is aimed to demonstrate that the investigated materials sustain the survival of cells and do not alter their proliferation.

3.3. Comet assay performed with the cells grown on the Ormocomp® surfaces

If the adherent cells used in this study can not attach, they react by reduction of the proliferation rate and even by inducing cell death, which correlates with an increase of the DNA damage effect, corresponding to an increase of tailmoment.

Table 1. The doubling time of cells cultivated on ORMOCOMP® and SU8 in comparison to that of cells cultivated under control conditions. The results are given as average (± SEM) for 5 experiments for each cell line are performed on ORMOCER® and 4 experiments with granulosa cell were performed on SU8

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ORMOCOMP®</th>
<th>SU8</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFSHR-17 granulosa cells</td>
<td>18.8 ± 2.2</td>
<td>17.5 ± 0.58</td>
<td>18.1 ± 0.6</td>
</tr>
<tr>
<td>SH-SY5Y neuroblastoma</td>
<td>26.1 ± 0.7</td>
<td>22.1 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>CHO cells</td>
<td>10.8 ± 0.3</td>
<td>8.4 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>GM-7373 endothelial cells</td>
<td>32.3 ± 2.5</td>
<td>28.7 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

The DNA damage effect is characterized by an increase in the sum of cells presenting a tail of the ‘comets’ (Figure 3a). By comparing the relative tailmoment, using Student’s t-test (p < 0.05, p < 0.01 and p < 0.001), it was shown that Ormocomp® does not significantly increase the incidence of DNA damage (Figure 3b). The results are given as a tailmoment ± the standard error of the mean (SEM). At least 1000 cells/treatment were evaluated.

The presence of Ormocomp® did not alter the proliferation parameters of the cells (Figure 2, Table 1) and did not increase the tailmoment (Figure 3). Therefore, one can conclude that Ormocomp® sustains the adhesion of cells. Whether cell adhesion to Ormocomp® is conditioned by the favourable charge distribution on the surface is not known; further studies are needed to clarify this specific topic.

3.4. Gap junction conductance measurement for cells grown on Ormocomp® surfaces

When the intercellular junctions of a new tissue are formed, the gap junctions are the last junctions to be established. Their formation is conditioned by cell–cell adhesion structures composed from other molecules, such as cadherins and cadherin-associated molecules (Fujimoto et al., 1997). Gap junctions are cell–cell adhesion structures, composed of direct cell–cell channels that allow a direct exchange of ions and small metabolites, such as second messengers between neighbouring cells. The channels are formed by association of connexins (connexons) which are present in membrane of adjacent cells. The connexons are hexamer structures consisting of connexins, which are polypeptidic products of a gene family with 19, 20 or 38 members in rodents, human or zebrafish, respectively (Willecke et al., 2002; Eastman et al., 2006).

The compatibility of Ormocomp® with the formation of cellular junctions appropriate for living tissue, such as gap junction coupling, has been studied for GFSHR-17 granulosa cells using the double whole patch-clamp technique (Ngezahayo et al., 2005; Ngezahayo et al., 2003). The conductance of the gap junction (GJ), which was measured within 5 min after establishment of the double whole-cell configuration, is shown in Figure 4.

Figure 3. DNA breaking, demonstrated by Comet assay. (a) Comparison of Comet with DNA breaking and normal DNA; (b) tailmoments of GFSHR-17 granulosa cells grown on Ormocomp® and on control coverslip (n = 4; average, SEM)

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Figure 4. Conductance of gap junction (GJ) in pairs of GFSHR-17 granulosa cells cultivated on coverslips coated with Ormocomp® and on control glass coverslips

The experiments were performed for 10 cell pairs grown on three coverslips with Ormocomp® and nine cell pairs grown on three coverslips under control conditions; the error bars indicate SEM; 10 of 12 and 9 of 12 cell pairs, cultivated on Ormocomp® and on control coverslips, respectively, showed gap junction coupling. Student’s t-test (p < 0.05) was used to compare the magnitude of G_J of both populations. No statistically significant effects of Ormocomp® presence on the value of G_J was observed (Figure 4). The use of the double whole-cell patch-clamp technique has the advantage of permitting a quantitative evaluation of the functionality of the cell–cell junctions. It was observed that the materials sustained the formation of functional intimate tissue junctions, such as the gap junctions. Based on these results, one can conclude that the cultivation of cells on Ormocomp® is compatible with the formation of tissue.

3.5. Cultivation of cells on 3D structures fabricated by two-photon polymerization technique

When cells are cultivated on a vertical/steep surface, the first challenge was to avoid the sedimentation of the cells so that they could adhere over the whole surface. In order to create 3D tissue, CHO cells, GFSHR-17 granulosa cells, GM7373 endothelial cells and SH-SY5Y neuroblastoma cells were cultivated on 3D structures standing on the coverslips. To avoid cell adhesion to the bottom of the Petri dishes and in order to keep the cells in suspension, the Petri dishes were placed on a shaking table (Figure 5). Under these conditions, the cells could adhere to the steep surfaces and began to proliferate along the surface (Figure 6). Microscopic

Figure 5. System used for cell growth on 3D structures fabricated from Ormocomp®. The coverslips containing the 3D structures are inserted into a holder made of Plexiglas, which is placed into a Petri dish containing cell culture medium. The Petri dish is mounted on a shaking table in a cell incubator

Figure 6. Stereomicroscope images: top view of cylindrical structure fabricated from Ormocomp®. SH-SY5Y neuroblastoma cells grow on the vertical/steep surface of the structure
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analysis of the cells after 3–4 days in culture showed that the cells were able to adhere to the vertical/steep surfaces and to form layers, which spread from the bottom to the top of the 3D structures (Figure 6). These results demonstrate that the 2PP technique applied on Ormocomp® in combination with a proper cell cultivation method allowed the fabrication of tissue with a well-controlled shape.

Conclusions

The fabrication of scaffolds with a desired structure in 3D is a very important task in tissue engineering. It has been demonstrated that CAD-designed complex 3D structures can be generated by the two-photon polymerization (2PP) technique. Using different cell lines, it was shown that two important polymers used for 2PP, Ormocomp® and SU8, support cell growth and are biocompatible at the cellular level. Cells are able to form cell–cell junctions, such as gap junctions, characteristic of functional tissue. It was demonstrated that cells can be grown on the vertical surfaces of Ormocomp® structures generated by the 2PP technique. These results demonstrate the great potential of 2PP technology for the fabrication of 3D scaffolds and test structures for tissue engineering and systematic cell interaction studies in 3D.

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