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Laser Photofabrication of Cell-Containing Hydrogel Constructs

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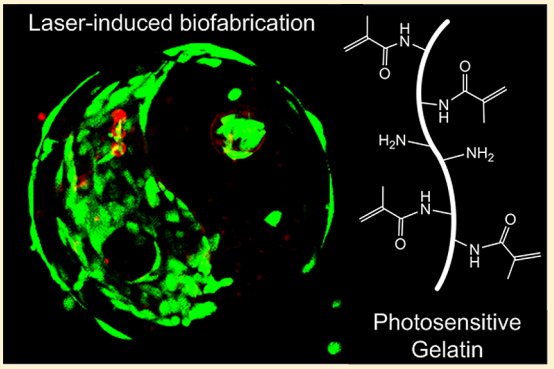
Supporting Information

ABSTRACT: The two-photon polymerization (2PP) of photosensitive gelatin in the presence of living cells is reported. The 2PP technique is based on the localized cross-linking of photopolymers induced by femtosecond laser pulses. The availability of water-soluble photoinitiators (PI) suitable for 2PP is crucial for applying this method to cell-containing materials. Novel PIs developed by our group allow 2PP of formulations with up to 80% cell culture medium. The cytocompatibility of these PIs was evaluated by an MTT assay. The results of cell encapsulation by 2PP show the occurrence of cell damage within the laser-exposed regions. However, some cells located in the immediate vicinity and even within the 2PP-produced structures remain viable and can further proliferate. The control experiments demonstrate that the laser radiation itself does not damage the cells at the parameters used for 2PP. On the basis of these findings and the reports by other groups, we conclude that such localized cell damage is of a chemical origin and can be attributed to reactive species generated during 2PP. The viable cells trapped within the 2PP structures but not exposed to laser radiation continued to proliferate. The live/dead staining after 3 weeks revealed viable cells occupying most of the space available within the 3D hydrogel constructs. While some of the questions raised by this study remain open, the presented results indicate the general practicability of 2PP for 3D processing of cell-containing materials. The potential applications of this highly versatile approach span from precise engineering of 3D tissue models to the fabrication of cellular microarrays.



1. INTRODUCTION

Additive manufacturing technologies (AMT) were demonstrated to be relevant for fabrication of scaffolds for tissue engineering and regenerative medicine. With these methods, complex 3D constructs for cell growth can be produced from a variety of materials and in accordance to a computer-aided design (CAD) model.[1−3] Among other AMTs, two-photon polymerization (2PP) stands out as a photochemistry-based approach, facilitating superior spatial resolution and relatively mild processing conditions.[4] In recent years, it was used by a number of groups around the world for the fabrication of 3D scaffolds for tissue engineering.[5−12] The 2PP is based on localized cross-linking of photopolymers, induced by two-photon absorption (TPA) of femtosecond laser pulses. The 2PP achieves a minimum feature size below 100 nm as TPA and the threshold characteristic of the process result in a strongly confined polymerized material region.[4] Since the processed material is essentially transparent to the utilized laser wavelength, the structures can be produced within the volume of the sample, without the need to deposit the material layer-by-layer, as it is done in other AMTs. This feature also enables 2PP processing of effectively solid material samples, such as the photosensitive gelatin, used in the present study, or Zr-based organic−inorganic hybrids, used in other recent reports.[9,12]

The current portfolio of 2PP-compatible biopolymers is fairly versatile; it spans from modified natural proteins to purely synthetic nonbiodegradable and biodegradable materials.[4] Structures fabricated from hydrophilic polymers, such as methacrylamide-modified gelatin (gelMOD) and poly(ethylene glycol) diacrylate, swell in water, essentially becoming a hydrogel.[13−15] Furthermore, our recent study demonstrated that by using appropriate water-soluble photoinitiators (PI), 2PP processing of formulations with high initial water content of up to 90% is possible.[16,17] Hydrogels are widely used as 3D matrices for cell growth due to the similarity of their mechanical and diffusivity properties to the natural cell

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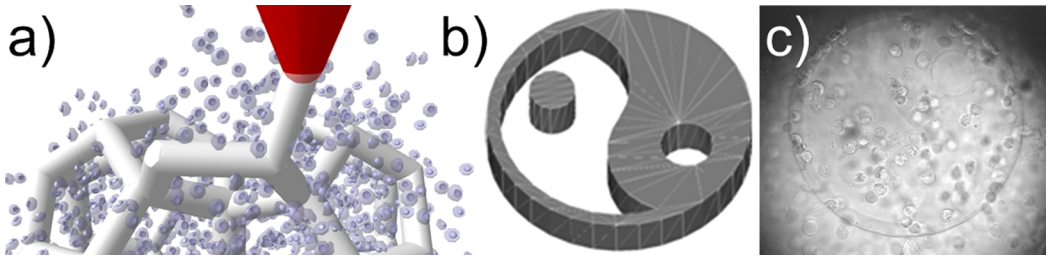

Figure 1. Two-photon polymerization (2PP) in the presence of living cells: (a) schematic illustration showing 2PP structures (gray) fabricated by a focused laser beam (red) within the suspension of cells (blue); (b) CAD model used for laser cell encapsulation (the disklike lid is not shown for the purpose of illustrating the inner structure); (c) an optical online image of a 2PP-produced 400 μm wide Yin−Yang hydrogel structure with cells inside (an according video is provided as Supporting Information).

environment—the extracellular matrix (ECM).[18,19] Furthermore, encapsulation of living cells within the hydrogel produces constructs with high initial cell loading and intimate cell−matrix contact, similar to that of the natural ECM. A number of synthetic and natural hydrogels have been developed and used for studies of cell behavior.[20] The main requirement toward such materials are cytocompatibility, adequate biodegradation behavior and features facilitating cell adhesion and/or migration. A recent review by Seliktar summarized the properties crucial for the use of hydrogel as cell−culture matrices and discussed the guidelines for their design.[18] Photoinduced polymerization can be carried out at temperature and pH conditions suitable for cells and living organisms.[21] The main concern, associated with the conventional photoencapsulation of cells, is the possible damage induced by UV light and free radicals.[22−24] Therefore, both the UV irradiation time and the intensity of the UV light applied are typically limited during conventional photoencapsulation. For example, Nichol et al. exposed fibroblast-encapsulated gelMOD to 6.9 mW/cm^2 UV light (360−480 nm) for 15 s.[14] Their results indicated that after 8 h encapsulation, a cell viability of 92% was obtained. In addition, Aubin et al. also applied gelMOD as starting material to encapsulate fibroblasts, endothelial cells, hepatocytes, and myoblasts using conventional UV polymerization for 50 s (6.9 mW/cm^2, 360−480 nm).[25] They mainly focused on evaluating the occurrence of cellular alignment occurring within gelMOD based micropatterns. Conversely, most of the currently reported 2PP-fabrication systems are using femtosecond lasers operating in the near-IR spectral range at around 800 nm. Tissues and cells exhibit a transparency window at this wavelength. This property is utilized in multiphoton microscopy for imaging cells and tissues, including the living ones.[26]

Here, we report the use of the 2PP technique for the fabrication of 3D hydrogel structures from photopolymers containing living cells. The following of the above-mentioned 2PP characteristics make this technique suitable for the fabrication of cell-containing 3D hydrogel constructs: (i) utilization of near-IR laser radiation at 800 nm, capable of penetrating deep into the cell-containing materials without damaging the cells; (ii) photopolymerization can proceed under mild, cell-friendly, pH, and temperature conditions; (iii) materials with high water content can be processed by 2PP.

Despite the similarities to conventional cell encapsulation, the 2PP is different in at least two major aspects: (i) the photopolymerization is initiated sequentially in a small volume of the material, and (ii) most of the reported PIs are unusable due to their low TPA efficiency and poor water solubility. In this work, two benzylidene cycloketone-based PIs G2CK and P2CK, specially developed by our group for 2PP of water-based materials, were employed.[17] An MTT assay was used to study the effect of these PI solutions on the metabolic activity of the cells. The results identified P2CK as a suitable candidate, performing similar to Irgacure 2959—a reference PI often used for UV encapsulation of cells.[22−24] Because of large TPA cross sections and good water solubility of these novel PIs, gelMOD formulations containing 80% of cell culture medium were successfully processed. Interestingly, previous research already indicated the potential of gelMOD to be applied as cross-linkable hydrogel precursor to develop porous scaffolds using 2PP.[9,27] The results indicated that 2PP enabled the control over the microtopography of gelMOD scaffolds with great precision. Moreover, the scaffolds developed maintained their shape during several weeks upon cell seeding. The present work demonstrates the potential of 2PP to engineer 3D cell-holding hydrogel constructs. Human osteosarcoma cells (MG63) were used; this cell line was recently shown to be suitable for the preliminary evaluation of materials, which have potential for bone tissue engineering applications.[28] By using a starting material containing living cells, the 2PP is entering the realm of a true biofabrication technique. The potential applications of this approach range from fabrication of customized 3D tissue models to drug screening, tissue engineering, and regenerative therapies.

2. MATERIALS AND METHODS

2.1. Two-Photon Polymerization (2PP). In our work, a Ti:sapphire femtosecond laser (High Q, Femtotrain) emitting pulses with duration of about 80 fs at 75 MHz repetition rate around 800 nm is used for 2PP. An acousto-optical modulator is employed for fast switching of the laser beam and for adjusting its intensity. In order to produce stable structures by 2PP, the gelMOD with a quite high number (~50) of double bonds per gelatin chain was utilized. Hydrogel precursors were prepared by dissolving 20% of gelMOD in cell-culture medium containing 1.827 mM of the PI. Both these gelMOD parameters are expected to result in a relatively high cross-linking density. The density of MG63 cells was set to 10^7 cells/mL suspension. The material-cell suspension was deposited into the microwell consisting of two cover glasses separated by a 1 mm thick silicon spacer. The sample is mounted on an assembly of three linear translation stages for complete 3D movement. Figure 1 illustrates the 2PP processing of a cell-containing material formulation. The femtosecond laser beam is focused with a microscope objective (20×, N = 0.8) into the sample. The patterns are obtained by scanning the beam within the sample by a galvo scanner (HurryScan, ScanLab) in accordance to the input CAD model. The femtosecond pulses result in localized polymerization of material trapping the cells. For the presented experiments, a scanning speed of up to 7 mm/s and an average laser power of up to 330 mW were used. A CCD camera allows us to observe the process online and take snapshots of the

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sample. Control samples exposed to identical conditions but not processed by 2PP, were prepared for every experiment. After 2PP processing, the samples were immersed into the cell-culture medium at 37 °C in order to remove the nonpolymerized gelMOD.

2.2. Material Development. The gelatin applied was isolated from bovine skin by an alkaline process (Rousselot). The material possessed an approximate isoelectric point of 5 and a Bloom strength of 257. The synthesis of methacrylamide-modified gelatin (gelMOD, Figure 2) was performed as described earlier.[29] In brief, part of the amine functions of gelatin were reacted with methacrylic anhydride. For this work, a derivative with a degree of substitution of 65%, based on the lysine and hydroxylysine units, was used. Irgacure 2959 was kindly donated by BASF (Germany). The water-soluble photoinitiators (PI) for 2PP were synthesized according to a generic protocol.[17] The rheological measurements with the 20 wt % gelMOD formulation cross-linked by UV light, indicate the elastic modulus of around 77 kPa at 37 °C. While it is not possible to directly assess the mechanical properties of 2PP-produced microstructures with the currently available equipment, this value can be used as a guideline. Further studies, using nanoindentation and/or atomic force microscopy equipment, to characterize the mechanical properties in more detail, are planned.[30]

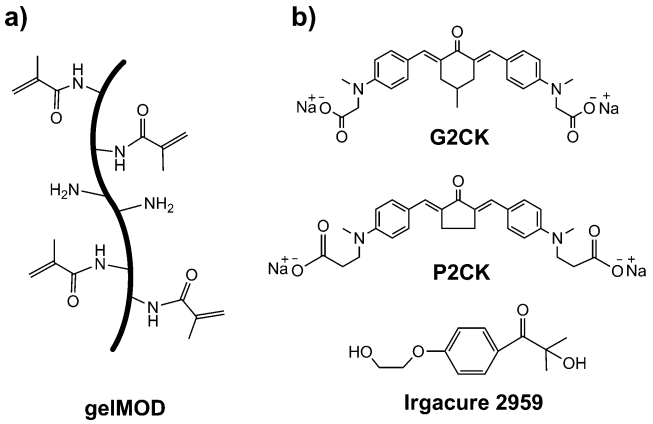

Figure 2. Chemical structures of (a) methacrylamide-modified gelatin (gelMOD) and (b) the water-soluble photoinitiators (PI) used in this study.

2.3. Cell Culture and Staining. The osteosarcoma cell line MG63 was cultured in DMEM containing 10% FBS and used for all encapsulation experiments. To determine the viability of embedded cells, 2PP processed hydrogels were incubated in 1× PBS containing 1.5 μM propidium iodide (Invitrogen) and 2 μM calcein-AM (Invitrogen) for 20 min at room temperature. After a washing step with 1× PBS, green (live) and red (dead) fluorescent cells were observed using a confocal laser scanning microscope (Zeiss LSM700). The cell counting was performed using ImageJ software.

2.4. MTT Assay. The viability of the cells in contact with the PIs was evaluated by means of MTT assay, which is an indicator of the relative metabolic activity. MG63 cells were seeded onto a 96-well tissue culture plate at a density of 1×10^4 cells per well in 200 μL of DMEM containing 10% of FBS. Photoinitiators (PI) were dissolved in 1× PBS without Ca^{2+} and Mg^{2+} to a final concentration 1.827 mM. Before exposing the cells to the PI solution, the supernatants were aspirated, and the cells were washed once with PBS. The sterile-filtered PI solution was added to the cells, and the plate was incubated for 10 or 30 min at 37 °C. The PI solution was removed, and cells were washed twice with PBS. Fresh growth medium was then added to the cells. All steps were performed without direct exposure to light. Following additional 24 h of incubation, the supernatants were removed and cells were washed three times with PBS. The sterile PBS containing no PI was used as a control solution in this experiment. MTT reagent (Sigma) was dissolved in 1× PBS (5 g/L). Before adding to the cells, this stock solution was diluted with the respective growth medium to 3.25 g/L and the plate was incubated for 1 h at 37 °C. The supernatant was removed, and formazan crystals were dissolved using dimethyl sulfoxide (Sigma). The plate was incubated on a shaker in the dark at room temperature for 20 min, and absorbance was measured at 540 nm with 650 nm as reference. Raw data were collected from two different experiments done in triplicate and analyzed using Prism 5 (Graphpad). The significance was determined using nonparametric, unpaired, two-tailed Mann−Whitney test (each PI group vs control group).

3. RESULTS AND DISCUSSION

Highly efficient hydrophilic two-photon initiators are necessary in order to produce structures from materials with high initial water content by 2PP. Two benzylidene cycloketone-based two-photon initiators G2CK and P2CK (Figure 2) were synthesized via classical Aldol condensation of appropriate aldehyde and cycloketones.[17] The TPA chromophores comprise typical D-π-A-π-D core structures with C_{2v} symmetry, where dialkylamino groups act as donors (D), vinyl as π-conjugated bridges, and carbonyl as an acceptor (A). Carboxylic sodium salts as hydrophilic functionalities were incorporated at the terminal amino group to ensure water solubility. Because of the long conjugation length and the presence of strong electron donor and acceptor groups, large TPA cross sections of 163 GM and 176 GM were obtained for aqueous solution of G2CK and P2CK, respectively.[17] These values surpass the TPA characteristics of commercially available PIs, most of which are not water-soluble, by a large margin.[31] Both PIs exhibited broad processing windows in 2PP hydrogel fabrication tests using formulations with water contents of up to 80%.

The results of an MTT assay showed that while the G2CK had a significant impact on the metabolic activity of MG63 cells, the effect of P2CK was comparable to the control (Figure 3). As a reference PI, a commercially available Irgacure 2959, conventionally used for UV cell encapsulation and considered cytocompatible, was employed (Figure 2).[22−24] Because of its molecular structure and spectral absorption characteristics, Irgacure 2959 is not suitable for 2PP at 800 nm. Therefore, only the G2CK and P2CK compounds were utilized as PIs for the laser fabrication of cell-containing hydrogel constructs.

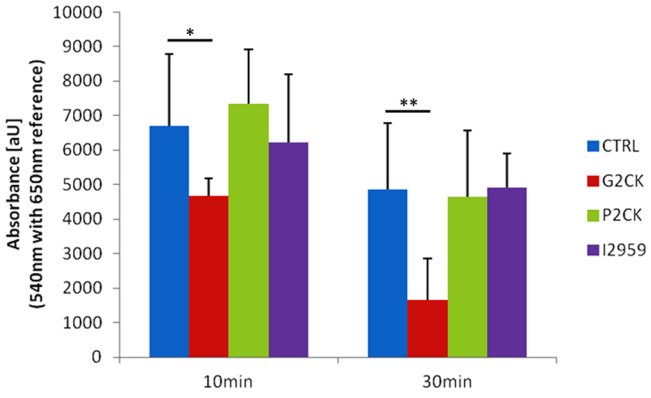

Figure 3. Relative metabolic activity of MG63 cells exposed to the solutions of photoinitiators (PI) for 10 and 30 min, examined by an MTT assay (*$p < 0.05$; **$p < 0.01$).

Human osteosarcoma cells (MG63) employed in the presented experiments are uncomplicated in handling and maintenance. Nevertheless, a recent study demonstrated their basic suitability for the preliminary *in vitro* evaluation of bone tissue engineering materials.[28] Further, *in vitro* studies using

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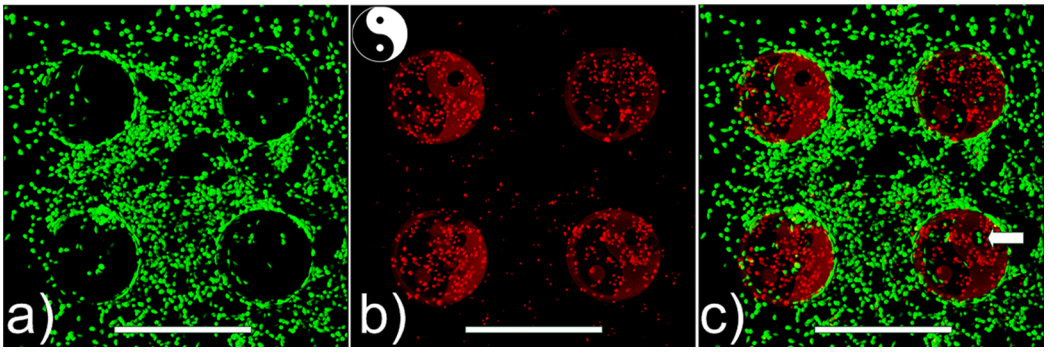

Figure 4. Viability of cells encapsulated in a gelatin-based hydrogel structures produced by two-photon polymerization (2PP): (a) green-stained live cells; (b) red-stained dead cells; (c) overlay, with an arrow indicating two live cells trapped within a cylindrical void. Scale bar represents 500 μm.

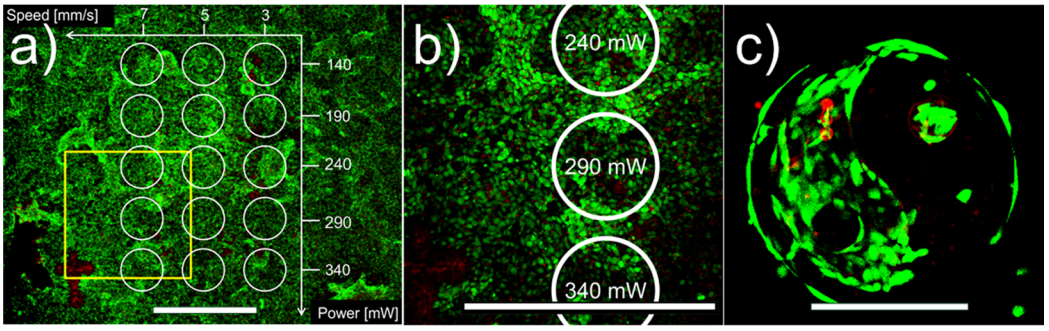

Figure 5. Viability of cells: (a) exposed to laser radiation conditions used for 2PP encapsulation; (b) magnified view of the lower left part; and (c) proliferation of MG63 cells trapped within the voids of a 2PP hydrogel construct after 3 weeks in culture. Scale bar is 200 μm in image (c) and 1 mm in (a, b).

primary cells have to be performed to confirm the results obtained with the MG63 cells.

The considerable practical advantage of gelMOD-based formulations for cell encapsulation is that it is a physical gel at room temperature and becomes a liquid above 35 °C. The samples of cell-material suspension were prepared at 37 °C and then cooled down to room temperature for 2PP. Therefore, the cells are essentially immobilized within the gel throughout the 2PP processing, ensuring optimal cell distribution. This gelMOD's property also proved to be important for the final evaluation of the encapsulated cell viability. Figure 4 shows results of the live/dead staining of the sample containing four Yin−Yang structures produced by 2PP in the presence of cells. The majority of the green-stained cells visible in the Figure 4a are located outside the structures. These cells were in contact with the material formulation but were not exposed to the laser radiation. Therefore, these cells essentially represent a control group for general handling conditions and exposure to the photopolymer and PI. Although some red cells can be observed outside the structures in the Figure 4b, most of the cells from this control group proved viable (Figure 4c). The red autofluorescence signal from the hydrogel allows to observe the actual shape of the 2PP-produced structures (Figure 4b). The red regions represent the bulk of the construct along with the circular walls, while the dark regions are the voids. The majority of cells located within the structure, and in particular the bulk material regions, are also stained red. A few cells located in the immediate vicinity of the bulk within the construct, but not embedded into the material, are stained green. While the cell survival outside the exposed regions is around 95%, only 25.7 ± 1.4% of viable cells are found in the voids of the Yin−Yang structures. In particular, in the lower right structure, two live cells were captured in a small cylindrical void of the Yin−Yang (indicated by an arrow in Figure 4c). Since the structure design actually includes a disk-like lid, these cells are surrounded by polymerized material from the top and the sides, while residing on the glass slide at the bottom. Therefore, these surviving cells were present in the construct volume throughout the fabrication procedure and did not enter it at a later time point. The results of the cell 2PP encapsulation for the formulation containing different PIs (G2CK and P2CK) are very similar. These findings indicate that the cell damage is not caused by their contact with material *per se*, but either the exposure to the laser radiation or the reactive species, produced by activated PI.

On the basis of the previous reports, we do not expect that the utilized parameters of the laser can damage the cells.[32−34] Nevertheless, to verify this assumption, an identical to the above experiment was performed, with no PI being present in the material formulation. A very important concern in such an experiment is the localization of the exposed and unexposed regions. Since without the PI the material would not be cross-linked and cells would not be embedded or confined within the structure, it was necessary to guarantee the immobilization of the cells otherwise. Therefore, instead of a cell suspension in the material, we have prepared confluent layers of the cells adhered to the cover glass. In the sample configuration, identical to the previous experiment, the cells were covered by the layer of gelMOD formulation containing no PI. The sample was processed in the same fashion; i.e., the cells were exposed to the identical laser radiation conditions. The results of the experiment are shown in the Figure 5a,b. In order to mark the position on the sample, a cross pattern was produced using an excessive exposure dose, i.e., focusing on material−glass

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interface at a maximum of laser intensity for a few seconds until bubble formation occurred. The live/dead staining indicated that all the cells within the cross region are critically damaged and stained red. Herewith, they represent a dead control for staining and a mark for the localization of the region exposed to laser. Next to this mark, a program producing an array of cylinders at different average laser powers and scanning speeds was executed. The cylinders are designed to have the size and thickness identical to that of the Yin−Yang structures. Our results indicate that at even at the highest exposure dose used for 2PP, no impact of the laser radiation on the cell viability is observed in this experiment. Since no actual difference of the laser exposed regions to the surrounding is visible, the position of the deployed patterns is indicated by the white circular overlays (Figure 5a,b). These findings confirm our previous assumptions towards the harmlessness of the utilized parameters of laser radiation and indicate the critical role of the PI for the process.

The PI activated by the laser can result in the formation of reactive oxygen species and radicals. Reactive oxygen species are known to be harmful to cells and are employed for targeted cell damage in the photodynamic therapy (PDT). A recent study on the structure−property relationship of bis(arylidene)-cycloalkanone moieties indicates that the singlet oxygen generation greatly depends on the size of the central cycloalkanone ring of the PI. The singlet oxygen quantum yields of hydrophobic chromophores with cyclopentanone are 10 times larger than that of its counterpart with cyclohexanone in chloroform.[35] Therefore, from the PDT perspective, the chromophore with a six-member ring, as in G2CK, is expected to exhibit less cytotoxicity than one with a five-member ring, as in P2CK. However, our results did not yield any evidence to support such hypothesis.

Most photosensitizers used in PDT are required to possess long lifetime of triplet state to ensure the efficiency of the energy transfer between the excited photosensitizers and oxygen molecules to form singlet oxygen.[36] Earlier studies have demonstrated that Irgacure 2959 can have a phototoxic effect on cells.[23,24] However, Irgacure 2959 is an efficient Type I PI, which undergoes direct fragmentation to generate active free radicals for initiating the polymerization. Generally, this type of PIs has a very short-lived triplet states to ensure fast fragmentation. Therefore, the triplet quenching by a molecular oxygen is negligible under most conditions.[37,38] Considering that the efficiency of Irgacure 2959 to produce the reactive oxygen species is limited, the cell damage can be mainly attributed to the radicals, induced after this PI is activated with light. Indeed, it is known that active radicals can react directly with cellular components such as cell membrane, proteins, and DNA.[23] Therefore, another credible scenario in our study is the cell damage by free radicals produced by activated PI.

While the cytotoxic effects from one-photon-activated Irgacure 2959 have been established,[22] the mechanisms of cellular damage by G2CK and P2CK are unexplored. The currently accepted initiating mechanism in 2PP involves charge-transfer interactions between the two-photon excited initiator and the macromer (e.g., gelMOD), resulting in macromer radicals responsible for the subsequent polymerization (Figure 6).[39,40] Although these macromer-based radicals are expected to be cytocompatible,[14] the PI radicals might form peroxyl radicals in the presence of oxygen,[41] which possibly induce cell damage. By abstracting hydrogen from unsaturated fatty acid, peroxyl radicals have been generally thought to induce lipid

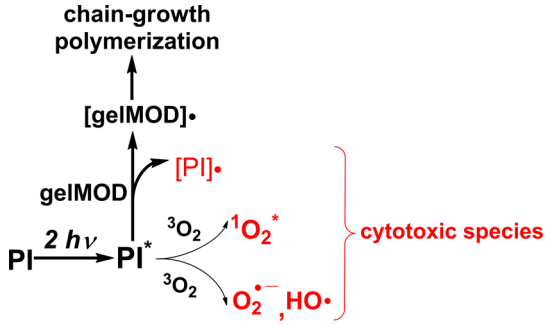

Figure 6. Proposed initiating mechanism of 2PP and molecular pathways for generating cytotoxic species during 2PP cell encapsulation.

peroxidation and related human disease.[42] On the other hand, two-photon-excited PI (with a quite long triplet lifetime compared to cleavable one-photon initiators like Irgacure 2959) can undergo two pathways to generate different cytotoxic reactive oxygen species. First, it can transfer its energy to molecular oxygen (triplet) and form excited singlet oxygen ($_1O_2$*) which has proven effective for killing tumor cells in PDT. Second, oxidative species such as superoxide anion ($O_2^{\bullet-}$) could also be generated by electron transfer from the excited initiator to molecular oxygen. In addition, superoxide could further produce hydrogen peroxide and highly reactive hydroxyl radicals (OH$^\bullet$). It is well established that all these species can cause detrimental damage to plasma membrane, mitochondria, and subsequent apoptosis.[43]

The size of the central cycloalkanone ring affects not only the singlet oxygen generation but also the quantum yield of the active radicals for polymerization. Our recent tests showed that, despite slightly smaller TPA cross section, G2CK is a more efficient PI for 2PP than P2CK. The enhancement is attributed to the higher active radical quantum yields of G2CK than those of P2CK. The higher number of formed active radicals may potentially cause greater damage to the cells at the identical laser exposure dose. However, our experiments did not show any marked difference for the 2PP-encapsulated cell viability for these PIs. A possible reason for essentially similar outcome in cell viability of both investigated PIs, is the combined effect of the two harmful species (singlet oxygen and active radicals). In order to design an experiment allowing a direct quantitative evaluation of possible cell damage, it is necessary to deliver the laser-activated PIs to the cells, while monitoring the cell viability. This is hard to realize with the currently available equipment. Therefore, our future experiments will include evaluation of possible cell damage mechanisms by addition of different radical scavengers to the material formulation. These compounds would serve to deactivate the PI in a concentration-dependent manner.[44] This way it would be possible to indirectly verify if the radicals are the main cause of cell damage.

The viable MG63 cells trapped within the voids of the Yin−Yang structures (Figure 3c) were allowed to proliferate further. After 3 weeks of culture almost all the available space was occupied by viable cells, which stayed confined within construct (Figure 4c). Also, the small cylindrical void of the Yin−Yang accommodated at least ten viable cells, in place of initial two. Live/dead staining does not provide any information about the DNA damage, and cell proliferation does not completely exclude possible mutations. In order to exclude this possibility,

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additional experiments analyzing individual cells or groups of cells would have to be conducted in the future. Nevertheless, such cell-entrapping 3D structures can potentially be used to study the behavior of isolated cell groups. Cellular microarray platforms are becoming a popular tool for drug discovery and stem cell research.[45] Since the hydrogels are permeable to soluble factors, cell response to the outside chemical signals can be investigated. Such precisely engineered cell-containing constructs can be used to emulate conditions of stem cell niche microenvironment *ex vivo*.[46] By using multiple cell types, the samples for co-culture experiments can be realized. Free-standing hydrogel constructs can be even made implantable. Previous reports have demonstrated that the enzymatic degradation capability is preserved for constructs produced from gelMOD.[9] Therefore, it is to be expected that the cells will eventually remodel this material or even be released from the structure altogether. On the other hand, this property can be used for incorporating and gradual release of growth factors or other biomolecules from the walls of the produced 2PP construct. The flexibility of the 2PP method provides a possibility to study the effect of geometrical constraints on the cell behavior in 3D.[12] The high-resolution of 2PP allows fabrication of scaffolds with features at multiple length scales in one step, such as few-100 μm pores and topography on the μm scale.[9,11] Hence, with 2PP it should be possible to reconstruct the complexity of the natural ECM, including its chemical and mechanical aspects, in a controlled and systematic fashion.

Alternative microscale biofabrication technologies capable of additive manufacturing include inkjet printing, robotic dispensing, and laser-induced forward transfer.[47] These technologies impose certain requirements toward the properties of materials processable into a stable 3D construct. Two important aspects are the necessity to deposit the material through the nozzle and/or the required rapid material gelation kinetics. The situation is somewhat different for 2PP, since instead of depositing the material in accordance to the CAD design, the cell containing formulation is cross-linked locally via 2PP in order to define the 3D shape. This way the handling of small material volumes in a form of droplets or strands is avoided. The nonpolymerized material is removed by immersing the sample in the cell-culture medium. Therefore, the process is more gentle when it comes to material handling and places less restrictions on the physical properties of the initial material formulation. The 2PP microfabrication of hydrogel structures is still a largely unexplored topic. There is still a lot of room for optimization of processing and material formulation parameters. Swelling, degradation, mechanical, and cell-adhesion properties of constructs produced by 2PP of different materials, will have to be studied systematically. The availability of water-soluble PI suitable for 2PP will facilitate advances in this direction.[4]

4. CONCLUSIONS

The first use of the two-photon polymerization (2PP) technique for the fabrication of 3D hydrogel structures from a material containing living cells is reported. The critical factor for this approach is the availability of efficient water-soluble PIs. The novel PIs developed by our group facilitate 2PP processing of photosensitive gelatin with up to 80% cell culture medium. It is observed that the MG63 cells present directly within the exposure region are damaged, while the cells located in the immediate vicinity and even within the 2PP-produced structures can survive the process. The control experiments demonstrate that the laser radiation itself does not damage the cells at the parameters used for 2PP. On the basis of our findings and on the analysis of the reports by other groups, we conclude that the reactive species produced during 2PP are the likely cause of the observed cell damage. In order to overcome this impediment, and develop a material formulation supporting the viability of cells encapsulated by 2PP, it is important to identify and eliminate the source of the photochemical cytotoxicity. Once the cell-damage issue reported here is solved, we plan to apply this method to the fabrication of hydrogel constructs containing primary cells and/or stems cells. Our results show that the produced 2PP constructs are stable in cell culture for at least 3 weeks. During this period the viable cells, trapped within the voids of the Yin–Yang structure, are able to proliferate. By week 3 viable cells occupy most of the empty space available within the 3D hydrogel construct. Such cell-entrapping 3D constructs can be used as designer cellular microarrays to study the behavior of the isolated cell groups. The capability of the 2PP technique to shape hydrogels in accordance to a CAD model provides a completely new perspective on precise engineering of 3D cell culture matrices. Processing of cell-containing materials puts 2PP into the realm of biofabrication. Among potential applications of this highly versatile approach are 3D tissue models for drug discovery, stem cell research, and tissue engineering.

■ ASSOCIATED CONTENT

Supporting Information

A video showing the 2PP-produced gelatin Yin–Yang structure containing cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

2PP, two-photon polymerization; PI, photoinitiator; AMT, additive manufacturing technologies; CAD, computer-aided design; TPA, two-photon absorption; ECM, extracellular matrix; gelMOD, methacrylamide modified gelatin; PDT, photodynamic therapy.

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