Enzyme-Degradable 3D Multi-Material Microstructures

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There exists a growing need for smart materials suitable for use in biomedical applications. Herein, a photosensitive material that can be used to fabricate a biocompatible material entirely degradable by the enzyme chymotrypsin is introduced. The photosensitive material is based on a crosslinker with a tyramine moiety that is recognized and cleaved by the enzyme chymotrypsin. Macroscopic films as well as microstructures are fabricated via the use of direct laser writing. Multi-material boxing ring microstructures are generated and selectively degraded by the enzyme. Cell biocompatibility studies indicate that cells are able to attach and proliferate over one week on the material. A photosensitive material that is biocompatible and can be entirely removed by a biocompatible stimulus such as an enzyme can potentially be used as an easily removed tissue engineering scaffold and is especially promising for basic cell biology research.

1. Introduction

3D printing, also known as additive manufacturing, is a comprehensive term for all manufacturing processes in which a 3D object is constructed from a computer-aided design model. Since the patents of Swainson and Hull from 1974 and 1986, respectively, a variety of 3D printing techniques have been developed enabling feature sizes from the millimeter to the micron and even nanometer length scale. Due to the many advantages of 3D printing, such as cost-effective production of prototypes with short lead fabrication time, 3D printing holds advantages of 3D printing, such as cost-effective production of prototypes with short lead fabrication time, 3D printing holds

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Biodegradability is often essential for optimal tissue regeneration so that the scaffold can be absorbed by the surrounding tissue and eliminated without the need for subsequent surgical removal of the material. In the optimal case, the degradation kinetics matches the rate at which the specific tissue type regenerates ensuring the defected tissue retains its biological function and mechanical integrity. Both synthetic polymers as well as biopolymers derived from natural sources are currently finding application in tissue engineering applications. Biologically derived polymers such as polysaccharides, proteins, and nucleic acids, do have advantages in that they already contain similar biochemical components to those which cells experience in actual tissue. This leads to good biocompatibility and is associated with typically low inflammatory response being observed in vivo. In addition, their degradation into nontoxic by-products...
such as sugars and amino acids, enables a safe clearance by the body through its natural physiological cycles.\textsuperscript{[21]} However, other key properties, such as printability, stiffness, and degradation rate often limit their practical application as tissue scaffolding and they have a large amount of variation between batches. For example, the amount of growth factors in the popular Matrigel material, an assortment of extracellular matrix (ECM) proteins, varies substantially between batches as a result of the natural origin.\textsuperscript{[22]} In comparison, synthetic polymers show less variation and thus enable key opportunities in fabricating complex materials with well-defined and reproducible properties. Some notable examples of synthetic polymers used as scaffold material are polyactic acid, polyglycolic acid, and polycaprolactone (PCL).\textsuperscript{[23]} These polymers have desirable mechanical and degradation properties for specific anatomical applications, but also exhibit disadvantages including hydrophobicity or slow and uncontrolled degradation. One solution to this problem lies in the incorporation of hydrophilic and more biocompatible materials, such as polyethylene glycol (PEG), making them more effective for in vivo tissue engineering applications. However, the fact that they do not inherently possess biochemical components and the resulting issues with biocompatibility can limit their potential for tissue engineering applications.\textsuperscript{[24]} Herein we propose that a more promising approach from a tissue engineering perspective is to bring together synthetic polymers and naturally derived components into multicomponent systems, synergistically exploiting the advantages of both, while minimizing their limitations. We achieve this by basing the synthesis of the functional component of our photoresist on the biological components phenylalanine and tyramine.

Demand for scaffold materials with smart properties has stimulated substantial interest in designing photoresists with disparate properties.\textsuperscript{[25,26]} The use of enzymes to degrade materials is a highly attractive option for tissue engineering and regenerative medicine.\textsuperscript{[20,27]} Enzymes are highly chemo, regio- and stereoselective with the capability to work under mild, physiological, and aqueous conditions (pH 5–8, ambient temperature 25–37 °C). A popular approach is the incorporation of enzyme-cleavable peptide sequences into the scaffold material enabling protease degradation.\textsuperscript{[23,28]} Although natural proteins such as gelatine have indeed been utilized to fabricate degradable scaffolds with DLW\textsuperscript{[29,30]} and microstructures have been fabricated from synthetic polymers that are degradable through chemical stimuli,\textsuperscript{[31]} synthetic polymers containing enzymatically cleavable linkages have not, to our knowledge, been explored as an option to alter the degradation and mechanical properties.

A promising enzyme that can be utilized to cleave a scaffold is chymotrypsin. Chymotrypsin is an endopeptidase enzyme secreted by the exocrine pancreas and preferentially cleaves peptide bonds of aromatic amino acid like tyrosine, tryptophan, or phenylalanine. In addition to its known preference for cleaving amide bonds of hydrophobic amino acids, several groups have reported a range of esters based on hydrophilic and charged amino acids which can be hydrolyzed by chymotrypsin in high yields.\textsuperscript{[32–34]} We identified chymotrypsin as an enzyme that can be valuable for degradable scaffolds in two ways. First, chymotrypsin is known to cleave the cell surface proteins, which can be used to remove the biologic tissue with an undamaged ECM from the artificial scaffold. Second, chymotrypsin can simultaneously degrade the artificial scaffold material when an appropriate photoresist with a chymotrypsin-recognizable linkage is employed.\textsuperscript{[35]}

Herein, we introduce a biocompatible and photocurable resist for scaffold fabrication that can be readily degraded by chymotrypsin. We first outline the synthesis of a degradable chymotrypsin-specific crosslinker based on the naturally occurring components phenylalanine and tyramine, followed by investigating the photoresist formulation in terms of degradability using the synthesized chymotrypsin-specific crosslinker and dithiol polyethylene glycol. The polymer network studied herein takes advantage of the mixed-mode polymerization of thiols with acrylamides. We further demonstrate biocompatibility of the photoresist by cell viability tests showing that fabricated materials support cell adhesion and proliferation. To illustrate the suitability of the photoresist for tissue engineering applications, we print multi-material 3D scaffolds. We chose a boxing ring type design, i.e., four pillars connected by free-standing bridges, for these microstructures, as it can serve well as a model for larger scaffold structures.\textsuperscript{[36–38]} DLW as a manufacturing technique lends itself particularly well to altering the feature sizes and design of the microstructures and consequently the degradation behavior. Scheme 1 depicts the fabrication and degradation of the multi-material boxing ring microstructures.

\section*{2. Results and Discussion}

As our interest focuses on the development of a chymotrypsin-cleavable photoresist for light-based 3D printing, we initially synthesized a photocurable crosslinker containing an enzyme-specific linkage that could be hydrolyzed by chymotrypsin. Figure 1 shows the synthesis of the chymotrypsin-cleavable crosslinker in a three-step procedure. We started with the naturally occurring tyramine, a trace amine derived from the amino acid tyrosine. Tyramine has been reported as a hydrophobic motif that enables a chymotrypsin-selective hydrolysis of poly(amine acid)s.\textsuperscript{[39]} Tyramine was initially functionalized with an acrylamide group 1\textsuperscript{)} and subsequently converted into the precursor molecule 2 via a Steglich esterification with a phenylalanine having a tert-butyloxy carbonyl protecting group (boc) 2\textsuperscript{). A small molecule

\begin{figure}[h]
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\includegraphics[width=\textwidth]{image1}
\caption{Fabrication and degradation of multi-material boxing ring microstructures. Initially, DLW was used to print the frames from commercial IP-S resist. After developing and washing the samples, the bridges were printed in a second step from the ED resist. The bridges can subsequently be completely degraded by exposure to the enzyme chymotrypsin while leaving the frames intact.}
\end{figure}
study of considerable significance showed complete degradation of the precursor molecule 2 to the starting compounds 1 and boc-phenylalanine when dissolved in a solution containing chymotrypsin (Figure S9, Supporting Information). In contrast, no degradation products were detected in the absence of chymotrypsin under the same conditions after one day demonstrating a chymotrypsin-specific degradation. Finally, we removed the protective group with trifluoracetic acid and functionalized the resulting compound with a second acrylamide group to receive a photocurable crosslinker 3. Structural confirmation of the final molecule 4 was obtained by $^1$H and $^{13}$C NMR spectroscopy (Figures S6–8, Supporting Information).

After the successful synthesis of the chymotrypsin-cleavable crosslinker, we fabricated disks by one-photon polymerization to optimize the photoresist formulation for degradability. In a typical procedure, the photoresist formulation of interest with 1 wt% Irgacure 369 as the initiator species was sandwiched between two glass slides and exposed to 20 mW cm$^{-2}$ of 385 to 515 nm light for 5 min using a commercially available light-emitting diode (LED) lamp. Irgacure 369 as photoinitiator at a concentration of 1 wt% has been reported to be suitable for fabricating scaffold material. To assess the degradability, each disk was subjected to a pH 7.4 phosphate buffer solution (PBS) at 37 °C containing 1 mg mL$^{-1}$ chymotrypsin. The results were always compared to control experiments in which a disk received identical treatment, but without chymotrypsin. Our initial studies revealed that a photoresist formulation solely composed of our synthesized molecule 3 with a small quantity of solvent, e.g., DMF, DMSO, PEG, among others, resulted in non-degradable disks. Although the disks were incubated for one week in a solution of chymotrypsin, they remained mechanically stable without noticeable signs of degradation. As our small molecule study showed that the precursor molecule 2 is readily degraded into 1 and boc-phenylalanine by chymotrypsin (refer to Figure S9, Supporting Information), we hypothesized that the high crosslinking density accompanied by a short distance between consecutive crosslinks may make it difficult for chymotrypsin to access the cleavable ester bond. To increase the distance between cross-links, which is associated with better accessibility of the cleavable ester bond, we added dithiol polyethylene glycol to the photoresist formulation, which forms elastically active chains between cross-links in the network.

Incorporating PEG into the polymer network not only increases the network mesh size, thus decreasing the network stiffness, but also serves as a biocompatible motif that promotes cell adhesion and migration. All photoresist mixtures were formulated at thiol:acrylamide functional group ratios of 1:1 to incorporate one PEG linker between each crosslink in an ideal formed network. In the following, this material will simply be referred to as the enzyme-degradable (ED) material. Adding a dithiol-functionalized co-monomer to an acrylamide has significant impact on the polymerization mechanism. In this case, the network is formed by a mixed-mode polymerization mechanism that is a combination of radically mediated chain-growth and step-growth reactions. Previous work indicated enhanced writing performance in DLW, in terms of average usable laser power (LP) and fabrication speed, when a thiol was added to an acrylate resist. In addition to improving performance in DLW, hybrid polymerization is particularly valuable from the perspective of degradability, since hydrogel networks fabricated through the mixed-mode mechanism are readily hydrolysable at physiological pH through cleavage of

![Figure 1](image-url). Preparation of the chymotrypsin-cleavable crosslinker in a three-step synthesis.
ester linkages.[42,45,47,48] Our degradation studies with chymotrypsin showed complete visual disappearance of disks fabricated with the optimized photoresist formulation in an enzyme solution after 4 h, whereas control disks were unaffected even after one week of treatment at identical conditions but without enzyme. Of note was the rapid degradation rate, which indicates excellent accessibility to the enzymatically recognizable linkages in the polymer network. Although we focus on chymotrypsin to degrade in this report, we have also assessed several other notable cleaving conditions, which might be useful for other applications. A summary of different cleaving conditions is presented in Table 1. For example, another common biodegradation mechanism used for regenerative medicine is hydrolysis of esters. Disks exposed to basic conditions in a 1 M NaOH solution degraded, whereas disks remained stable to acidic conditions in a 1 M HCl solution for 1 week. This is attributed to the high reactivity of the phenolic ester towards nucleophilic addition, which is in line with reports showing higher hydrolysis rates of phenolic esters at basic conditions than at acidic conditions.[49,50] In addition to base-catalysed hydrolysis, disks can be completely degraded at 50 °C within 1 h via aminolysis when immersed in a solution of ethanolamine.

Having identified a photoresist formulation that was selected for the intended application, multiple tests with MC3T3-E1 (passage 19) cells, an osteoblast precursor cell line derived from mouse skull bones, were conducted in order to ascertain whether these cells could attach to and proliferate on the material.[51] The MC3T3 cell line is a reliable cell model for in vitro studies and widely used in medical research.[52] In all tests, disks of the ED material fabricated in the same way as the disks used in the degradability studies, as well as disks made from PCL were investigated as control samples. PCL was chosen as a widely utilized non-cytotoxic (or biocompatible) material for tissue engineering scaffolds. Both a qualitative assay, in the form of an Alamar blue assay, and a quantitative cell viability assay, in the form of a LIVE/DEAD assay, were used to assess the cytotoxicity of the ED material. Additionally, the cell morphology was visualized using the immunofluorescence of a DAPI/phalloidin stain.

In a LIVE/DEAD assay, disks seeded with cells were stained with calcein and ethidium revealing live cells fluorescing green from intracellular esterase activity and dead cells fluorescing red owing to ethidium penetrating compromised cell membranes and binding to the cell nuclei. Figure 2a shows that the majority of cells were alive one day and seven days after cell seeding on the PCL control, as well as on the ED material. These results indicate that the ED material is not cytotoxic, which is crucial for the intended application.

4',6-Diamino-2-phenylindole (DAPI)/Phalloidin staining was performed to evaluate the morphology of the cells seeded onto the ED material.[53,54] The cells were stained with Alexa Fluor 488 phalloidin, staining the cytoskeleton green, and DAPI, staining the nucleus blue. The DAPI exhibited some non-specific binding to the ED material itself, leading to background fluorescence, however the shape of the cells’ cytoskeleton can be clearly identified and the cell stretching indicates adhesion to the material and spreading, a favorable response to the surface and a good indicator of biocompatibility (Figure 2b).

The most important result came from an Alamar blue assay, which assesses the cell viability and metabolic activity.[55] Here, the fluorescence intensity Alamar blue dye reduced by live cells was stained with calcein (green) and ethidium (red), indicating live and dead cells, respectively. Scale bars: 100 µm. b) DAPI/phalloidin stain. Cells were stained with Alexa Fluor 488 phalloidin, staining the cytoskeleton green, and DAPI, staining the nucleus blue. The DAPI exhibited some non-specific binding to the ED material itself, leading to background fluorescence, however the shape of the cells’ cytoskeleton can be clearly identified and the cell stretching indicates adhesion to the material and spreading, a favorable response to the surface and a good indicator of biocompatibility (Figure 2b).
cells was evaluated and quantified on day 1 and day 7 of cell culturing (Figure 2c). This gives an indication of metabolic activity. The test showed that metabolic activity per unit area was significantly higher on the ED material than on the PCL control on day 1. On day 7, this difference is smaller, but still present. Importantly, the metabolic activity on the ED material increased over the course of the test, just like it did on the PCL control, indicating cell proliferation. In summary, the results from the three tests indicate cell viability, attachment, and proliferation on the ED material. It thus indicates a good level of biocompatibility and qualifies for further investigation for use in biological applications.

Once the biocompatibility of our photoresist formulation was established, we investigated the performance of the photoresist in DLW by fabricating line patterns to determine the minimum feature size (i.e., the linewidth). It is essential for a tissue engineering application to be able to print at least on a length scale relevant to cells, which is typically in the micron range. All experimental DLW was carried out with a commercially available 3D DLW lithography system (Photonics Professional by Nanoscribe GmbH) using a 63 × oil immersion objective (NA = 1.4). The linewidth at the fabrication threshold was found to be close to 800 nm. Cell scaffolds of many different dimensions have been utilized and this minimal feature size falls well within that range.[18]

We subsequently fabricated simple block structures from our resist formulation using a range of different writing parameters, i.e., LP and scan speed (SS) (Figure 3). This test was performed to assess what writing parameters would lead to stable structures. Choosing the writing parameters for DLW presents a balancing act, as too much laser exposure of a certain voxel per unit of time will lead to micro-explosions, causing uncontrolled polymerization (top left Figure 3), while too little laser exposure will lead to no structures being written at all. We fabricated block structures using LP between 8 and 16 mW and SS between 200 and 5000 µm s⁻¹. At the extreme edges of those parameters indeed either no polymerization or microexplosions were observed, while most parameters in between produced acceptable structures. The notches on the block microstructures mostly serve to make it easier to distinguish structures written using different parameters, but their shape can also serve as another indication of fabrication quality.

Having determined the writing properties of our resist, we subsequently constructed more elaborate boxing ring structures (Figure 4) that served as models for scaffold structures. In all cases the frames of the microstructures were constructed from commercial IP-S resist. After washing and developing the samples, the free-standing bridges were constructed in a second writing step from the ED resist. We attempted constructing bridges of several lengths and found that while bridges with a length of 100 µm tended to collapse, bridges with a length of 75 µm were consistently stable, which led us to construct all further microstructures with those dimensions (see Figure S10, Supporting Information). Bridges with cross-sections of two different areas were constructed, both to showcase the versatility of the structures that can be fabricated, but also to investigate how features of different dimensions degrade. This particular boxing ring design was chosen because the ribs and platform increase the overall stability and adhesion to the substrate.

The boxing rings were degraded in the same way as the film samples. The degradation was monitored using optical microscopy (refer to Figure 4 and Movie S1, Supporting Information). The sample had bridges of two different dimensions (∼3 µm vs ∼9 µm in thickness) to assess the difference in
The first signs of degradation can be seen after around 40 min, when the first tears in the bridges start to appear. 20 min later, the thinner bridges already collapse. After 80 min of exposure to chymotrypsin even the thicker bridges have collapsed or snapped. Overall, complete disappearance of the bridges varies between 60 and 180 min depending on the bridge thickness. Crucially, the frame not constructed from the ED resist is completely intact after the procedure, showing that this is a valid method to selectively degrade parts of microstructures.

It is interesting to observe where exactly the degradation commences. The optical microscopy images already give a good indication of that, however, to obtain a more accurate impression we analyzed a sample that had been partially degraded for 60 min using SEM (Figure 5). Here it is immediately clear that the bridges do not degrade evenly across the entire structure.
but that degradation initially occurs at the connection points between the frames and bridges, as well as at the very center of the bridges. We assume that these points experience the most structural stress and therefore collapse first.

We further fabricated two slight variations of the boxing ring microstructures (Figure S11, Supporting Information). Multi-material boxing rings with bridges with a programmed breakpoint in the middle should increase the tendency of the bridges to first degrade in the center even further, leading to faster collapse. If the only goal is for the bridges to collapse and not for full degradation to occur—which might very well be the case for cell scaffold applications—this would be highly desirable. Multi-material boxing rings with bridges with two separate breakpoints could not be achieved by simply modifying the files used to create the structures, as the bridges collapsed during the DIW process. Instead we had to employ a technique sometimes referred to as grey-tone lithography.[59] Here, the material properties of microstructures are modified by varying the writing parameters during the writing of (a certain part of) the microstructures. We employed a LP of 12 mW to fabricate the thick parts of the bridges and 6 mW to construct the thin parts. These bridges should degrade in a different manner from the standard bridges.

3. Conclusion

We have introduced a photoresist allowing the fabrication of materials that can be completely degraded by the enzyme chymotrypsin. The degradability is based on a crosslinker we introduce herein that contains a functional group which is recognized and cleaved by the enzyme. The material is highly versatile as it can not only be degraded by the enzyme, but also by base and through aminolysis. Both simple films via LED-curing and elaborate microstructures containing free-standing elements via DIW have been fabricated. Multi-material boxing ring microstructures have been generated, showing that degradation only occurs in those parts of the microstructures generated from the ED material, while leaving other parts completely intact. The degradation behavior can be modified through the design of the microstructures, simply by varying the feature sizes, or by designing breakpoints that are to be degraded first. It is critical that the material is biocompatible, as proven through cell viability assays, qualifying it for development towards future biomedical applications. A biocompatible material that can be degraded readily and mildly in this manner is an ideal candidate as cell scaffold material. Small boxing ring structures as presented herein may be used to study the reaction of individual cells to the degradation of their scaffold, possibly leading to answers for basic questions of biology, while larger scaffold structures could be used for tissue engineering. Being able to engineer tissue in a scaffold which can be removed at a chosen point in time without affecting the tissue itself is highly desirable and moves towards enabling 3D tissue constructs to be created to heal patients with tissue loss or to guide tissue regeneration.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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