Bioprinting can be defined as the art of combining materials and cells to fabricate designed, hierarchical 3D hybrid constructs. Suitable materials, so-called bioinks, have to comply with challenging rheological processing demands and rapidly form a stable hydrogel postprinting in a cytocompatible manner. Gelatin is often adopted for this purpose, usually modified with (meth-)acryloyl functionalities for postfabrication curing by free radical photopolymerization, resulting in a hydrogel that is cross-linked via non-degradable polymer chains of uncontrolled length. The application of allylated gelatin (GelAGE) as a thiol–ene clickable bioink for distinct biofabrication applications is reported. Curing of this system occurs via dimerization and yields a network with flexible properties that offer a wider biofabrication window than (meth-)acryloyl chemistry, and without additional nondegradable components. An in-depth analysis of GelAGE synthesis is conducted, and standard UV-initiation is further compared with a recently described visible-light-initiator system for GelAGE hydrogel formation. It is demonstrated that GelAGE may serve as a platform bioink for several biofabrication technologies by fabricating constructs with high shape fidelity via lithography-based (digital light processing) 3D printing and extrusion-based 3D bioprinting, the latter supporting long-term viability postprinting of encapsulated chondrocytes.

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Biofabrication technologies provide a processing pathway for automated production of cell-material composites that resemble the complex hierarchical architecture of tissues and possess functional features. Bioprinting, the direct processing of these composites into 3D constructs, is one of the main biofabrication strategies.[1] Suitable bioinks must meet demanding processing criteria, such as cytocompatibility, rheological profiles, and rapid mechanical recovery or cross-linking postextrusion, to ensure high shape fidelity.[2] The biofabrication window and processing viscosity requirements vary significantly between the multiple fabrication methods available, often necessitating the optimization of an individual bioink for each individual biofabrication technique.

Gelatin is one of the most frequently used hydrogel materials for bioink formulations. It is water soluble, contains bioactive cues such as cell-binding motifs (e.g., Arg–Gly–Asp (RGD)), and is enzymatically degradable.[3] Since most cell culture experiments are required to be at physiological temperature (37 °C), gelatin has to be generally modified with functional groups enabling covalent network formation postfabrication, independently on whether the hydrogel is simply cast into a mold or printed into a 3D structure using a robotic dispensing system. Functionalization of gelatin with methacryloyl moieties (GelMA) is the most widely adopted approach, enabling cytocompatible postfabrication curing by free radical photopolymerization via different photoinitiators.[4] Free radical polymerization does, however, result in formation of polymer chains that act as chemical cross-linkers between gelatin molecules to form a rather heterogeneous network.[3b,5] Thiol–ene click chemistry herein represents an interesting alternative cross-linking mechanism, given that it is based on dimerization of thiols with reactive carbon–carbon double bonds (“enes”). This reaction follows a step-growth radical mechanism with high conversion of functional groups, low polymerization shrinkage and stress, while requiring lower radical initiator concentrations.[3b,5a,b,6] Thiol–ene coupling of multifunctional molecules thus yields more homogeneous networks with characteristics determined by the molecular architecture of the used educts.[5a,b,6,b,c] Using
this approach, norbornene-modified gelatin has recently been applied to successfully encapsulate cells by thiol–ene cross-linking.\cite{3b,7} Another advantage of thiol–ene chemistry over free radical polymerization is the generally rapid reaction kinetics.\cite{5a–c} Gel points for thiol–ene cross-linked systems, such as PEG4NB (PEG norbornene), are reached within few seconds (3 ± 1 s) compared to free radical polymerizations of PEGDA (PEG diacylate) hydrogels with 125 ± 7 s.\cite{8} Furthermore, we have also demonstrated the suitability of thiol–ene clicked poly(glycidol) polymers for postfabrication curing of biofabricated hydrogel constructs.\cite{6a,9}

The present study aims at synergizing the properties of thiol–ene cross-linking with the favorable biological properties of gelatin to yield a flexible bioink platform able to be produced in large quantities for biofabrication. We characterized allylated gelatins (GelAGE) regarding the degree of modification (DoM), possible modification sites, and molecular weight distributions as a result of degradation under required alkaline synthesis conditions.\cite{10} Furthermore, we compared two different photoinitiator systems throughout this study, UV-initiated (320–390 nm) 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (I2959) and visible-light-initiated (390–500 nm) tris(2,2′-bipyridyl)dichloro-ruthenium(II) hexahydrate with sodium persulfate (Ru/SPS). Based on the systematic characterization of the products and the resulting hydrogel properties, we aimed to develop gelatin-based thiol–ene photoclickable materials as a bioink capable of meeting the demands of several biofabrication techniques, with demonstrated applicability via lithography-based (digital light processing; DLP) 3D printing and extrusion-based 3D bioprinting.

GelAGE synthesis was optimized based on previous published protocols\cite{11} by reacting gelatin with allyl glycidyl ether (AGE) at 65 °C under alkaline conditions and performing a systematic reaction parameter study by using 0.4, 2.0, and 10 mmol NaOH per g gelatin and 2.4, 12, and 60 mmol AGE per g gelatin (Figure 1A). Throughout this paper, the products are termed according to their synthesis parameters, representing low, medium and high concentrations of NaOH and AGE. For example, GelAGE_{1MM} refers to 1 h reaction time, medium NaOH concentration (2.0 mmol), and medium AGE concentration (12 mmol). GelAGE products were thoroughly characterized, including the influence from the alkaline reaction conditions on the degradation and the assessment of most probable gelatin reaction sites (Table S1 and S2, Figure S1–S3, Supporting Information). For comparison to the current gelatin-based bioink gold standard, GelMA was used as a reference bioink throughout this study.

To demonstrate the difference in network composition (Figure 1B), we fabricated GelMA and GelAGE_{1MM} hydrogels with the same irradiation dosage, followed by microwave-assisted acidic hydrolysis of the obtained hydrogels and gel permeation chromatography (GPC) analysis of the residual polymer fraction (Figure 1C, Figure S4, Supporting Information). For hydrolyzed GelMA hydrogels, an increased UV absorbance at an earlier retention volume was detected compared to GelMA and GelAGE_{1MM} precursors and to the hydrolyzed GelAGE_{1MM} hydrogels. This peak occurred in the same

Figure 1. A) Synthesis of GelMA and GelAGE. B) Scheme of the free radical polymerization of GelMA in comparison to the controlled dimerization with thiol–ene click chemistry for GelAGE. C) GPC elugrams of gelatin, pMA, hydrolyzed GelMA and GelAGE_{1MM} hydrogels (20 wt%; 0.05 wt% I2959; allyl:SH = 1:12) and the corresponding precursors.
retention volume range as the GPC standard pMA and thus proves the presence of nondegradable higher molecular weight components in GelMA hydrogels as a consequence of the free radical photopolymerization reaction.

We then compared GelAGE and GelMA hydrogels regarding their mechanical properties in order to elucidate whether the systematic variation of GelAGE synthesis could be correlated to concurrent tuning of the mechanical properties of the hydrogels prepared thereof. We first investigated different batches of GelAGE cross-linked with DTT (allyl:SH = 1:1.5–1:12) with conventional UV-initiated polymerization using the photoinitiator I2959, to analyze whether gelatin degradation would impact the mechanical or swelling properties of the formed hydrogels due to alkaline synthesis conditions. Since cytotoxic effects of the commonly used photoinitiator I2959 have been reported at concentrations above 0.1 wt%,[12] we worked with a concentration of 0.05 wt% to mitigate undesirable cytotoxicity. No noticeable differences in hydrogel mechanical properties were detected for different GelAGE batches (Figure S5, Supporting Information), and we concluded that negative effects arising from degradation were compensated by increasing DoM values. Based on these measurements, we chose GelAGE1MM for subsequent studies due to the balance of DoM to the molar weight distribution and the increased mechanical stiffness and low swelling behavior at lower thiol concentrations (allyl:SH = 1:3).

Since the application of UV irradiation for cell encapsulation raises concerns regarding potential long-term damage of cellular DNA,[13] initiator systems harvesting light in the more cytocompatible visible-light wavelength range have recently gained attention. One alternative visible-light-initiating system with improved cytocompatibility is the ruthenium-complex-based Ru/SPS initiator system.[3a] We thus compared this initiator system with UV (I2959) for photopolymerization of GelMA and GelAGE1MM to investigate their effect on the mechanical and physicochemical properties of the resulting hydrogels. For both initiating systems, the lowest allyl:SH ratio resulted in the weakest hydrogels, and increasing cross-linker concentrations led to maximum compressive Young’s moduli at ratios of 1:3 for I2959 (53.2 ± 5.8 kPa) and 1:6 for Ru/SPS (103.6 ± 13 kPa) (Figure 2A,B). The optimum cross-linking efficacy for UV conditions was supported by the lowest sol fraction and mass swelling ratio (Figure S6A,B, Supporting Information). In contrast, the visible-light system displayed comparable sol fraction and mass swelling ratios for 1:3–1:6 allyl:SH ratios (Figure S6C,D, Supporting Information). Notably, with cytocompatible initiator concentrations, the visible-light system allowed further tuning of hydrogel stiffness in the range of 2–110 kPa by varying Ru/SPS concentrations (0.2/2–1/10 × 10⁻³ M Ru/SPS) at fixed allyl:SH ratios (1:6) and polymer weight percentages (Figure 2B). It generally appeared that higher excess of thiols (allyl:SH = 1:12) led to excess coupling of DTT to GelAGE which saturated the AGE functionalities resulting in a reduction in cross-link formation and thus decreased mechanical properties.

Specifically for UV conditions, increasing polymer weight percentage represented the only possibility to significantly enhance the properties of both hydrogel systems, and hence 30 wt% hydrogels were included in the comparison (Figure 2C). While GelAGE1MM and GelMA showed similar mechanical properties for I2959, the visible-light system displayed superior properties for GelAGE1MM with comparable weight percentages to GelMA. For GelAGE1MM, the highest Ru/SPS concentration resulted in improved hydrogel mechanical properties compared to I2959. We further demonstrate cytocompatibility of the GelAGE1MM system over a wide range of allyl:SH ratios for visible-light cross-linked hydrogels, irrespective of differences in mechanical stiffness (Figure 2C,D and Figure S7 and S8, Supporting Information). An upper limit for excess DTT was identified, where 1:12 allyl:SH molar ratios significantly impaired both cell viability and metabolic activity, explained by the concurrent presence of thiol groups saturating the allyl groups without further cross-linking the network. In addition, a negative effect of UV irradiation damage was evident by the significant difference in cell viability between both the 20 wt% 1:3 and 1:6 allyl:SH molar ratio GelAGE1MM conditions cross-linked with UV-light as compared to visible-light (Figure 2D). Similarly, a significantly lower viability was observed in GelMA hydrogels cross-linked with UV as compared to visible-light, comparable to our previous findings.[3a] Overall, the visible-light system appeared favorable due to its decreased potential for DNA damage by avoidance of irradiation under UV-light[14] and especially as it displayed an increase in metabolically active cells compared to the UV-initiation of GelAGE1MM polymerization (Figure 2E).

In general, bioinks currently in use are developed and optimized in individual formulations to serve the selected biofabrication technology. This originates from the significant differences that each fabrication technology imposes on a potential ink. For example, whereas non-Newtonian fluid behavior such as shear thinning is crucial for cell laden extrusion-based printing of bioinks, low-viscosity Newtonian behavior is needed for biobased resins used in lithography-based DLP 3D printing applications. Moreover, the temperature-dependent physical gelation of gelatin is often exploited in extrusion-based processes, whereas it is critical for lithography-based DLP bioresins to be non-thermosetting to achieve the necessary flow properties to yield high print fidelity. The resulting demand for individual bioink development for each technology is a significant drawback.

In contrast, the herein presented control over degradation and DoM of gelatin during GelAGE synthesis together with the tunability of the hydrogel properties enables the application of this system for different biofabrication approaches. For application as bioresin in lithography-based DLP 3D printing, GelAGE1MM possesses advantageous properties due to the lack of physical gelation and the low viscosity of 10–20 wt% solutions at room temperature. Using this solution, a micropore arrangement with porous structures (250 µm in diameter strands) was obtained in combination with 0.5/5 × 10⁻³ M Ru/SPS (Figure 3A,B). Furthermore, addition of photoabsorbers, such as Orasol Orange G, is commonly required for successful lithography-based DLP 3D printing in order to control radical propagation.[14] However, in this study, complex GelAGE1MM constructs with high shape fidelity were successfully fabricated via lithography-based DLP 3D printing without the need for any photoabsorber. In contrast, GelMA (10 wt%) was inherently not applicable for lithography-based DLP 3D printing due to its physical gelation at room temperature.
Since GelAGE 8MM solutions remain liquid even upon cooling, this specific product cannot be used as a bioink in extrusion-based 3D bioprinting. However, a less degraded product (GelAGE 1MM) with remaining thermogelling properties was proven suitable for extrusion-based 3D bioprinting. Rheological experiments confirmed that 30 wt% GelAGE 1MM

Figure 2. A,B) Overview of compressive Young’s moduli for 20 wt% GelAGE$_{1\text{MM}}$ hydrogels photopolymerized via UV (0.05 wt% I2959) (A) and varying Ru/SPS visible-light-initiator concentrations (0.2/2–1/10 × 10$^{-3}$ μM Ru/SPS) (B). C) Young’s moduli for different weight percentages of GelAGE$_{1\text{MM}}$ (allyl:SH = 1:3) compared to GelMA for both initiator systems. D,E) Effect of different hydrogel compositions and photoinitiators on cell viability (D) and change in metabolic activity (correlation of 7 d to 1 d) of HACs encapsulated in hydrogels (E). Conditions marked with (#) in (B) and (C) represent nonmeasureable hydrogels (too soft) and (∼) assigns lack of gelation.
Lithography-based DLP 3D printing

Extrusion-based 3D bioprinting: Vis; 30 wt% GelAGEIM; 1:3

Extrusion-based 3D bioprinting

UV; 30 wt% GelAGEIM; 1:3

Vis; 20 wt% GelAGEIM; 1:3

Vis; 10 wt% GelAGEIM; 1:12

Experimental Section

See the Supporting Information for further details.

Syntheses of Modified Gelatins: GelMA was synthesized as described before.\[^{11}\] Synthesis of GelAGE was performed based on modifications of previous protocols.\[^{11}\] Gelatin was dissolved in deionized water (10 wt%, 65 °C) and different amounts of AGE (2.4–60.0 mm) and 2 wt% NaOH (0.4–10 mmol) were added. The reactions were continued for 1–24 h at 65 °C and afterward dialyzed (molecular-weight cutoff (MWCO) = 1 kDa) against deionized water. The products were lyophilized and stored at 4 °C until use. The DoM was based on 1H-NMR spectroscopy for modified gelatins.

Hydrogel Fabrication, Characterization, and Cell Encapsulation: All hydrogels were prepared in PBS and irradiated with UV- or visible-light for 3 min at 30 mW cm\(^{-2}\). GelAGE precursor solutions were mixed with DTT to obtain functional group ratios of 1:1.5–1:12 (allyl:SH) and photopolymerized in an open environment, whereas GelMA hydrogels (I2959) were covered with glass slides to reduce oxygen-inhibiting effects. Sol fraction and mass swelling ratio (q) were determined. Mechanical properties were recorded in linear compression using a BOS 5500 system (ElectroForce). For aqueous GPC analysis, hydrogel samples were hydrolyzed in 6 M HCl (200 μL) for 30 min at 300 W (CEM discover SP-D) and dissolved in GPC eluent overnight prior to GPC analysis of the hydrolyzed hydrogels, the precursors, as well as pMA.

Human articular chondrocytes (HACs) were encapsulated in hydrogels (15 × 10\(^6\) cells mL\(^{-1}\)) and cultured (7 d) in chondrogenic differentiation medium. Cell-free hydrogels served as a negative control group. Viability was assessed by Live/Dead staining and metabolic activity by AlamarBlue assay. Articular chondrocytes were harvested from either macroscopically normal regions of articular cartilage following informed consent from human patients undergoing anterior cruciate ligament reconstruction surgery (New Zealand Health and Disability Ethics Committees, URB/07/04/014/AM02).

Lithography-Based DLP 3D Printing: A 4 Standard apparatus (EnvisionTec, Perfactory Standard) was used to fabricate GelAGEIM constructs (10–20 wt%, 0.5/5 × 10\(^{-3}\) M Ru/SPS, allyl:SH = 1:12 in PBS). The constructs were fabricated with the depth of each layer set at 50 μm.

Extrusion-Based 3D Bioprinting: 3D printing of 30 wt% GelAGEIM (allyl:SH = 1:3, 0.05 wt% I2959 or 1/10 × 10\(^{-3}\) M Ru/SPS) in PBS) was achieved by robotic dispensing at 4–7 mm/s with a 3D discovery printer (RegenHU) and 23 G needle tips. The constructs were irradiated while printing (5 × 2 s intervals) with UV-light or after printing (3 min) with visible-light and analyzed with a stereomicroscope (SteREO Discovery V20, Carl Zeiss). For Vis-light extrusion-based bioprinting, porcine weight components was proven, as well as the possibility to tune its synthesis to result in formulations that can be used for different biofabrication technologies. The commonly used UV-photoinitiator I2959 was compared to an alternative initiator system based on visible-light (Ru/SPS). A broader, cytocompatible concentration range of Ru/SPS enables tuning of the physicochemical and mechanical properties of GelAGE hydrogels in dependence of photoinitiator concentrations and varying cross-linker concentrations. GelAGE cytocompatibility was demonstrated over a wide hydrogel composition and polymer percentage range. The versatility of our system as a potential bioink was proven for both high-fidelity lithography-based DLP 3D printing and extrusion-based 3D bioprinting purposes. Extrusion-based 3D bioprinting of chondrocyte-laden constructs promoted long-term viability for up to three weeks, further underlining the applicability of our approach toward tissue engineering applications. These results demonstrate that GelAGE exhibits a generic applicability and can serve as platform bioink for different biofabrication techniques.
chondrocyte-laden constructs (3.0 × 10^6 cells mL⁻¹) were cultured for up to 23 d in chondrocyte growth medium. Porcine chondrocytes were obtained from healthy cartilage tissue of patellofemoral joints of three-month-old pigs. Material was obtained from a local slaughterhouse. Live/Dead staining was used for visualization (LEICA TCS SP8, Leica Microsystems AG).

Statistical Analysis: Differences between hydrogel groups were assessed by one-way ANOVA with post hoc Tukey (SigmaPlot 12.5). Viability data are expressed as the results from t-test analysis between time points. All values are presented as the mean ± standard deviation. Significant differences between the represented groups are assigned with * (p < 0.05) and ** (p < 0.001).

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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