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Light-Based 3D Printing of Thiol-Ene and Methacrylate Hydrogels

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Abstract

Light-based 3D printing of photocrosslinkable hydrogel resins allows fabrication of complex scaffolds for tissue engineering and medical devices including the incorporation of cells. Fast and controlled crosslinking is necessary to fabricate scaffolds with customized properties, adequate resolution and high cell viability. This thesis investigates hydrogels formed by photo-initiated thiol-ene and methacrylate crosslinking and compares their properties and performance as (bio)resins in two light-based 3D printing methods. Photopolymerizable resins based on hyaluronic acid modified with allyl ether, methacrylate or norbornene groups are developed and their photocrosslinking kinetics, mechanical properties and printability are evaluated and compared using oscillatory shear rheology and layer-by-layer digital light processing. Low viscosity, fast crosslinking variants are further explored for tomographic volumetric bioprinting of cell-loaded scaffolds with internal channels without addition of viscosity enhancers. Finally, the performance of methacrylate and thiol-norbornene polyvinyl alcohol-based resins for volumetric printing of high-resolution hydrogel devices is assessed. The results demonstrate the benefits and limitations of thiol-ene and methacrylate-based resins for light-based 3D printing of hydrogels of relevance for biomedical applications.

Zusammenfassung

Der lichtbasierte 3D-Druck von photovernetzbaren Hydrogel-Tinten ermöglicht die Herstellung komplexer Strukturen für Tissue-Engineering und Medizinprodukte, in die auch Zellen eingeschlossen werden können. Eine schnelle, kontrollierte Vernetzung ist essenziell, um Strukturen mit definierten Eigenschaften, Präzision und hoher Zellviabilität zu gewährleisten. Diese Arbeit untersucht Hydrogele, die durch lichtinitiierte Thiol-En- oder Methacrylat-Vernetzung entstehen und vergleicht deren Eigenschaften als (Bio-)Tinten in zwei lichtbasierten 3D-Druckmethoden. Photovernetzbare Tinten auf der Basis von Hyaluronsäure, modifiziert mit Allylether-, Norbornen- oder Methacrylatgruppen, werden entwickelt und deren Vernetzungskinetik, mechanischen Eigenschaften und Druckbarkeit werden mittels oszillatorischer Scherrheologie und schichtweisem Digital Light Processing verglichen. Niedrigviskose, schnell vernetzende Varianten werden zusätzlich im tomographischen volumetrischen Biodruck von Strukturen mit internen Kanälen und eingeschlossenen Zellen ohne den Einsatz von Viskositätsmodifikatoren untersucht. Zuletzt wird die Leistungsfähigkeit von Methacrylat- und Thiol-Norbornen-funktionalisierten, Polyvinylalkohol-basierten Tinten für den volumetrischen Druck hochauflösender Hydrogel-Medizinprodukte untersucht. Die Ergebnisse zeigen Vorteile und Limitierungen von Thiol-En- und Methacrylat-Tinten für den lichtbasierten 3D-Druck von Hydrogelen für biomedizinische Anwendungen.

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List of Abbreviations

α	absorption coefficient	LAP	lithium phenyl-2,4,6-trimethylbenzoylphosphinat
δ	chemical shift	MA	methacrylate
ε	molar extinction coefficient	MALLS	multi-angle static light scattering
A	absorbance	hMSC	human mesenchymal stem cells
AE	allyl ether	MW	molecular weight
BOP	benzotriazol-1-yloxytris(dimethyl amino)phosphonium hexafluorophosphate	NB	norbornene
c	concentration	NHS	N-hydroxy succinimide
CAD	computer-aided design	OCT	optical coherence tomography
CGT	critical gelation threshold	PBS	phosphate-buffered saline
CL	contact lens	PEG	polyethylene glycol
DAPI	4',6-diamidino-2-phenylindole	PEGDA	polyethylene glycol diacrylate
DLP	digital light processing	PI	propidium iodide
DMA	dynamic mechanical analysis	PVA	polyvinyl alcohol
DMAP	4-(dimethyl amino)pyridine	RI	refractive index
DMSO	dimethyl sulfoxide	ROI	region of interest
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium	ROS	radical oxygen species
DPBS	Dulbecco's phosphate-buffered saline	RU	repeating unit
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)	SD	standard deviation
DTT	dithiothreitol	SLA	stereolithography
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide	TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxyl
EWC	equilibrium water content	t_{gel}	gelation time
F	degree of functionality	UV	ultraviolet
FBS	fetal bovine serum	VP	tomographic volumetric printing
FDA	fluorescein diacetate		
FITC	fluorescein isothiocyanate		
FWHM	full width half maximum		
G'	storage modulus		
G''	loss modulus		
GPC	gel permeation chromatography		
HA	hyaluronic acid		
HEMA	hydroxy ethyl methacrylate		
HUVEC	human umbilical vein endothelial cells		

All other abbreviations, including physical and chemical units, have their usual meanings if not stated otherwise.

Motivation

Biofabrication technologies have progressed in recent years and headlines such as “organs from a 3D printer” have attracted significant attention beyond the scientific community.^[1, 2] 3D bioprinting multicellular architectures is expected to generate tissue models with better predictive potential for drug testing and personalized therapies, thereby reducing the reliance on animal experiments and improving the outcome of therapeutic treatments. Bioprinted tissues are expected to impact regenerative medicine and contribute to the pressing need of engineered organs in an ageing population. Hydrogel design is a relevant step in the biofabrication process. Hydrogels have physicochemical and mechanical properties that can be tailored to the specific tissue type and biomedical need through material selection and a versatile, readily available chemical toolbox. 3D printing enables the fabrication of complex structures, for example incorporating channels for vascularization, multi-material compositions with cell-specific properties, or designs based on patient-derived data. In this context, light-based 3D bioprinting technologies offer the highest spatial resolution, as well as in-operando modulation of light properties (i.e. wavelength, irradiance, exposure time) for fine-tuning of the physicochemical characteristics of the generated scaffolds from aqueous, photoreactive resins.^[3]

Printing speed, fidelity (i.e. feature accuracy, resolution) and biological performance (i.e. cell viability and function) of 3D bioprinted hydrogels depend, among other factors, on the efficiency, kinetics and biorthogonality of the photocrosslinking reaction, and its robustness to external conditions. Photo-initiated radical polymerization of (meth)acrylate groups remains the gold standard for light-based bioprinting. The reaction is sufficiently rapid for bioprinting within reasonable timescales, but it is sensitive to the presence of oxygen and produces inhomogeneous network structures. To overcome oxygen inhibition, a high photoinitiator concentration, high light intensity and/or longer exposure time are required in the photoprocessing of (meth)acrylate resins. This also implies a higher concentration of radicals in the reaction mixture and therefore a higher risk of cytotoxicity. It is thus important to explore alternative photopolymerization reactions. Here, the radical thiol-ene reaction has gained increased interest as it (i) is not sensitive to the presence of oxygen, (ii) offers faster crosslinking, (iii) allows processing

at lower light doses, thereby (iv) minimizes the exposure of cells to harmful radicals, and (v) produces more homogeneous networks than (meth)acrylate systems.

Although thiol-ene based resins have been explored for 3D printing, the current literature lacks a quantitative, side-by-side characterization of the advantages and limitations of photopolymerizable thiol-ene vs. methacrylated hydrogel resins in the context of bioprinting beyond comparison of photocrosslinking kinetics. In this thesis, the properties of thiol-ene vs methacrylated resins are systematically evaluated using compositions and printing conditions relevant for biomedical scenarios.

The study focuses on the following hydrogel biomaterials:

- i. **Hyaluronic acid (HA)**, a natural biopolymer frequently used in tissue engineering and regenerative medicine. It is available in different molecular weights and can be functionalized with photoreactive groups. Aqueous solutions of HA with adjustable viscosity for hydrogel processing can be prepared by varying the polymer concentration and molecular weight.
- ii. **Polyvinyl alcohol (PVA)**, a synthetic hydrophilic polymer widely used for medical applications, including cell encapsulation for tissue engineering, drug delivery or ophthalmological devices like contact lenses.

and compares their properties and performance using two different light-based (bio)printing technologies:

- iii. **Digital light processing (DLP)** for layer-by-layer 3D processing of hydrogels. The systematic analysis of the printing performance in correlation with the underlying crosslinking mechanism is expected to be of interest to a broad community and help (bio)resin development and optimization.
- iv. **Tomographic volumetric printing (VP)** for layer-free curing of photoreactive resins is largely restricted to high-viscosity and thermogelling systems. This limitation motivates the exploration of alternative formulations and photocrosslinking systems.

This thesis is organized according to the following structure:

Chapter 1 presents the state of the art of photocrosslinkable hydrogels with focus on hydrogels processed by light-based biofabrication methodologies. It also describes light-based 3D printing technologies of relevance for this work.

Chapter 2 presents thiol-ene (norbornene, allyl ether) and methacrylated HA-based photocrosslinkable resins. The synthesis and characterization of storage stability, rheological properties and photocrosslinking kinetics of the resins are described, and their relationship to the respective crosslinking mechanism is discussed. The printing performance of the different resins in DLP is compared in terms of printing speed and quantifies the resolution for the norbornene (NB) resin. This chapter provides a quantitative measure of the benefits of the thiol-ene resin bearing allyl ether (AE) or NB groups compared with methacrylate-based systems and supports further investigation of this material for light-based 3D printing.

Chapter 3 investigates low-viscosity norbornene and methacrylate HA resins for tomographic volumetric printing without thickening additives or post-curing steps. Besides photocrosslinking kinetics, the optical and physicochemical properties relevant for the printing technology are characterized. Printability, shape fidelity and the ability to fabricate scaffolds with internal channels with corresponding channel width are quantified as function of light dose, and physicochemical scaffold properties will be evaluated. The printability of HA-NB-based scaffolds with internal channels in medium and loaded with living cells is assessed. The findings in this chapter pave the way for a new class of resins based on HA with low viscosity and fast crosslinking kinetics suitable for volumetric bioprinting under cell-friendly conditions without adverse effects on encapsulated cells.

Chapter 4 presents thiol-ene and methacrylated PVA-based photocrosslinkable resins and evaluates their optical, physicochemical and rheological properties and photocrosslinking kinetics. The volumetric printability for discs with different thicknesses and for contact lens geometries is evaluated as a function of light dose and absorption compensation settings. Optical coherence tomography is used for cross-sectional

imaging of the printed contact lens and quantification of the printed geometries. This chapter provides an initial assessment of the potential of PVA-based resins for soft contact lens fabrication and discusses potential optimization strategies.

Chapter 5 summarizes the obtained results, highlights the main conclusions of this Thesis and discusses their relevance in the context of research and development of (bio)fabricated biomedical devices.

1. Introduction

1.1. Hydrogels for biomedical applications

Hydrogels are three-dimensional polymeric networks with the capability of holding substantial amounts of water. These materials are typically soft (Young's modulus <MPa) and in many cases biocompatible.^[4, 5] They allow size-dependent diffusion of water-soluble molecules across the network. These properties render hydrogels interesting materials for biomedical applications such as carriers for drug delivery^[6], scaffolds for tissue engineering^[5, 7], substrates for biosensing^[8], optical fibers for photodynamic therapies^[9, 10] or transparent medical devices like contact lenses^[11, 12].

Biomedical hydrogels can contain hydrophilic polymer chains of natural origin, i.e. alginate, collagen, dextran, gelatin, hyaluronic acid (HA), or silk.^[13, 14] Naturally derived biomaterials can mimic part of the physicochemical microenvironment for encapsulated cells, especially if they are components of the extracellular matrix like collagen, gelatin or HA, and present bioactive groups for cell attachment or stimulation.^[13] They typically contain amine, hydroxyl or carboxylic acid groups which can be modified with reactive groups to expand their bifunctionality. Depending on the source, natural biomaterials can show large molecular weight distributions, batch-to-batch variability and immunogenic potential, which can limit their application in biomedical contexts. Further, synthetic polymers like polyethylene glycol (PEG), Pluronic® or polyvinyl alcohol (PVA), can also be used or combined with natural polymers to synthesize biomedical hydrogels.^[13, 14] Synthetic polymers can be synthesized with desired and tailored molecular structure and molecular weight distribution, and functional groups can be integrated at desired density.^[13]

1.2. Crosslinking of hydrogels with focus on photocrosslinking strategies

The mechanical integrity of a swollen hydrogel relies on persistence of the 3D, crosslinked network structure. This can consist of physical or chemical bonds, or a combination of both. Physical crosslinks hold the polymeric chains in the network by reversible, dynamic interactions and rely on the entanglement of the long polymeric chains, or on reversible attractive forces between groups at different polymeric chains, i.e. hydrogen bonding, hydrophobic or ionic interactions (**Figure 1A**).^[15] To trigger the

gelation process, changes in pH, ion content or temperature of the hydrogel precursor solution can be exploited, as these can be used to change the properties of the molecule (charge generation or shielding, solubility) and trigger the interactions. Divalent cations mediated crosslinking of alginates or thermoreversible gelation of gelatin are two examples of triggerable physical crosslinks.

Although light is not typically used as a trigger for initially forming physically crosslinked networks, it has been used to modulate the properties of light responsive physical crosslinks.^[16] For example, in polymer chains containing azobenzene (host) and β -cyclodextrin (guest) groups, photoirradiation with visible light triggers *cis-trans* isomerization of the azobenzene groups and crosslinking of them with β -cyclodextrin rings by formation of hydrophobic guest-host complexes (**Figure 1B**). Upon ultraviolet (UV) irradiation, the *trans*-azobenzene units are photochemically converted into their *cis* configuration which leads to dissociation from the cyclodextrin rings rendering the physical crosslinking reversible.^[17] Another photophysical gelation mechanism involves the ring-opening transformation of spiropyran which changes from a compact, non-polar structure to a planar, zwitterionic isomer after UV irradiation (**Figure 1C**).^[18] With a photo switchable crosslinking mechanism, a light-based modulation of hydrogel properties is possible.

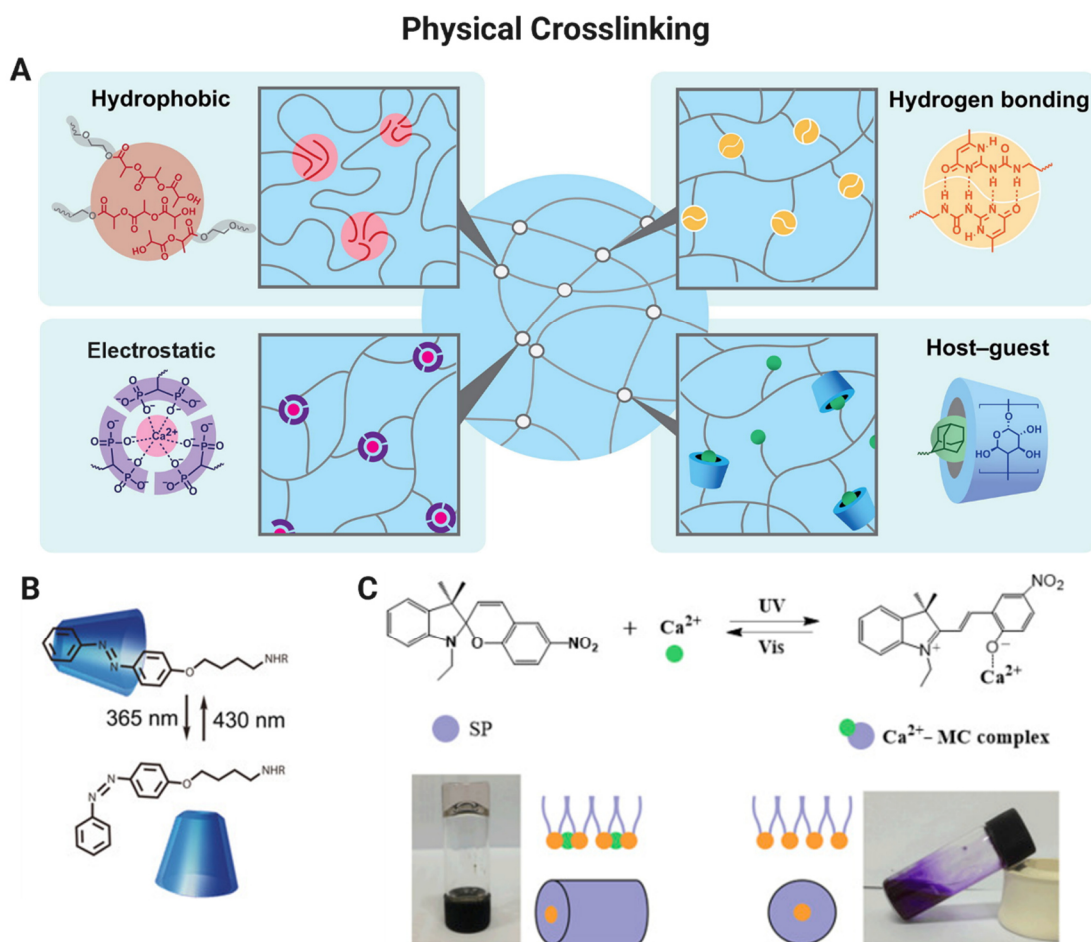


Figure 1. Hydrogel containing physical crosslinks induced by changes in temperature, pH, ion content or light exposure. **A.** Examples for physical crosslinks depending on hydrophobic or electrostatic interactions, hydrogen bonds or guest-host complexes. Adapted from Bernhard et al., 2021, licensed under CC BY 4.0.^[19] **B.** Light triggers the *cis-trans* isomerization of azobenzene which changes the formation of guest-host complexes. Adapted with permission from Xie et al.^[20] Copyright © 2018 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. **C.** Light-based transformation of spiropyran and merocyanine. Reproduced with permission from Chang et al.^[21] © 2016 Elsevier Ltd. All rights reserved.

In chemically crosslinked hydrogels, the network structure is maintained by covalent bonds which usually lead to hydrogels with higher mechanical properties than the physically crosslinked analogues. Precursor prepolymers and crosslinkers contain reactive groups and can form covalent bonds by different reaction mechanisms. Common chemical crosslinking reactions of interest for biomedical hydrogels involve nucleophilic additions, for example the thiol-Michael addition, Schiff base, oxime or hydrazone formation, active ester-amine coupling, or the thiol-aryl sulfone reaction.^[22-26] Ring-opening reactions like the thiol-epoxy or amine-epoxy reaction, disulfide bond

crosslinking or cycloadditions like Diels-Alder, 1,3-dipole or metal catalyzed reactions have also been used to prepare biomedical hydrogels (**Figure 2**).^[27-29]

Chemical Crosslinking without Light

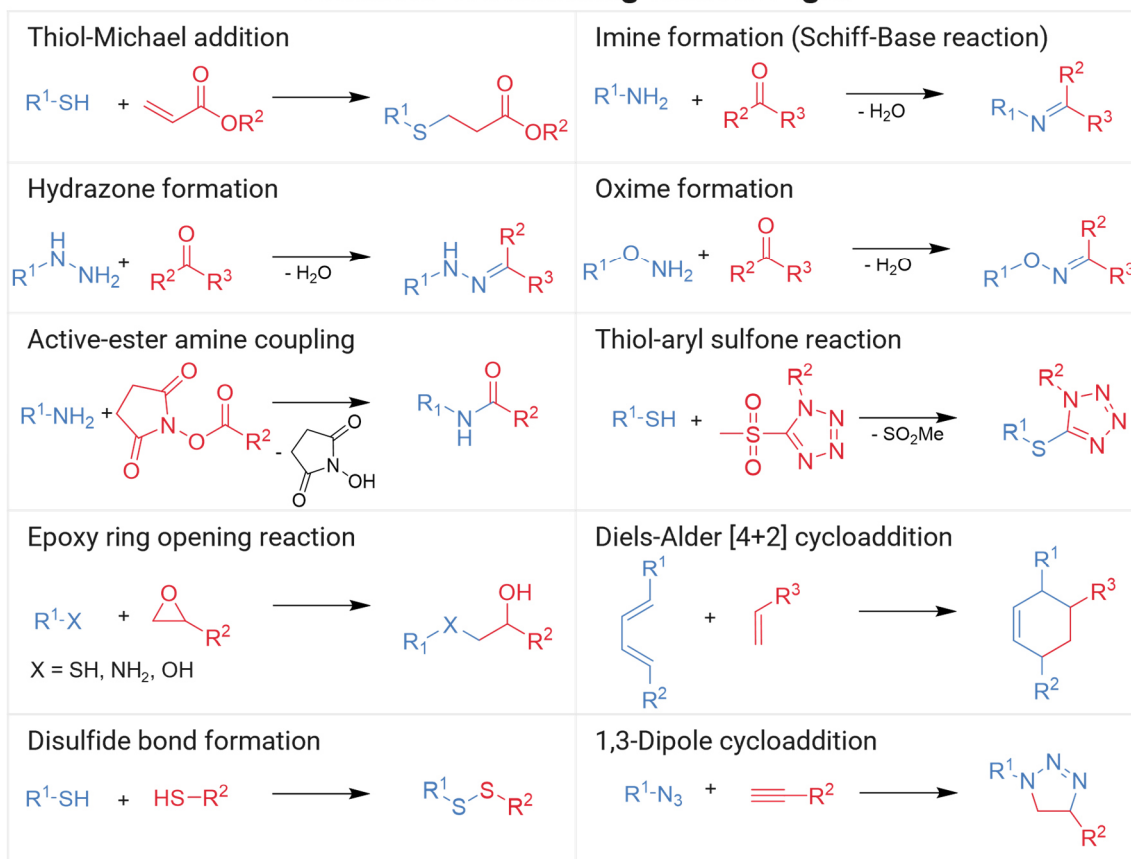


Figure 2. Example of common reactions to introduce chemical crosslinks in hydrogels without light.^[22-29]

In biomedical scenarios, hydrogels need to be in contact with biological molecules or even living cells. In some cases, like in 3D culture-based assays, drug delivery carriers or cell-based therapies, the hydrogel needs to be prepared in the presence of biological molecules or living cells. Therefore, crosslinking reactions that occur efficiently in water and in mild conditions and do not require a toxic catalyst are necessary for the synthesis of biomedical hydrogels. Photo-initiated covalent crosslinking reactions which are activatable at biocompatible light doses are interesting in this context (**Figure 3**). Light-triggered crosslinking reactions are often used for preparing hydrogels in the presence of cells, like in 3D (bio)printing.^[30] Light can be readily available and easily spatially controlled, and this allows modulation of the hydrogel formation process and the final hydrogel properties with spatiotemporal resolution.^[16] Photochemical reactions can be performed at doses and wavelengths with minimal interference with native biochemical

processes (bio-orthogonality).^[16] In addition, the employed wavelengths can be tailored to the application from UV to near-infrared, and the use of wavelength-selective photoreactions provides further control over the photochemical process.^[31, 32]

The photo-initiated free radical crosslinking of methacrylated monomers or prepolymers is the most used photo-initiated reaction for covalent crosslinking of hydrogels.^[13, 16, 27, 33] In this reaction, the methacrylate groups react with photo-initiated radicals to form covalent polymethacrylate crosslinks. More recently, the radical thiol-ene based photocrosslinking has also attracted interest. Upon irradiation in the presence of a photoinitiator, a thioether linkage is formed between thiol and vinyl group bearing polymer precursors. A radical thiol-yne reaction proceeds in a similar manner but allows the subsequent addition of two thiol moieties to the alkyne. The radical coupling of phenol groups in precursors containing tyrosine or tyramine moieties is less used.^[34, 35] To initiate tyramine photopolymerization, visible light and a photoinitiator like Eosin Y^[34] or Ruthenium complexes with sodium persulfate have been used.^[35] Upon irradiation, phenoxy radicals are generated which form covalent dityramine or dityrosine crosslinks. The reaction usually proceeds slower than the classical photopolymerization of carbon double bonds but is considered to be highly cytocompatible.^[33, 34] The light-triggered cycloadditions like the [2+2] photodimerization of cinnamate or coumarin groups is also described.^[16]

Chemical Crosslinking with Light

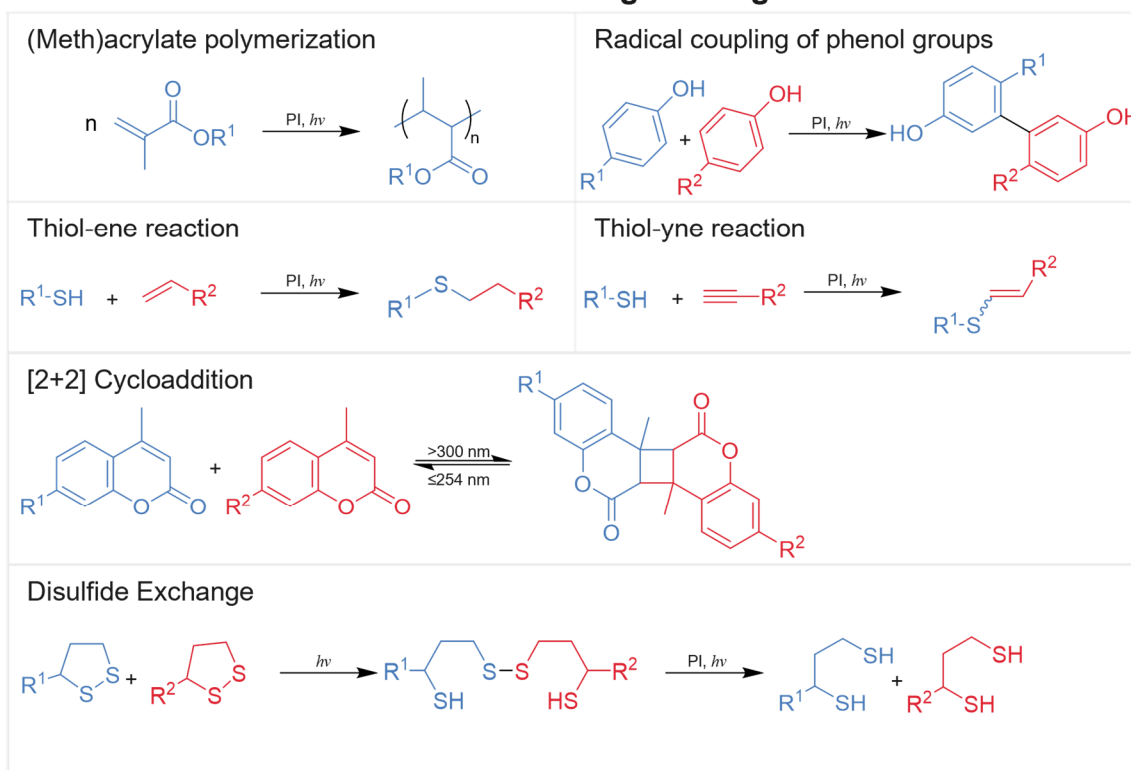


Figure 3. Examples of phototriggered reactions to form chemical crosslinks in hydrogels. The reactions proceed upon irradiation and usually require a photoinitiator (PI).^[13, 16, 27, 33, 36]

Besides physical reversible and covalent permanent crosslinks, dynamic covalent bonds can also be used to form hydrogel networks. Such crosslinks can break and re-form upon application of a specific stimuli like pH, temperature, mechanical load, reducing agents or competing molecules and are interesting to form hydrogels with self-healing properties.^[37] Examples of such crosslinking reactions are covalent bonds formed by Diels-Alder or Schiff base reaction, boronic esters or disulfides. Light can also be used as trigger for reversible, dynamic covalent bonds (**Figure 3**). For example, the crosslink formed by photodimerization of coumarin groups can be cleaved by shorter-wavelength UV light (≤ 254 nm).^[16] The formation and degradation of disulfide crosslinks can also show a phototriggered dynamic behavior. Dithiolanes are ring-strained, 5-membered cyclic disulfides which can undergo light-induced ring opening to form intermolecular crosslinked disulfide bonds.^[38] Although the dithiolanes do not reform, irradiation induces radical-mediated degradation of the crosslinks.^[36]

Light can also be used to trigger the irreversible cleavage of covalent bonds incorporated into hydrogel networks. A common example are hydrogels modified with photocleavable

groups in their main chain, like *ortho*-nitrobenzyl derivatives (**Figure 4**). Irradiation with UV light (typically 300–365 nm) leads to intramolecular photorearrangement and fragmentation of the *ortho*-nitrobenzyl moiety to generate a 2-nitrosobenzaldehyde or ketone by breaking the C-O or C-N bond attached to the polymer backbone which results in degradation of the hydrogel network.^[39, 40] Notably, photocleavable groups can also serve as photocages that expose reactive intermediates to activate crosslinking and lead to the formation of new covalent bonds. For example, the release of alkoxyamine and aldehyde moieties can initiate photomediated oxime ligation^[41] or a generated photobase can trigger thiol-Michael photopolymerization^[42].

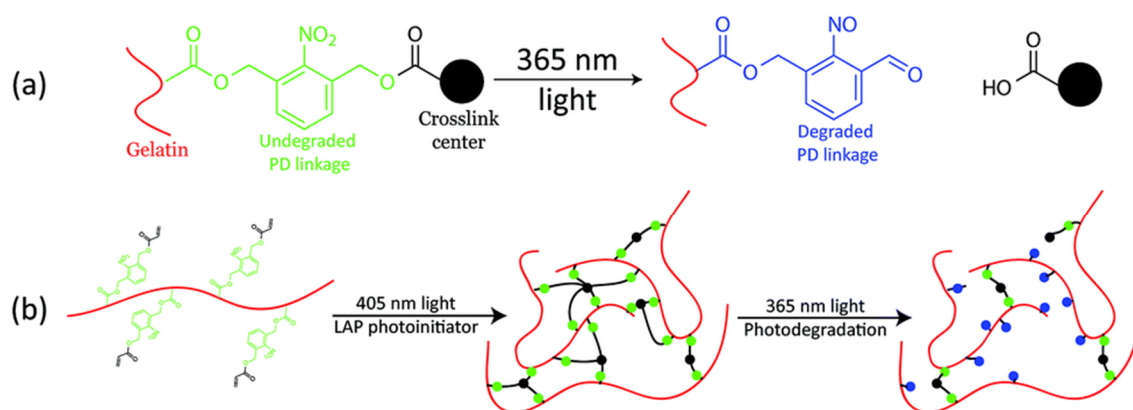


Figure 4. Schematic representation of gelatin hydrogels containing *ortho*-nitrobenzyl groups. (a) Upon irradiation with UV light (365 nm), the *ortho*-nitrobenzyl group gets cleaved into an aldehyde and carboxylic acid. (b) Hydrogel formation and degradation occurs at orthogonal wavelengths. Pendant acrylate groups are photocrosslinked at 405 nm while the *ortho*-nitrobenzyl groups are degraded at 365 nm which leads to degradation of the hydrogel network. Reproduced with permission from Norris et al.^[39] © 2019 Royal Society of Chemistry.

1.2.1. Radical-initiated photocrosslinking reactions

Photo-initiated radical reactions require a photosensitive molecule (photoinitiator) to form reactive radical species upon irradiation and initiate the reaction. Compared with non-radical photoinduced pathways such as photoacid-/photobase-triggered reactions, cycloadditions or photocage-mediated ligations, radical photopolymerizations often proceed faster and generally less sensitive to moisture, enabling light-triggered curing in aqueous media.

Non-toxic photoinitiators commonly employed in hydrogel formation for biomedical applications are Irgacure 2959, lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP) and Eosin Y (**Figure 5**).^[13, 43] Irgacure 2959 is typically activated with UV light (365 nm), LAP with UV or blue light (365–405 nm) and Eosin Y with visible light (400–500 nm) in the presence of a co-initiator.^[44] All are type I photoinitiators, meaning that only one photon is needed to form the radical species. Recently, type II photoinitiator systems which require two photons for activation have gained interest for their higher absorption in the visible range (400–500 nm).^[44] The main example are Ruthenium complexes with additional co-initiator.

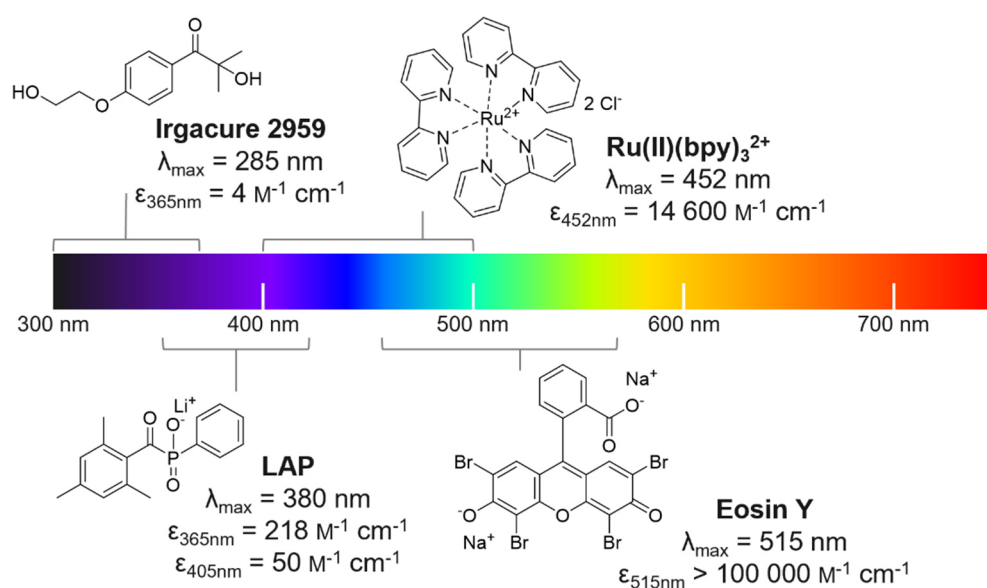


Figure 5. Structure and specifications of photoinitiators commonly employed in photocrosslinking of biomedical hydrogels.^[43, 45]

After photoinitiation, the photocrosslinking reaction can follow a chain-growth or a radical step-growth mechanism depending on the photoreactive functional groups of the precursors.

1.2.1.1. Photo-initiated radical chain-growth reaction mechanism

The polymerization of (meth)acrylate groups is the most common photo-initiated radical reaction used in biomedical hydrogels. This reaction follows a chain-growth mechanism and includes three steps: initiation, propagation and termination (**Figure 6A**).^[46] In the initiation step, the photogenerated free radical adds to the polymerizable vinyl group. The vinyl group can be part of a monomer or a functional group integrated into a polymer

backbone. Second, the new radical propagates by successive addition of vinyl groups to generate long polymer chains or a crosslinked network (**Figure 6B**). Termination occurs when two propagating radicals react with each other resulting in non-reactive ends, either by recombination or disproportionation. The successive addition of functional groups to the growing macroradicals as well as termination and chain-transfer reactions lead to the formation of heterogeneous networks with a non-uniform distribution of crosslinks.^[16, 47]

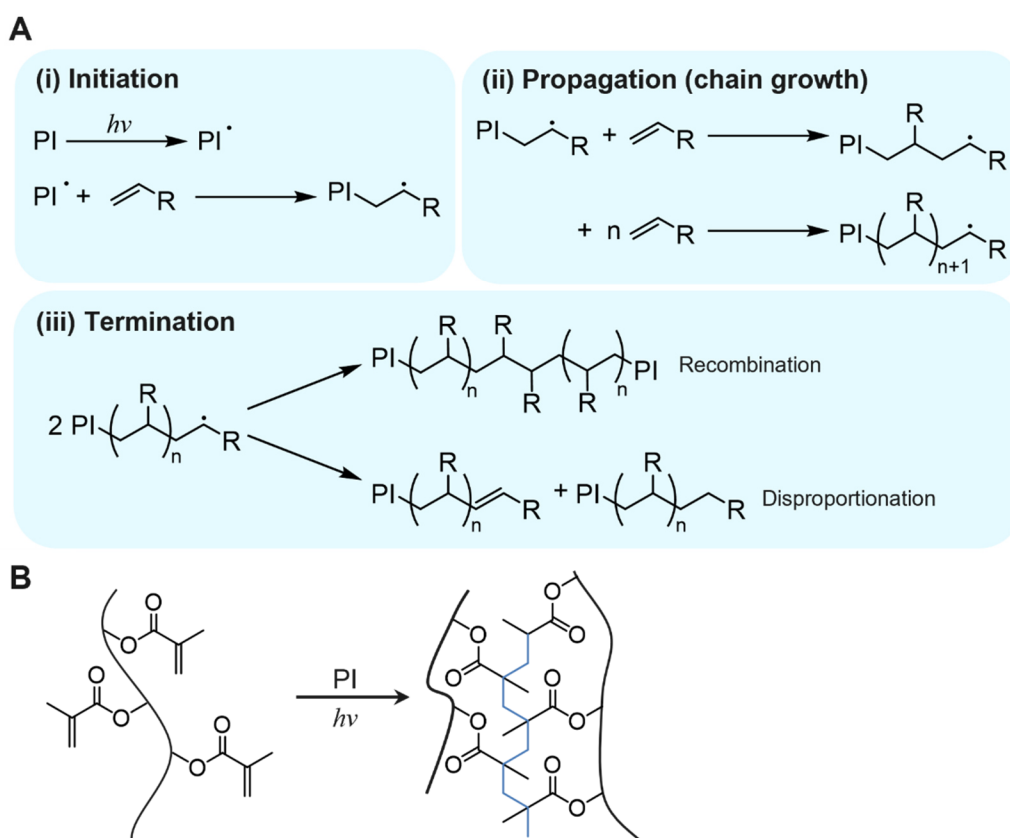


Figure 6. Chain-growth mechanism. **A.** Photo-initiated polymerization and crosslinking via chain-growth mechanism involving the three steps (i) initiation, (ii) propagation and (iii) termination. **B.** Radical-initiated photocrosslinking of methacrylate groups and final network structure. Multiple methacrylate groups are connected through the kinetic chain. PI stands for the photoinitiator.

The propagation rate and therefore the speed of polymerization or crosslinking depends on the stability of the formed radical. In the crosslinking of acrylate or acrylamide groups, which form a secondary radical as intermediate, the reaction rate is faster than in methacrylate (MA) or methacrylamide groups, where more stable tertiary radicals are involved.^[46] Resonance stabilization slows down the polymerization rate. The crosslinking kinetics and the reaction speed increase when the radical concentration is

increased, for example with increasing temperature or initiator concentration.^[46] In a photo-initiated chain-growth reaction, the radical concentration can be tuned with light intensity and irradiation time to shorten the gelation time.^[48]

During a radical chain-growth crosslinking process, the viscosity of the solution increases, thereby reducing the mobility and diffusion of radical species and radical chain ends. At this point, the termination becomes diffusion-limited. In a radical polymerization reaction, especially in bulk polymerization, this translates into an auto-acceleration of the polymerization (Trommsdorff-Norrish effect).^[48] In a photo-initiated chain-growth radical crosslinking reaction, the retarded termination leads to the extension of the propagation reaction beyond the light exposure time. This phenomenon is called dark curing.

Important for hydrogel fabrication by radical chain-growth mechanism is the role of oxygen. Radical species react with oxygen to form an inactive peroxy radical which terminates the polymerization or crosslinking reaction.^[49, 50] The inhibitory effect of oxygen is responsible for a lag phase between the onset of the illumination and the start of the reaction in a photo-initiated radical polymerization, which starts once the oxygen molecules in the hydrogel precursor mixture have been consumed. Common strategies to avoid oxygen inhibition involve (i) degassing of the polymerization mixture, (ii) working under inert conditions, and/or (iii) by adding additives like amines, *N*-vinyl amines or silanes which can react with peroxy radicals to regenerate radical species.^[13, 50, 51] In the biomedical field, the applicability of these strategies needs to consider their potential cytotoxic effects. The period of oxygen inhibition can be reduced by generating a higher number of radicals, e.g. by increasing photoinitiator concentration or applied light dose as long as it remains below the phototoxicity threshold. For biomedical applications which involve the in situ encapsulation of cells, increasing the radical concentration might result in increased cytotoxicity.^[49]

1.2.1.2. Photo-initiated step-growth reaction mechanism

A typical example of a radical-mediated step-growth mechanism is the radical thiol-ene reaction. In this step-growth reaction, a covalent bond is formed between a thiol- and an ene-functionalized species after initiation by a (photo-initiated) radical (**Figure 7A**). The

radical unit is transferred to the thiol moiety to form a sulfur-centered thiyl radical. This radical adds to a vinyl group to form a new covalent bond and a carbon-centered radical intermediate. After chain-transfer to another thiol, the thioether with anti-Markovnikov orientation is released.^[52] The newly generated thiyl radical propagates the crosslinking reaction by sequential radical addition and transfer between vinyl and thiol groups in a click-reaction manner. Termination occurs once all radicals or functional groups are consumed. The radical thiol-ene reaction is insensitive to the presence of oxygen as the generated peroxy radical readily abstracts a hydrogen atom from a thiol which can then proceed in the thiol-ene polymerization.^[53] The crosslinking via step-growth mechanism results in homogeneous networks with higher consumption of functional groups (**Figure 7B**).^[3, 49, 52]

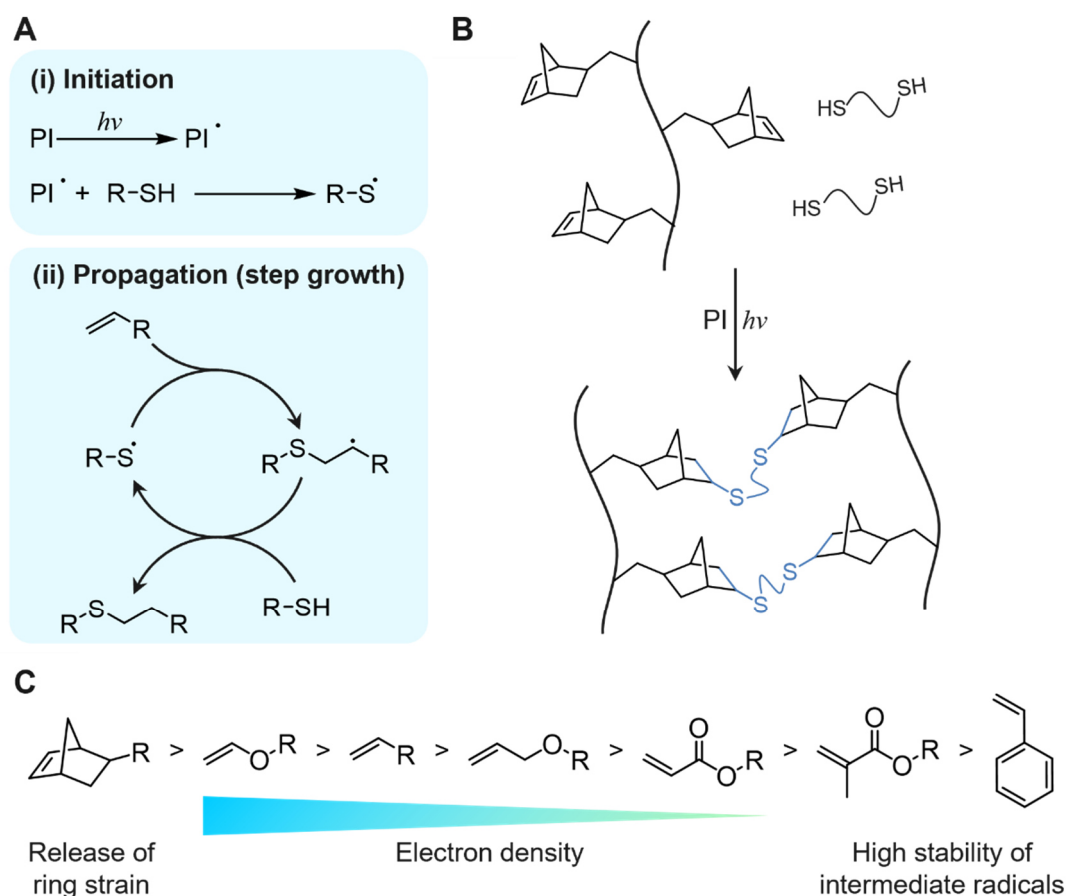


Figure 7. Step-growth mechanism exemplified by thiol-ene crosslinking. **A.** Photo-initiated polymerization and crosslinking via step-growth mechanism involving the two steps (i) initiation and (ii) propagation. Termination occurs once all radicals and functional groups are consumed. **B.** Radical-initiated photocrosslinking of norbornene and thiol groups and final network structure. Two norbornene groups are connected by one dithiol. PI stands for the photoinitiator. **C.** Reactivity of different vinyl groups towards

radical thiol addition depends on ring strain, electron density and intermediate radical stability.^[52]

The addition of the thiyl radical to the vinyl group is faster in the case of electron-rich or strained vinyl groups, like vinyl ether, allyl ether (AE) or norbornene (NB) groups, and slower with electron-poor vinyl groups, or vinyl groups where the intermediate radical is stabilized, as in methacrylates (MA) (**Figure 7C**).^[49, 52, 54] NB groups show the highest reactivity towards thiyl radical addition, because the high electron density of the ene and the release of ring strain upon thioether formation favor the thiol-ene reaction. On the other hand, vinyl groups with intermediate radical stabilization slows down the H-abstraction from thiols. In a radical-initiated thiol-ene polymerization with slower systems, like with methacrylated polymers, both step-growth thiol-ene and chain-growth vinyl polymerization occur in parallel. Fast thiol-ene systems like AE or NB exclusively undergo the radical thiol-ene reaction without significant homopolymerization.^[55, 56] Besides the vinyl group, the structure of the thiol can influence the reactivity of radical thiol-ene reactions. Due to steric hindrance, tertiary and secondary thiols react slower than primary thiols.^[57] The reactivity of thiols increases with decreasing electron density and with the addition of electron withdrawing groups.^[58] Radical thiol-ene crosslinking only shows minor disulfide bond formation as those are readily cleaved by radicals.^[53]

A side reaction which has to be considered for thiol-ene reactions is the thiol-Michael addition (**Figure 2**). Under basic conditions or in the presence of a nucleophilic catalyst, a thiolate anion can be formed which reacts with electron-poor vinyl groups such as methacrylates.^[59] AE and NB groups are not activated in this sense and therefore cannot undergo thiol-Michael addition. This makes the AE and NB alkenes ideal systems for photo-initiated radical thiol-ene crosslinking as their reactivity can be solely controlled by the light-initiated radicals and, therefore, it can occur with accurate spatiotemporal control. This also prevents side reactions with other nucleophiles in biomedical contexts, e.g. on the cell surface, and can lead to higher cytocompatibility.^[60, 61] These features are important for photo-initiated fabrication processes, as described in section 1.5.

1.2.1.3. Methacrylate vs. thiol-ene reaction for biomedical hydrogel synthesis

This section compares reported work on the photocrosslinking kinetics, physicochemical properties and network structure of MA and thiol-ene crosslinked biomedical hydrogels as well as differences in their performance as scaffolds for cell seeding or encapsulation. This information serves as basis for the comparison regarding their application as materials for (bio)printing discussed in section 1.5 and in the introduction of Chapter 2.

Thiol-ene crosslinking has been proposed as alternative to methacrylate crosslinking for biomedical hydrogels. The step-growth mechanism of the thiol-ene reaction leads to more homogeneous hydrogels in more cell-friendly conditions and in the presence of oxygen (**Figure 8**).^[13] The radical thiol-ene reaction offers faster photocrosslinking kinetics and higher conversion, and it requires lower light doses and fewer radicals than methacrylate polymerization^[62-64], leading to lower generation of radical oxygen species (ROS)^[65] and higher cell viability^[65-67]. Step-growth mediated thiol-ene reactions proceed highly efficiently with nearly quantitative consumption of the functional groups leading to the formation of well-defined networks. Especially with electron-rich vinyl groups like NB or AE which are not prone to side reactions like thiol-Michael reaction, the reaction proceeds selectively with the employed thiol and ene moieties in a click-reaction manner. The step-growth mechanism offers superior spatiotemporal control compared with the chain-growth photocrosslinking of MA groups, in which diffusion of radicals and auto-acceleration limit the control over the reaction.^[48] The chain-growth mechanism further leads to clustering of crosslinks as polymerization occurs through multiple MA groups while the thiol-ene reaction selectively connects one thiol and vinyl group by the formation of a thioether bond. In contrast to MA crosslinking, the radical thiol-ene reaction is not affected by the presence of oxygen.

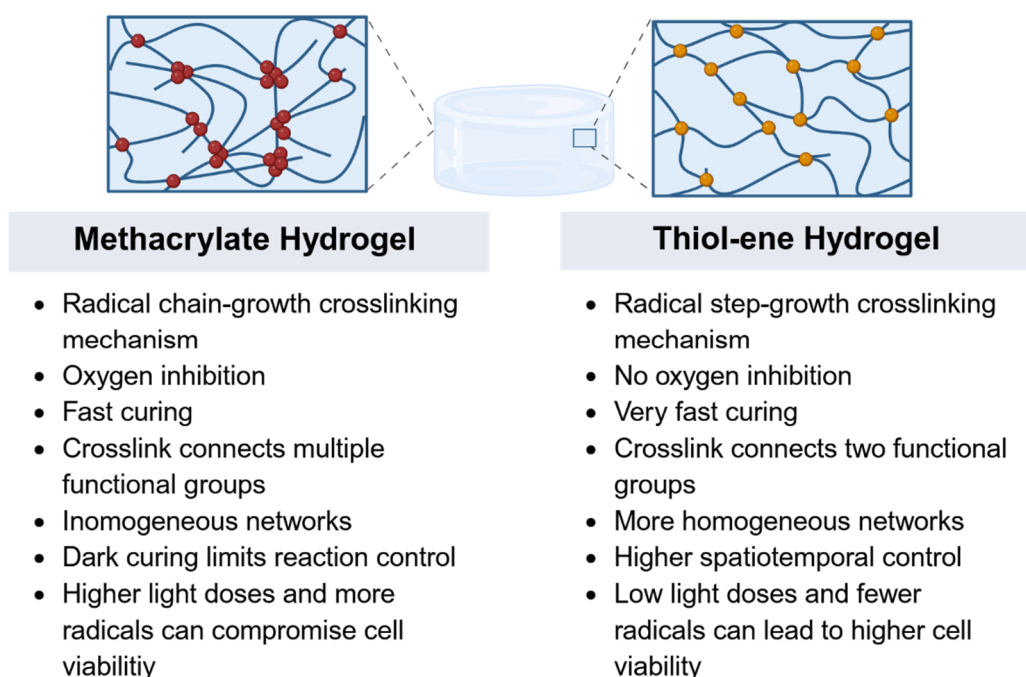


Figure 8. Comparison of MA and thiol-ene hydrogels. Photocrosslinking via radical chain-growth or step-growth mechanism leads to differences in curing kinetics, network structure and cytocompatibility. *Created with BioRender.com.*

The first hydrogels crosslinked using radical thiol-ene chemistry were presented in 2009. The group of Anseth developed hydrogels based on PEG4NB (20 kDa) crosslinked with a dithiol enzyme cleavable peptide. Analysis of the decrease of alkene and increase of thioether signals demonstrated that crosslinking proceeds via an ideal step-growth mechanism according to the Flory-Stockmayer theory without homopolymerization where only NB and thiol groups crosslink equally in a 1:1 reaction.^[68] The gelation time was tunable with the LAP photoinitiator concentration (50 μM –11 mM) and the crossover of storage and loss modulus, i.e. gelation point t_{gel} , occurred within 1 s to 6 min (365 nm, 10 mW cm^{-2}) in hydrogels with 10% w/w polymer content as demonstrated in photorheology experiments. Shorter illumination periods quantified that radical thiol-ene crosslinking enables temporal control over the network formation without autopolymerization as no increase in G' was observed once the light was switched off. Therefore, the radical thiol-ene reaction requires active radical formation for crosslinking. Human mesenchymal stem cells (hMSCs) encapsulated in situ showed high cell viability (>95%) and additional photopatterning with a thiolated cell-adhesive peptide led to localized cell spreading. In a follow-up work, the group used 10% w/w PEG4NB (5 kDa) hydrogels along with another bis-cysteine enzyme cleavable

peptide to demonstrate hydrogel degradation in the presence of human neutrophil elastase for protein release. Encapsulated proteins did not alter the crosslinking kinetics and retained >90% bioactivity.^[69] Shih et al. studied the photorheology of 10% w/w NB- or diacrylate-functionalized poly(ethylene glycol) (PEGDA) hydrogels.^[63] The gelation point of the PEG4NB (20 kDa) precursor solution was reached 2-fold faster in rheology experiments compared to the acrylate-based PEGDA (10 kDa) system. Both systems contain the same number of functional groups, but the internal polymer architecture is different. In the final network, the star structure of the PEG4NB generates double number of crosslinks than the linear PEGDA hydrogel. A higher cell viability was demonstrated for the NB hydrogels.

A few examples in the literature have compared the final properties of thiol-ene vs. MA photocrosslinked hydrogels based on the same polymer backbone. Initial comparison of 6–11% w/w acrylate and thiol-ene PEG hydrogels for the in situ encapsulation of chondrocytes was presented by Roberts and coworkers in 2013.^[70] The initial double bond content was adjusted so that it was similar for both systems. The thiol-ene hydrogel-based on PEG4NB (5 kDa) and PEG2SH (3.4 kDa) exhibited a 10-times faster gelation i.e. shorter t_{gel} in photorheology experiments in comparison to PEGDA (4.6 kDa). The addition of cells did not alter gel point. While no significant change was observed in the final shear storage modulus of thiol-ene hydrogels, the PEGDA hydrogels showed a ~45% lower final G' when crosslinking was carried out in the presence of cells. The hydrogel compositions were prepared to have a similar concentration of reactive ene groups (28–28.5 mM). The authors further studied the immediate cell response during the encapsulation by measuring the concentration of ROS. The chain-growth crosslinking mechanism led to a high intracellular ROS concentration which was not observed in step-growth thiol-ene crosslinking. In addition, thiol-ene hydrogels showed a more homogeneous network. McCall and coworkers studied the bioactivity of proteins encapsulated in 10% w/w PEG hydrogels based on thiol-ene or acrylate crosslinking. Following exposure, the bioactivity of proteins in the acrylate hydrogels reduced by 50%, while the proteins encapsulated in thiol-ene hydrogels retained their activity by approx. 100%.^[71]

Other groups compared thiol-ene and MA hydrogels based on gelatin, an animal-derived biopolymer with polypeptide structure which intrinsically contains the RGD motif as cell-adhesive domain.^[59, 64-67, 72-75] In contrast to others, Munoz and coworkers did not observe a significant difference in t_{gel} of 5% w/w Gel-MA and Gel-NB hydrogels (Functionality $F \sim 45\%$) in photorheology experiments.^[67] However, Gel-MA hydrogels showed approx. 2-times higher stiffness in comparison to Gel-NB hydrogels which were crosslinked with DTT. The differences were found as consequence of (i) the short dithiol crosslinker, (ii) orthogonal crosslinks in thiol-ene networks and (iii) the long (poly)-MA linkages in chain-growth hydrogels. The storage modulus of 5% w/w Gel-NB hydrogels was tunable with the DTT concentration, reached a maximum value at a condition for which the effective thiol:ene ratio was expected to be close to 1, and decreased with an excess of thiol groups. Exchange of the DTT crosslinker against PEG4SH (5 kDa) led to hydrogels with >10-times higher stiffness, denser networks and lower swelling. Crosslinking chemistry also influenced the cell-material interactions; hMSCs encapsulated in 4 or 8% w/w Gel-NB/DTT hydrogels showed a higher cell viability and increased cell spreading in comparison when encapsulated in Gel-MA hydrogels. Van Hoorick et al. studied the gelation of 5–15% w/v Gel-NB/DTT or Gel-MA ($F \sim 65\%$).^[64] A 24-fold shorter gelation time was demonstrated for Gel-NB in photorheology experiments. Under full exposure conditions, complete conversion of the NB groups was observed, while the conversion for Gel-MA was found to be 50–80%. When solutions were physically gelated before illumination, Gel-MA hydrogels showed a higher storage modulus and a higher mass swelling ratio than Gel-NB hydrogels after photocrosslinking. Similar to other studies, the higher modulus was attributed to the different crosslinking mechanisms: in the MA hydrogels, the growing radical macromer can connect many gelatin chains while in the thiol-ene system each NB function can only connect to one other NB unit of the same or another polymer backbone via the bifunctional DTT crosslinker. The lower mass swelling ratio in the Gel-NB system was attributed to the homogeneous network structure, the high conversion and the more hydrophobic nature of the NB functions inside the network. Interestingly, comparable storage moduli of Gel-NB and Gel-MA hydrogels were found at lower polymer concentration (5% w/v). In following studies, 10% w/v Gel-MA and Gel-NB hydrogels with higher degree of functionality $F_{\text{ene}} = 90\text{--}95\%$ were compared.^[59] Using LAP or Irgacure as photoinitiator, the gelation point in Gel-NB crosslinked with DTT was

reached in 0.36 s or 9.18 s, in contrast to 19.9 s or 49.5 s for Gel-MA, respectively. Increasing the number of functional groups or the molecular weight of the thiol crosslinker led to faster gelation and higher modulus in Gel-NB hydrogels. While slightly lower toxicity for the Gel-NB/DTT precursor solution was observed, no significant difference was found in cell viability in hydrogels crosslinked under the same irradiation conditions. In addition to the faster photocrosslinking kinetics, Zhao et al. compared 8% w/v Gel-MA and Gel-NB hydrogels with 50% functionalization.^[65] DTT or thiolated heparin were used to crosslink Gel-NB. The gel point was reduced 1.7-fold in time using DTT and 3.7-fold with thiolated heparin as crosslinker in comparison to Gel-MA in photorheology experiments. No significant difference was found in the final shear storage modulus for the hydrogel crosslinked with DTT, but a 3.2-fold higher stiffness was obtained using thiolated heparin. After the exposure, an elevated ROS concentration was observed in Gel-MA hydrogels but no significant increase was found in Gel-NB. The increase in ROS and radical accumulation in the radical chain-growth reaction of MA groups further caused increased cell death in Gel-MA hydrogels while human umbilical vein endothelial cells (HUVECs) embedded in Gel-NB hydrogels maintained high cell viability. The faster photocrosslinking, lower light doses and higher cell viability in Gel-NB vs Gel-MA hydrogels were also observed by others.^[66, 72, 73, 76]

In addition to NB modifications, AE groups have been introduced on gelatin to enable photocrosslinking via thiol-ene chemistry.^[74] Bertlein and coworkers compared 20–30% w/w Gel-AE against 10–30% w/w Gel-MA. In contrast to reports on NB, the highest Young's modulus was obtained when Gel-AE was crosslinked with DTT at thiol:ene ratio of 3:1 or 6:1 depending on the photoinitiator and irradiation wavelength.^[74] While Gel-AE and Gel-MA showed similar mechanical properties upon crosslinking with Irgacure and UV light, using a visible-light system with Ru/SPS as photoinitiator, higher stiffness was obtained for Gel-AE at comparable weight percentages. Encapsulated human articular chondrocytes showed slightly higher viability in 20% w/w Gel-AE hydrogels than in 10% w/w Gel-MA, particularly under visible-light initiation, whereas very high thiol excess (thiol:ene = 12:1) impaired viability. Genc et al. noticed that the introduction of AE alters the rheological profile of gelatin and shows a lower viscosity and easier handling in comparison to Gel-MA solutions.^[75] Also other groups report hydrogels

based on Gel-AE, but their performance is benchmarked only against Gel-MA values from literature rather than through a direct side-by-side comparison.^[77, 78]

Thiol-ene crosslinkable precursors have also been developed for other polymer backbones (e.g. alginate, collagen, HA, polyvinyl alcohol) to enable step-growth mediated hydrogel formation after photoinitiation.^[79-87] However, these studies have not performed a side-by-side comparison against the corresponding MA derivatives and, therefore, they will not be described in this section.

1.3. Hyaluronic Acid-based hydrogels

Hyaluronic acid (HA) is a naturally occurring linear non-sulphated glycosaminoglycan in the extracellular matrix and widely used in biomedical applications and tissue engineering. It is biocompatible, non-immunogenic and biodegradable by enzymes like hyaluronidase and possess other interesting properties such as hydrophilicity and viscoelasticity.^[6, 88] It consists of alternating units of repeating *N*-acetyl-D-glucosamine and D-glucuronic acid disaccharide (**Figure 9**). Although HA is synthesized in animals or natural HA-producing microorganisms, commercially available HA which is usually used in research and final biomedical applications, is produced biotechnologically via microbial fermentation in engineered safe microorganisms.^[88, 89]

HA can be obtained commercially in a range of different molecular weights (5–10000 kDa) which, in addition to the polymer concentration, influences physicochemical and biological properties.^[90, 91] High molecular weight HA (>500 kDa) shows antiangiogenic and immunosuppressive functions and provides space-filling and structural support.^[90] HA with intermediate MW (20–200 kDa) on the other hand is involved in tissue repair, ovulation and embryogenesis.^[90] HA oligosaccharides (6–20 kDa) show inflammatory, immuno-stimulatory and angiogenic effects and small MW HA (0.4–4 kDa) show anti-apoptotic effects and can induce heat shock proteins.^[90, 91] Notably, there is no clear division of the MW range and the classification can vary in different publications.^[89, 91]

The MW of HA hydrogel precursors is a primary consideration for biomedical applications because it significantly influences rheological properties of resins and mechanical

properties of resulting hydrogels. For example, 20 mg/mL high MW HA solutions show zero-shear viscosities >1.7 Pa s and a strong shear thinning behavior.^[92] In contrast, medium MW HA of 77 kDa shows a viscosity of 0.013 ± 0.003 Pa s and only a weak shear thinning with nearly Newtonian fluid behavior.^[92] At physiological pH, HA is negatively charged and usually counterbalanced with cations such as Na^+ , K^+ , Ca^{2+} and Mg^{2+} allowing for higher hydrophilicity and stabilization of the structure by additional hydrogen bonds.^[88]

HA-based hydrogels lack cell-adhesive sites. Therefore, they need to be functionalized with cell-adhesive peptides to be used for cell encapsulation.^[83, 93] Alternatively, HA can be blended with gelatin which intrinsically contains RGD binding motives.^[94]

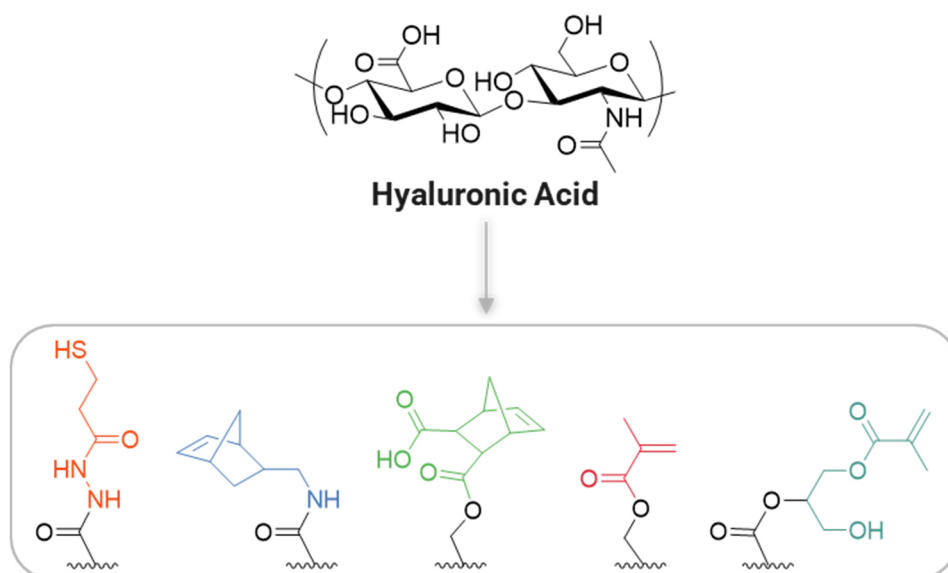


Figure 9. Chemical structure of hyaluronic acid (HA) and modification for photocrosslinking. Hyaluronic acid is based on disaccharide repeating units of *N*-acetyl-D-glucosamine and D-glucuronic acid. Photocrosslinkable moieties like thiols, NB or MA groups can be introduced to the carboxylic acid or hydroxyl groups via amide, ester or ether bonds.^[7, 22, 29, 82, 83, 86, 93-102]

1.3.1. Photocrosslinkable HA hydrogels

To render HA photocrosslinkable, the photoreactive moieties can be incorporated into the polysaccharide backbone using different modification strategies. Chemical functionalization of HA commonly target the carboxylic acid, the primary hydroxyl or, after deamination, also the *N*-acetyl group.^[6]

Numerous publications report the modification of HA with MA groups.^[7, 83, 94-99] This is usually done by modification of the primary hydroxyl group with methacrylic anhydride under basic conditions by an esterification or nucleophilic acyl substitution.^[7, 83, 95-99] Alternatively, the MA group can be introduced to carboxylic acid group by reaction with glycidyl methacrylate.^[94] The reaction proceeds via a base catalyzed ring-opening reaction. In both synthesis approaches, the obtained degree of functionality can be tuned with the equivalents of MA-reactant compared to HA repeating units.

Similar strategies can be used to functionalize HA suitable to react in a radical thiol-ene reaction. For example Galarraga et al. used NB anhydride to functionalize HA (88 kDa) with NB via ester bond at the primary hydroxyl group with tunable functionality (15–100%).^[84] The conventional strategy to introduce the NB functionality via ester or amide bond to the carboxylic acid group involves a two-step synthesis.^[82-84, 86, 100, 101] First, HA is converted into its tetrabutylammonium salt to be soluble in dimethyl sulfoxide (DMSO). Secondly, it is reacted with norbornene-methylamine or norbornene-carboxylic acid in the presence of a coupling agent or catalyst like benzotriazol-1-yloxytris(dimethyl amino)phosphonium hexafluorophosphate (BOP)^[82, 83, 100, 101] or 4-(dimethyl amino)pyridine (DMAP)^[84, 86]. Using BOP, functionalities between 15–45% were achieved for various molecular weights (61.8–150 kDa).^[82, 83, 100, 101] With DMAP, HA with a molecular weight of ~90 kDa was modified with 20–30% NB groups.^[84, 86] Notably, the modification in organic solvents requires additional synthesis steps, longer final purification processes and higher costs. An alternative strategy is the synthesis in aqueous media. For example, the amide derivative of HA-NB can be synthesized using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) mediated synthesis in buffered aqueous solution.^[93] A tunable degree of modification (4–48%) with NB groups has been reported for 61.8 kDa HA. Another common approach for the modification of carboxylic acid groups in HA with amides is the coupling using carbodiimide chemistry involving 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as co-activator.^[93] HA (61.8 kDa) was modified with 14% NB groups. The EDC/NHS coupling can also be applied to synthesize thiolated HA.^[22, 29] Here, various molecular weights (60–1000 kDa) were modified with thiol groups with a functionality between 11–61%.

1.4. Polyvinyl Alcohol-based hydrogels

Polyvinyl alcohol (PVA) is a synthetic polymer which is approved for biomedical applications and considered as non-toxic and biocompatible.^[13] It is industrially manufactured by the polymerization of vinyl acetate, followed by hydrolysis of the acetate groups rendering commercially available PVA of different molecular weights (~9–186 kDa) and with different hydrolysis degrees (70–72 mol%, 87–89 mol%, ≥99 mol%).^[103, 104] Although PVA hydrogels can be formed by physical bonds during repeated freeze-thaw cycles, covalent photocrosslinking allows the fabrication of stronger networks with high stability.^[13] Biomedical applications of crosslinked PVA hydrogels include drug delivery, tissue engineering, regenerative medicine and ophthalmology.^[13, 105] In the latter category, contact lenses are particular significance owing to the advantageous properties of PVA hydrogels, specifically their hydrophilicity, high transparency and low friction, which render them suitable and beneficial.^[12] Besides the use as contact lenses (CL) for vision correction, photocrosslinked PVA hydrogels allow the in situ encapsulation of drugs, lubricants or biofactories for ocular therapeutics.^[105, 106] The structure of PVA is shown in **Figure 10**.

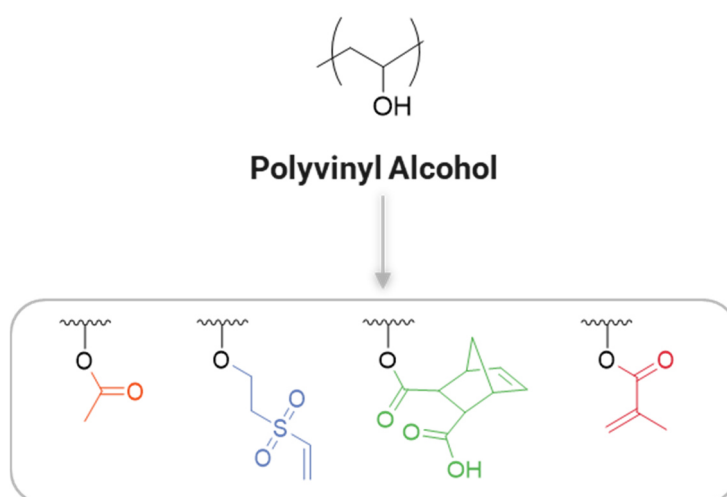


Figure 10. Chemical structure of polyvinyl alcohol (PVA) and modification for repeating unit in the starting material and for photocrosslinking. PVA is based on repeating units of [CH₂CH(OH)]. Depending on the degree of hydrolysis, repeating units can contain acetate groups. Photocrosslinkable moieties like vinyl sulfone, NB or MA groups can be introduced to the hydroxyl groups via ether or ester bonds.^[85, 106-108]

1.4.1. Photocrosslinkable PVA-based hydrogels

The hydroxyl groups on the PVA backbone allow modification with different photoreactive moieties. For example, methacrylated PVA (PVA-MA) can be synthesized using methacrylic anhydride under basic conditions (16 kDa PVA, $F_{\text{ene}} \sim 15\%$).^[107] PVA derivatives containing NB (PVA-NB) groups suitable for a radical thiol-ene reaction can be prepared using norbornene anhydride in the presence of catalytic amounts of *p*-toluene sulfonic acid in dry DMSO.^[85, 108] The degree of NB functionalization was reported as 7.8% for a 47 kDa PVA starting material.^[85] Qin et al. prepare PVA-NB using a 22 kDa PVA starting material with F_{ene} ranging from 5 to 50% by changing either the reaction time or the stoichiometry.^[108] The additional carboxylate groups increased the water solubility. An alternative photocrosslinkable PVA contains vinyl sulfone groups which can be introduced using divinyl sulfone under basic conditions.^[106] PVA (130 kDa) was functionalized with $1.5 \pm 0.2\%$ vinyl sulfone groups. Above a polymer concentration of 10% w/v in water, no homogeneous solutions were obtained.

1.5. Light-based (bio)fabrication

Additive manufacturing or 3D printing describes the process of fabricating objects by sequential addition of materials based on digital 3D models. Its implementation for processing biomaterials and cells, called bioprinting, offers several advantages, including:

- Fabrication of complex 3D geometries and multi-material assemblies
- Short processing times
- Easy customization using computer-aided designs (CAD)

Among 3D printing approaches, light-based 3D printing technologies typically offer the highest spatial resolution and allow fine-tuning of the physicochemical properties of the generated scaffolds by merely adjusting the exposure dose.^[3] For light-assisted 3D printing of a hydrogel, a liquid polymer precursor, eventually mixed with cells, is needed. The precursor solution contains photopolymerizable or photocrosslinkable moieties and is called “photopolymer resin” or in short “resin”.^[45] If cells are included in the

photopolymer resin, the terms “bioresin” and “bioprinting” will be used. In the presence of photoinitiator and light, the resin forms a crosslinked hydrogel network.

Light-based 3D printing techniques can be categorized according to the sequence of scaffold fabrication in point-by-point, layer-by-layer and volume-at-once processes (**Figure 11**). In the following sections, the mechanism and most important resin considerations will be presented. After that, examples of the application of light-based additive manufacturing to fabricate hydrogels for cell encapsulation will be discussed. The state of the art for the layer-by-layer and volume-at-once printing techniques will be focused on HA hydrogels which will be used in this thesis, as well as on gelatin hydrogels as they are the current standard for biofabrication. In addition, literature with side-by-side comparison of thiol-ene and MA resins will be highlighted.

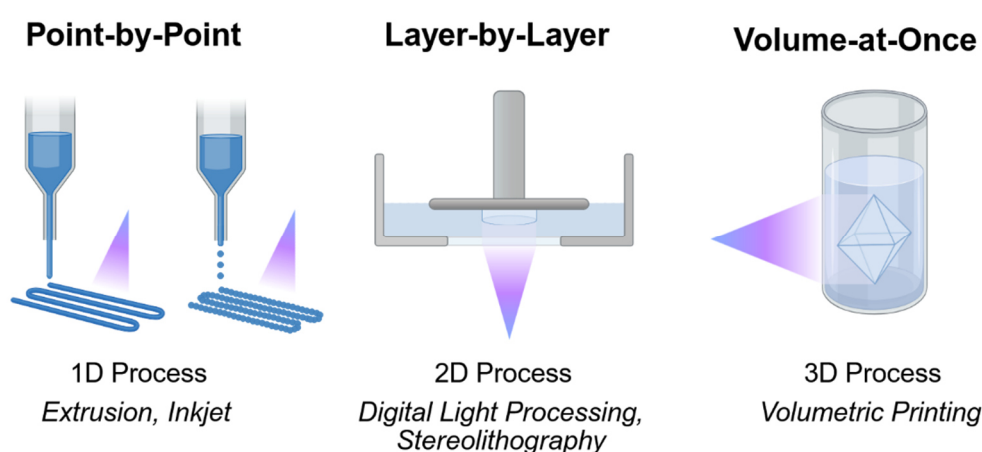


Figure 11. Overview of light-based 3D printing techniques. Point-by-point techniques deposit material along a 1D process to fabricate the structure (e.g. extrusion, inkjet). Layer-by-layer approaches enable layerwise (photo)crosslinking to build the scaffold sequentially via 2D operations (e.g. digital light processing, stereolithography). Volume-at-once techniques induce simultaneous materialization of the entire geometry, enabling a 3D fabrication process (e.g. volumetric printing). *Created with BioRender.com.*

1.5.1. Point-by-point: 3D printing using 1D processes

Light-based 3D printing techniques can be based on a 1D operating process for material deposition. The scaffold is built point-by-point using droplet-based or filament-producing techniques, such as inkjet or extrusion printing.^[109] The object is stabilized sequentially by photocrosslinking before, during or after the deposition.^[110] Resins in extrusion printing

usually exhibit a high viscosity to guarantee stability and integrity of the material after deposition.^[13] Ideally, the resin should further show a shear thinning behavior for smooth extrusion through the nozzle.^[73] The spatial control over the scaffold fidelity is rather controlled by the material deposition than the photocuring. Bioprinting can be achieved by including the cells in the resin before extrusion, however the applied shear stress during the extrusion can cause cell damage.^[73] Integration of different materials into one scaffold is possible by (i) switching between different print heads^[111] or (ii) using multi-axial nozzles^[9]. The printing techniques produces minimal material loss as the whole resin is deposited during the printing procedure.^[107] To improve the scaffold stabilization and printability of photopolymer resins, photocrosslinking could be combined e.g. with the formation of physical crosslinks triggered by temperature or the presence of ions.^[107] The nozzle-dependence of the technique usually limits the resolution to 200–1000 μm , restricts the printing to one object and shows the slowest print speed.^[112]

Gelatin-based resins crosslinkable via MA or thiol-ene chemistry remain the gold standard for extrusion (bio)printing.^[65, 66, 73] Gockler et al. fabricated 5% w/v Gel-MA and Gel-NB hydrogels with low, medium and high degree of modification by extrusion printing but did not elaborate concrete differences in the printing results from the thiol-ene vs MA resins.^[66] Tigner et al. reported a broader printable window for 10% w/v Gel-MA in contrast to the faster crosslinking Gel-NB resin, but the printed hydrogels showed a similar xy fidelity (**Figure 12**).^[73] Gel-NB/thiolated heparin and Gel-MA resins were used to print a grid structure by Zhao et al.^[65] The printing parameters were optimized for each resin.

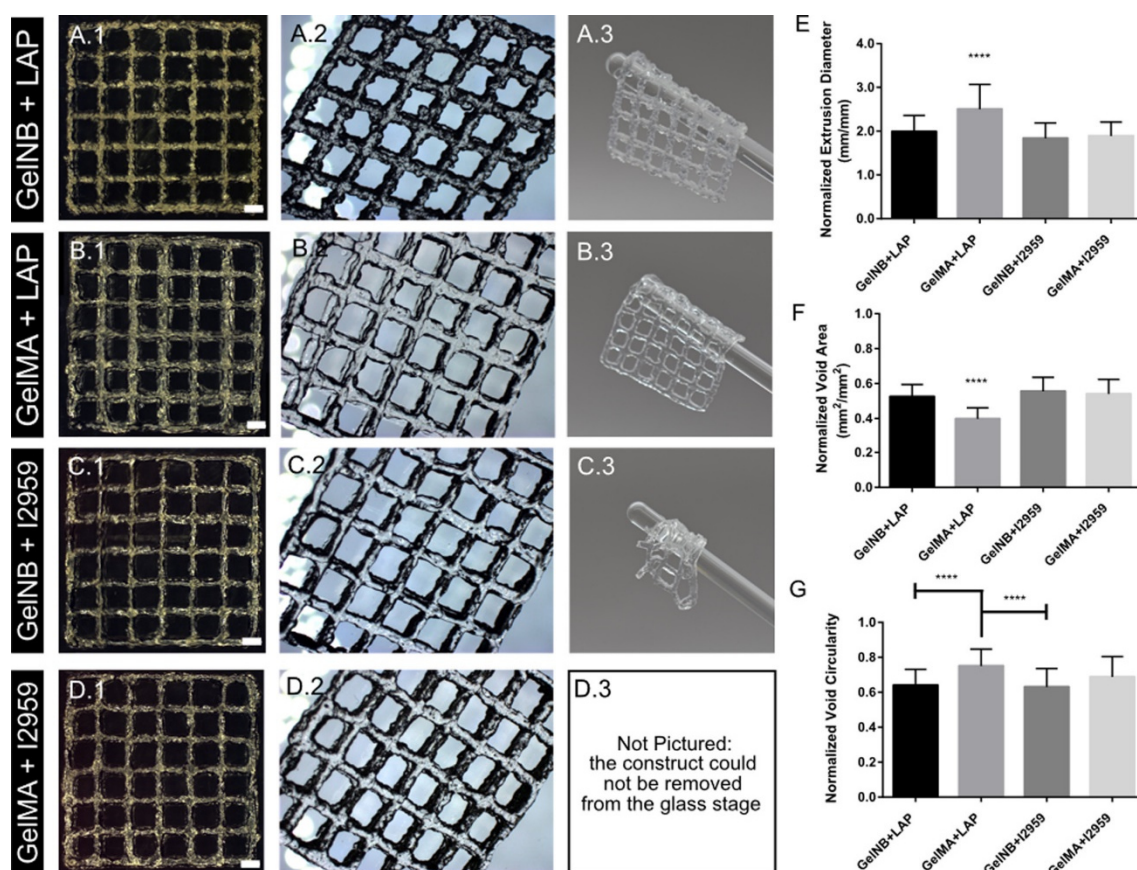


Figure 12. Comparison of printing grid structures using Gel-MA and Gel-NB resins by extrusion. The resins showed similar xy fidelity. Reproduced with permission from Tigner et al.^[73] Copyright © 2020 American Chemical Society.

Photocrosslinkable HA-based resins have also been explored for extrusion and inkjet printing.^[22, 81, 82, 97, 98] For example, Poldervaart and coworkers used 3% w/v HA-MA with high molecular weight (1700 kDa) with low degree of functionality ($F = 5-7\%$) for extrusion printing. Single-layer grids ($1.5 \times 1.5 \text{ cm}^2$) were (bio)printed in the presence of hMSCs. HA-MA with lower MW (90 kDa, $F_{\text{ene}} = 22\%$) was also used in extrusion printing by Ouyang et al.^[98] The printability of HA-MA was increased by the incorporation of guest-moieties into the backbone which increased the viscosity and led to a reduction in the filament size from $608.4 \pm 1.8 \text{ } \mu\text{m}$ to $321.8 \pm 9.5 \text{ } \mu\text{m}$. Dual-stage crosslinking of the hydrogel filaments allowed the fabrication of grid structures with >16 layers. A dual-stage crosslinking was also used by Hauptstein and coworkers to print HA-SH (51, 230 or 410 kDa) (**Figure 13A**).^[22] In a first step, the viscosity of the resin was increased by reacting 30% of the thiols with a PEGDA crosslinker via thiol-Michael addition. After extrusion, the scaffold with up to 21 layers was stabilized by orthogonal photo-initiated thiol-ene reaction with the remaining thiols and a PEG-diallyl crosslinker. Also HA-NB has been tested in extrusion printing (**Figure 13B**).^[82] A resin based on 2% w/w HA-NB (74 kDa, $F_{\text{ene}} = 40$) and DTT as

crosslinker was used to print multi-layered structures with in situ encapsulation of hMSCs. A 3% w/w HA-NB (60 kDa, $F_{ene} = 50\%$) resin with PEG2SH as crosslinker and with low viscosity (~ 8 mPa s) was also used for inkjet printing and allowed the fabrication of drug-loaded scaffolds.^[81]

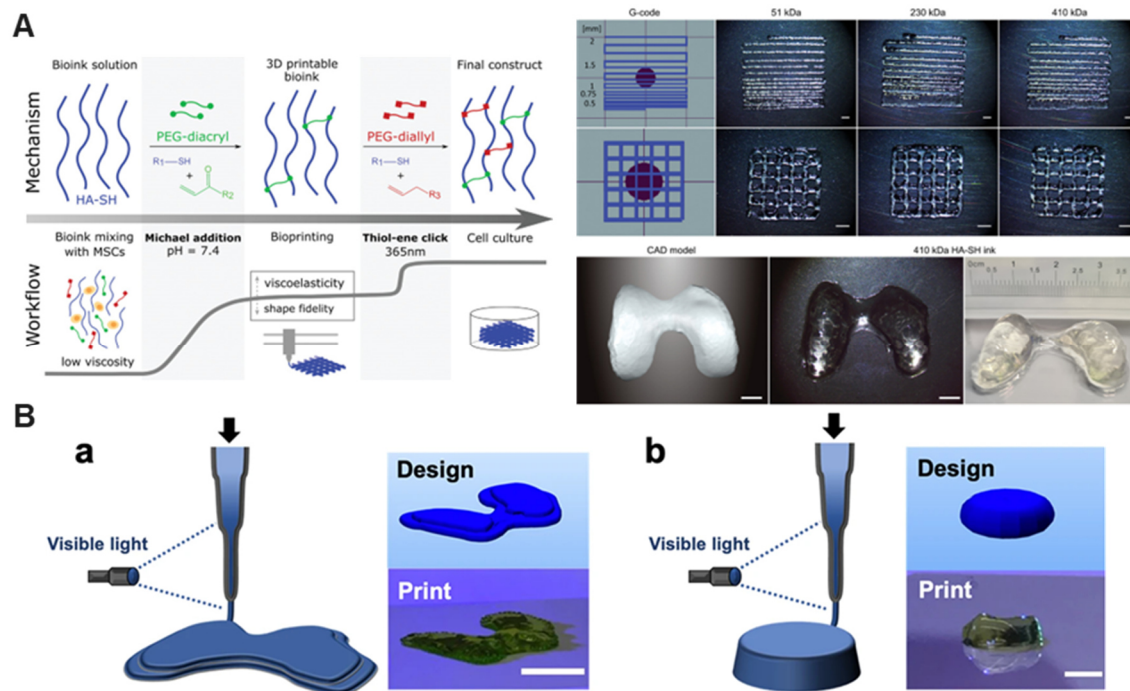


Figure 13. Extrusion printing with in situ curing of thiol-ene HA-based resins. **A.** Dual-stage crosslinking using HA-SH with (i) reaction with PEGDA by thiol-Michael addition to increase the viscosity of the resin and (ii) radical thiol-ene reaction with PEG-diallyl to stabilize the printed scaffold. The printability of different MWs was analyzed by printing filament and grid structures. A lung-model was printed using the high MW HA-SH. Scale bars represent 5 mm. Adapted from Hauptstein et al., 2022, licensed under CC BY-NC 4.0.^[22] **B.** Printing of multi-layered constructs using a HA-NB resin. Scale bars represent 1 cm (a) and 5 mm (b). Reproduced from Galarraga et al., 2019, licensed under CC BY 4.0.^[82]

1.5.2. Layer-by-layer: 3D printing using 2D processes

Increasing the complexity of the object fabrication by adding another dimension leads to a 2D process where the scaffold is fabricated layer-by-layer.^[109] Vat-based printing methodologies such as stereolithography (SLA) or digital light processing (DLP) fall into this category and are the most used light-based 3D printing technologies. The transition of the liquid photopolymer resin to the solid, crosslinked hydrogel network occurs at the

bottom of a transparent, resin-filled vat after light exposure and the crosslinked scaffold is fabricated sequentially on a stage (i.e. build platform) with vertical (z-axis) motion. In SLA, photocrosslinking is achieved by sequentially scanning the whole layer with a laser beam. In DLP printing, the whole layer is cured simultaneously by projection of a specific photomask which is modulated by a digital micro mirrors device.^[107] Layer-by-layer curing accelerates printing processes in comparison to nozzle-based techniques. Depending on the size of the build plate in comparison to the printable structure, multiple objects can be printed simultaneously. Multi-material objects containing distinct sections of different materials can be fabricated by switching resin vats or replacing liquid resins between the printing of consecutive layers.^[99, 113, 114] The printing procedure requires additional optimization to avoid cross-contamination and to obtain a smooth multi-material interface.^[113]

1.5.2.1. Workflow of the DLP printing process

The overall DLP printing process consists of the following steps: (i) slicing of the CAD model, (ii) loading of the resin, (iii) printing of the scaffold and (iv) post-processing.

Slicing. In this step, the parameters for printing are defined and according to those, the CAD model is processed into a procedure readable for the DLP printer. The 3D model is cut into slices along the z-axis which generates binary images and defines the pixels to be illuminated. Traditionally, the photomask only consists of black and white pixels, but recently also grayscale modifications are common.^[48] The number of slices or layers is defined by the layer thickness. The theoretical minimum layer thickness is conditioned by the precision of the motor which is moving the build platform along the z-axis. In this step, also the light intensity and exposure time are defined. Notably, exposure time and layer thickness must be adapted for each resin which will be discussed later. Other parameters include speed of the build platform movement and off time before starting the photomask projection. Especially for larger structures or scaffolds with delicate features, additional support layers or support structures can be added to the printed object to prevent demolition during the printing process. In addition, the first layer can be exposed for longer to ensure adherence to the build platform. However, those measures are usually of less interest for bioprinting.

Loading. Initially, the build platform is calibrated to ensure proper alignment with the bottom of the vat. The liquid photopolymer resin is loaded into the vat ascertain that enough volume is provided for full coverage of the build platform area and printing of the whole object.

Printing. The printing process is schematically depicted in **Figure 14**. Upon initiation of the printing procedure, the build platform moves towards the surface of the vat, forming a gap corresponding to the intended layer thickness. The area in this gap is exposed with the sliced photomask through the transparent bottom of the vat leading to solidification and crosslinking of the hydrogel material on the build platform. After the projection, the platform is raised. In sequential steps the build platform lowers back to the defined layer thickness curing consecutive layers onto the previous ones by varying the projection pattern based on the sliced 3D model. It is crucial that before illumination, the previous layer is coated with resin to prevent loss of shape fidelity.

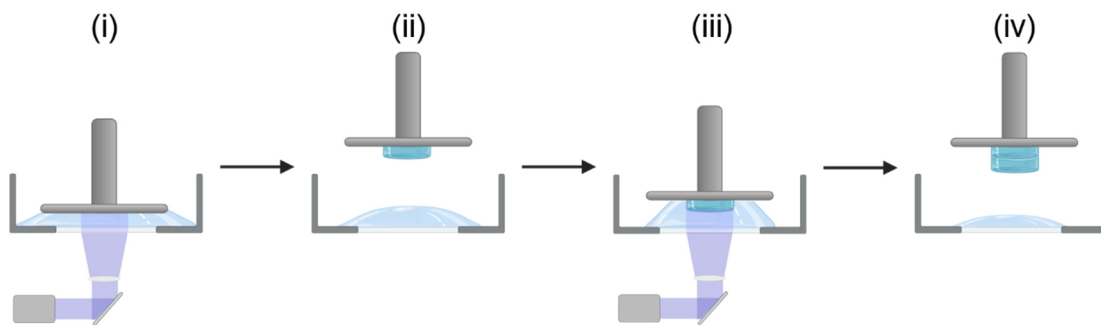


Figure 14. Schematic depiction of DLP-based printing process. (i) The build platform lowers into the resin-filled vat to the prescribed layer thickness. Illumination of the resin with a defined photomask crosslinks an entire layer. (ii) The build platform is lifted to separate the layer from the vat and allow uncured resin to flow away. (iii) The platform and scaffold are lowered back into the resin to re-establish the targeted gap. Exposure leads to photocuring of the next layer. (iv) Repetition of the processes leads to subsequent layer crosslinking directly onto the previous one to build the scaffold layer-by-layer. *Created with BioRender.com.*

Post-Processing. After the printed object has been finalized, the scaffold adheres to the build platform and needs to be removed manually. Uncured resin around the scaffold or in internal voids is removed by washing with water or buffer. If the photocrosslinking efficiency was insufficient to achieve adequate mechanical properties, a post-curing step can be added.

Mainly, gelatin-based resins are employed for (bio)printing using DLP. Besides Gel-MA, also thiol-ene Gel-NB, Gel-AE or Gel-SH resins have been reported.^[115, 116] In some cases, hybrid resins using a combination of photocrosslinkable gelatin and HA are used.^[7, 94, 95, 99, 117] A few reports have utilized photocrosslinkable HA derivatives as (bio)resins for DLP-based printing technologies. For example, Dhand and coworkers used a combination of HA-NB and HA-MA to print hydrogels with interpenetrating networks while Galarraga et al. used HA-NB only.^[7, 94, 99, 117, 118] Printing of thiol-ene and HA-based resins will be discussed in more detail in section 1.5.2.3.

1.5.2.2. Resin considerations for DLP printing

The resin requirements for DLP printing differ from nozzle-based techniques like extrusion. A recent review by Dhand et al. provides a step-by-step guide to DLP printing and highlights among other things the following resin properties which need to be considered for DLP photopolymerization: (i) resin viscosity, (ii) photoinitiators, (iii) photoinhibitors and photoabsorbers, (iv) polymer, (v) crosslinking chemistry and (vi) additional additives for optical tuning.^[119] Based on this, the following section will focus on the requirements and influence of viscosity, reactivity and absorbance.

Viscosity. An important consideration is the resin viscosity. During printing of the consecutive layers, resin needs to (i) flow away from the printed structure to avoid overcuring in voids and (ii) re-coat the printed object before illumination of the next layer. As discussed by Choi and coworkers, high-viscosity resins cause a mismatch between resin flow speed and build platform movement leading to interface breakage, entrapment of air bubbles, incomplete filling and print defects (**Figure 15**).^[120] A similar result was observed by Duong et al., where resins with higher viscosities led to the entrapment of air in the printed structure.^[94, 119, 120] Resins with medium viscosity (up to 1 Pa s) have been found suitable in DLP, but low-viscosity resins (<100 mPa s) are usually preferred.^[94, 119, 120] Some reports highlight that a higher viscosity could potentially limit the diffusion length of radicals thereby improving resolution.^[119] A slower build platform movement could prevent detachment between layers due to capillary forces arising during the separation speed in high-viscosity resins.^[119] The resin viscosity can be tuned with the

polymer type, concentration, molecular weight or the addition of rheology modifiers.^[119]

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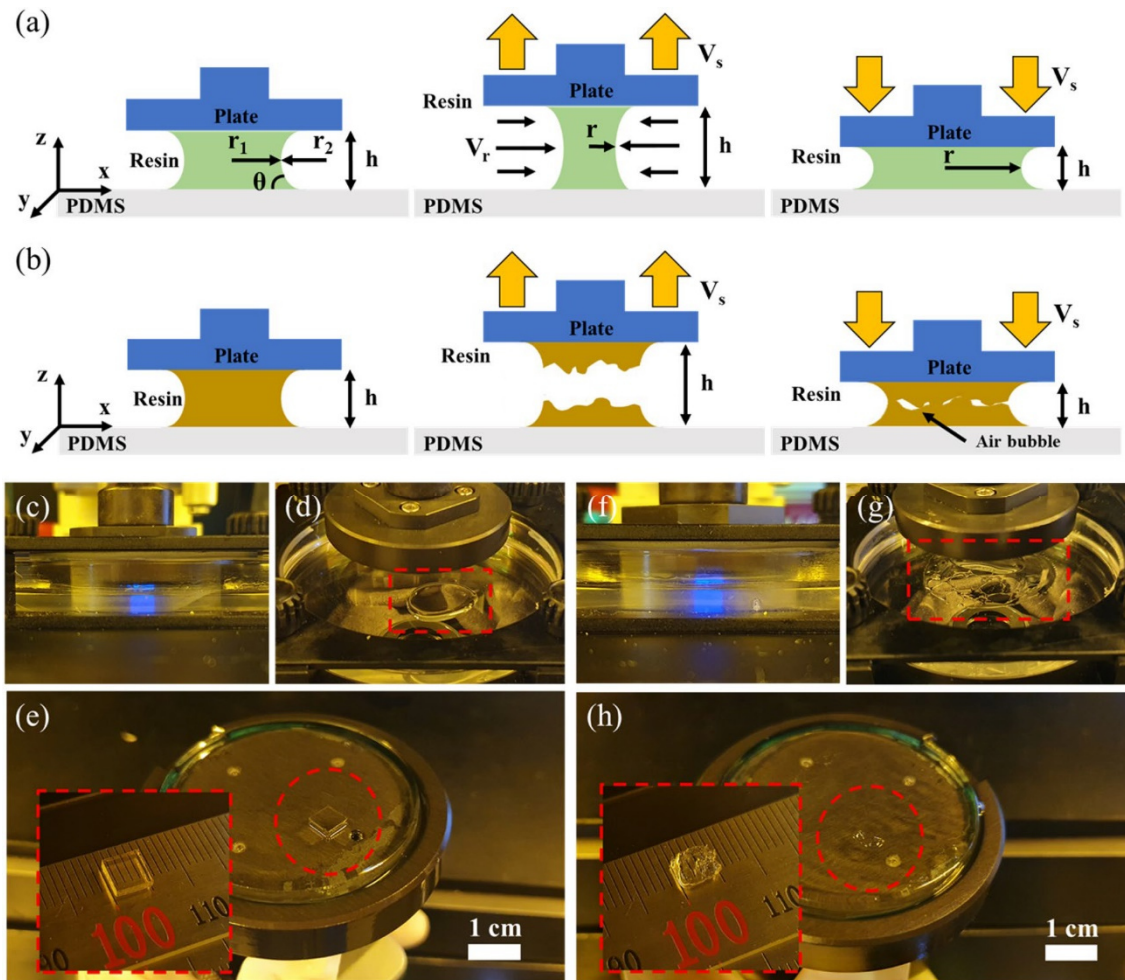


Figure 15. Considerations for resin viscosity. Build platform movement will not affect the printability of low-viscosity resins (**a**, **c-e**) while high-viscosity resins lead to interface breakage (**b**, **f**, **g**), entrapment of air bubbles (**b**) and poor recoating (**h**). Reproduced from Choi et al., 2022, licensed under CC BY 4.0.^[120]

Reactivity. The polymer concentration affects the resin viscosity, but is also essential to tune the crosslinking kinetics and the exposure time for polymerization and printing.^[122] Control of the resin reactivity is crucial to balance fast and efficient photocrosslinking with high shape fidelity, high resolution and high cell viability. On one hand, the resin should show fast crosslinking kinetics with fast gelation to enable high printing speeds with low exposure doses. This is especially important when printing is carried out in the presence of cells to minimize cell death and prevent cell sedimentation in low-viscosity resins.^[3] Options to tune the curing rate are the concentration of photosensitive prepolymer and photocrosslinkable groups. In general, a higher polymer concentration

and higher concentration of reactive groups will reduce the exposure dose required for gelation.^[84, 94] In addition, greater mechanical integrity of the printed scaffolds will be obtained.^[94, 123] However, a high polymer concentration can increase in viscosity of the resin, impair its printability, increase cell toxicity during in situ encapsulation and reduce cell viability in long-term studies.^[94, 96] Mostly, only the influence of the polymer concentration and degree of functionality are studied in photorheology experiments.^[115, 123, 124] However, Duong and coworkers studied the printability of a Gel-NB resin with stepwise variation of the polymer concentration. Increasing the Gel-NB concentration from 2.0 to 6.0% w/w decreased the required light intensity for printing of a perfusable construct from 28.9 to 18.7 mW cm⁻² at the same exposure time per layer.^[116] Wang et al. studied the printability of a Gel-MA resin with different concentrations. A concentration of 10% w/v was required to render the resin printable, while with 7.5% w/v only partially printed structures were obtained which collapsed during the printing process. With 5% w/v Gel-MA printing failed, the structures were too soft.^[117] In the same way, they analyzed the addition of different HA-MA content. They noted that not only the concentration of HA-MA but also the MW influenced printability. With low MW (10 kDa), the obtained mechanical properties were too weak to obtain a stable scaffold, while with higher MW (>500 kDa) the viscosity was too high. It has to be noted that for all tested formulations, the same exposure dose was used. Besides concentration, MW and degree of functionality F , batch-to-batch variability of the employed polymer backbone can compromise the reproducibility of the printing parameters.^[119]

As discussed in section 1.2.1.3, crosslinking chemistry influences the crosslinking kinetics and final properties of hydrogels. Hydrogels based on step-growth mediated thiol-ene crosslinking show faster crosslinking with lower t_{gel} . Thus, thiol-ene based resins should require lower exposure times per layer compared to MA resins based on the same polymer backbone and degree of functionality, leading to an overall faster printing process. Additional advantages could be expected in terms of resolution. Dark curing and radical diffusion of radical chain-growth crosslinking can lead to overcuring and blurring, causing loss of shape fidelity and accuracy.^[48] Ortiz-Cardenas and coworkers compared a Gel-SH/PEG-NB hydrogel system to Gel-MA in the fabrication of a microfluidic chip.^[125] Notably, the hydrogels were not fabricated by DLP printing but used a photomask for

controlled illumination and photocrosslinking similar to DLP light projection mechanism. Gel-MA required a 2.6-times longer exposure time in comparison to the thiol-ene system. While the thiol-ene hydrogels were obtained with sharp feature sizes down to 200 μm with less than 10% deviation from the theoretical structure, the feature sizes found in Gel-MA were 13–32% larger than expected, potentially due to dark curing and diffusion of radical species out of the illuminated areas.

As commonly demonstrated in photorheology experiments, the photoinitiator concentration, in addition to its absorptivity and efficiency, affect the photocrosslinking kinetics mainly in terms of the light dose required to induce gelation. For example resin development by Duong et al. involved monitoring of photocrosslinking kinetics with different photoinitiator concentrations by photorheology experiments.^[116] Increasing the LAP concentration from 5 to 10 or 15 mM shortened t_{gel} from 21 to 9 and 8 s, respectively. Interestingly, the employed LAP concentration further affected the final stiffness of the fabricated Gel-NB/PEG4SH hydrogel and was found to be lower at lower concentration. A shorter t_{gel} with increasing LAP concentration was also reported by Kim and coworkers.^[115]

Absorbance. In a DLP printing process, the photoinitiator concentration is not only crucial to balance the resin reactivity and exposure time per layer, but also influences the cure depth.^[122] Based on the Lambert-Beer law, the cure depth C_d can be described as follows^[126]:

$$C_d = D_p \cdot \ln\left(\frac{E}{E_c}\right)$$

D_p describes the light penetration depth, E the received light dose by multiplying exposure time and light intensity, and E_c describes the critical light dose needed to induce gelation. Increasing the exposure time leads to an increase in the cure depth of the exposed area which was shown in DLP experiments.^[107, 127] The importance of balancing exposure time and cure depth in DLP arises from the layer-by-layer fabrication technique. The cure depth must be high enough to ensure good interfacial adhesion of subsequent layers.^[126] Although the layer thickness is set in the printing parameters, a too high cure depth can lead to overcuring in previous layers, compromise the z-resolution and printability of

internal void structures.^[127] The cure depth further depends on the light penetration depth D_p which is dictated by the absorptivity of the resin, mainly caused by the photoinitiator concentration. Photoabsorbers are commonly added in DLP resins to reduce the light penetration depth, thereby improving axial resolution and prevent overcuring in previous layers by reduction of the cure depth (**Figure 16**).^[107, 117, 128] Usually, colored dyes like tartrazine or Ponceau 4R are employed, which need to be washed out after printing to obtain transparent structures.^[115, 116, 129] In rheology experiments, the addition of photoabsorbers increases the t_{gel} but also leads to a lower final stiffness.^[115, 116] If the photoabsorber concentration is too high, this can prevent curing and compromise layer adherence.^[116]

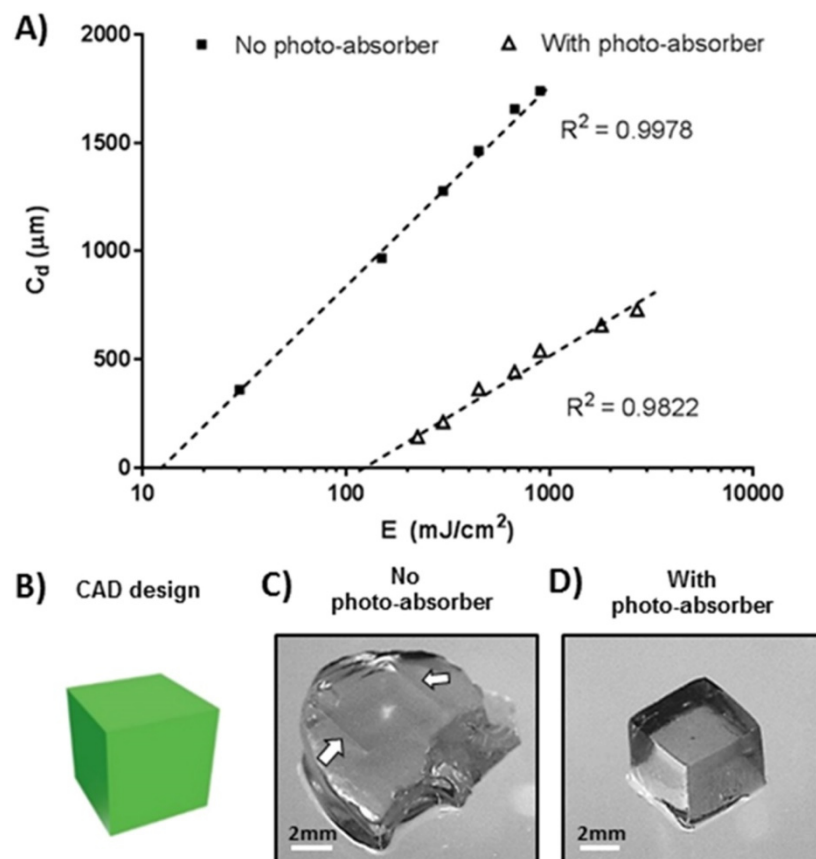


Figure 16. Influence of photoabsorbers. The addition of photoabsorbers alters the light dose needed for printing and the cure depth and can improve the printability. Reproduced with permission from Lim et al.^[107] Copyright © 2018 IOP Publishing Ltd. All rights reserved.

1.5.2.3. DLP-based (bio)printing using thiol-ene and HA-based resins

DLP-based bioprinting allows fabrication of 3D complex structures.^[107, 130-132] The printed scaffold is fabricated layer-by-layer by projecting a specific 2D pattern onto a liquid photopolymer resin which leads to spatially controlled photocrosslinking of the polymeric chains. As curing of one layer is independent of the complexity of the projected masks, DLP printing is more rapid than extrusion or inkjet printing where filaments or drops instead of whole layers are deposited.^[131] The printability and printing resolution of the liquid resin can be manipulated by altering chemistry and concentration of the functional groups, polymer content, viscosity and crosslinking kinetics as described in section 1.5.2.2.

The first gelatin-based thiol-ene crosslinkable resin was presented in 2017 by Bertlein and coworkers using Gel-AE (10–20% w/w, $F_{\text{ene}} = 68.9\%$) with DTT (thiol:ene = 12:1) as crosslinker and Ru/SPS (0.5/5 mM) as visible-light photoinitiator system.^[74] No photoabsorber was incorporated in the resin. At 30% w/w, the Gel-AE solutions exhibited a viscosity <100 mPa s. Grid structures with 250 μm strands were printed with one or two layers with a layer thickness of 50 μm . Interestingly, the authors note that the comparable Gel-MA resin was not printable using DLP due to physical gelation. Bioprinting of the Gel-AE resin was only demonstrated using extrusion printing. A few years later, Yu et al. reported on a Gel-NB resin (3.33% w/v, $F_{\text{ene}} = 44\%$) crosslinked with Gel-SH (6.67% w/v, $F_{\text{SH}} = 22\%$) for single-layer DLP-based printing.^[133] Various scaffolds with 2 x 2 mm² width and 250 μm or 1 mm thickness were fabricated using a customized DLP setup with 20–60 s exposure time at 365 nm with 9.5–10.15 mW cm⁻² irradiance. The printed structures showed features down to 10–30 μm resolution. After printing, thiolated proteins or peptides were covalently attached to residual NB groups via orthogonal photoconjugation with approx. 50 μm precision to engineer tailored microenvironments. Bioprinted iPSCs or HUVECs showed high cell viability, and the cell response, e.g. endothelial migration and vascularization, was tunable with orthogonal photopatterning. The authors highlight that in contrast to the benchmark Gel-MA, the hydrogel properties were tunable with the radical photoinitiator and crosslinker concentrations. Gel-NB (2–6% w/w, $F_{\text{ene}} \sim 82\%$) was also used with PEG4SH (1–7% w/w) as thiolated crosslinker by Duong and coworkers to print soft, cell-laden constructs ($G' < 2000$ Pa).^[116] The resin

contained LAP (10 mM) as photoinitiator and tartrazine (1.2 mM) as photoabsorber. To improve hydrolytic stability of the hydrogels, PEG4NB (0.5% w/w) was added to the resin. Constructs were printed with 100 μm layer thickness, 12–13 s exposure time per layer and with 24.4–28.8 mW cm^{-2} light intensity at 405 nm. The average deviation of the prints from the CAD model was reported as <3%. With 3% w/w Gel-NB, $\sim 5 \times 10 \times 2 \text{ mm}^2$ scaffolds containing perfusable channels with 300, 500 and 1200 μm diameter were printed (**Figure 17**). HUVECs were encapsulated during bioprinting, and additional cells were seeded into the fabricated channels. Orthogonal photoconjugation of angiogenic QK peptide to NB groups led to high cell viability and promoted vascular morphogenesis in the bioprinting scaffolds.

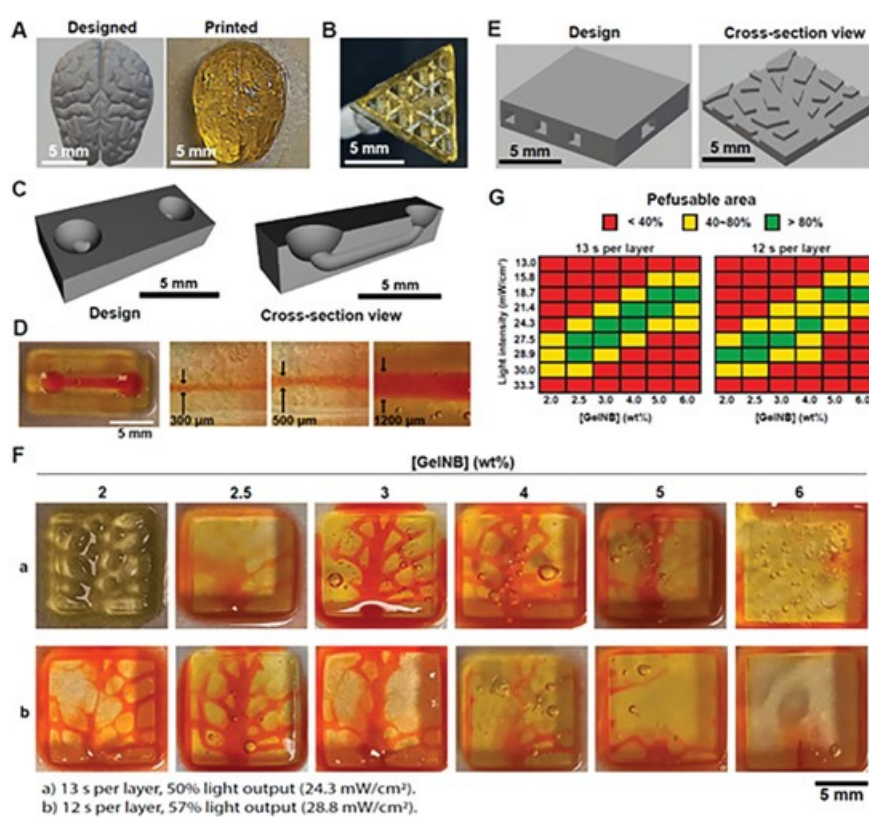


Figure 17. DLP printing of thiol-ene Gel-NB resin. **A-D.** Photographs and CAD model of scaffolds resembling a mini human brain (A), grid structure (B) or containing microchannels (C-D). **E-G.** Printability assessment using channel-containing structure as function of light intensity and Gel-NB concentration. CAD model (E), photographs (F) and corresponding printability window (G). Reproduced from Duong et al., 2023, licensed under CC BY-NC-ND 4.0.^[116]

In the literature, HA-MA-based resins are often found in combination with Gel-MA to incorporate cell-adhesive sites. For example, Shopperly et al. used a hybrid bioresin composed of Gel-MA (5% w/w, $F_{\text{ene}} = 60\%$) and HA-MA (0–4% w/w, 120 kDa, $F_{\text{ene}} = 14\%$) to

print ~1 mm thick constructs with 300 μm layer height in the presence of chondrocytes to fabricate cartilage-like constructs.^[95] Hossain-Rakin et al. used high molecular weight HA-MA (2% w/v, 1500 kDa, $F_{\text{ene}} \sim 90\%$) with Gel-MA (0–6% w/v, $F_{\text{ene}} = 90\%$) and a resin viscosity of ~50–300 mPa s for layer-by-layer printing.^[94] The scaffolds were fabricated with features ranging from 500–1000 μm by sequential photocrosslinking of the liquid bioresin by DLP-based pattern projection. Addition of gelatin was crucial to induce cell adhesion in the bioprinted scaffolds. Hybrid Gel-MA/HA-MA (5% w/v, $F_{\text{ene}} \sim 82\%$ / 3% w/v, 100 kDa, F_{ene} n.d.) bioresins were further studied by Wang et al. to fabricate cleavable cell-laden constructs. The incorporation of HA-MA allowed on-demand modulation of the mechanical properties by enzymatic cleavage by hyaluronidase which reduced the matrix stiffness.^[117] To decrease the cure depth, Ponceau 4R was added as photoabsorber. Thus, the cure depth decreased from 576.0 ± 32.7 and 1162.3 ± 67.0 μm to 34.7 ± 3.7 and 354.0 ± 16.1 μm for 5 or 30 s exposure time. With a layer thickness of 100 μm and 15 s exposure time per layer, scaffolds with 200 μm resolution, pyramid structures, cured surfaces and embedded channels (1 mm diameter) were printable (**Figure 18A**). The overall print time varied between 10 to 20 min. Lam et al. printed hemispherical constructs (8 mm diameter, 1 mm height) using 1% w/w HA-MA (~120 kDa, $F_{\text{ene}} = 14\%$) resins with 0.1% w/w LAP, 300 μm layer thickness and 30 s exposure time per layer.^[7] Cartilage-like structures were fabricated with the in situ encapsulation of chondrocytes. Cell-free and cell-laden scaffolds showed high stability in shape and dimension over 14 days under cell culture conditions. Thomas and coworkers evaluated different HA-MA and Gel-MA resin compositions for printability, material properties, digestibility and biocompatibility.^[99] All resins showed a viscosity <20 mPa s. A multi-material construct with 6 mm diameter and 1.2 mm height was printed using 6% w/v Gel-MA and 1.5% w/v HA-MA by intermediate switching of the resin container (**Figure 18B**). An internal channel structure was printed with the HA-MA resin in the presence of HUVECs. Digestion of HA-MA by hyaluronidase allowed fabrication of vasculature-like channels.

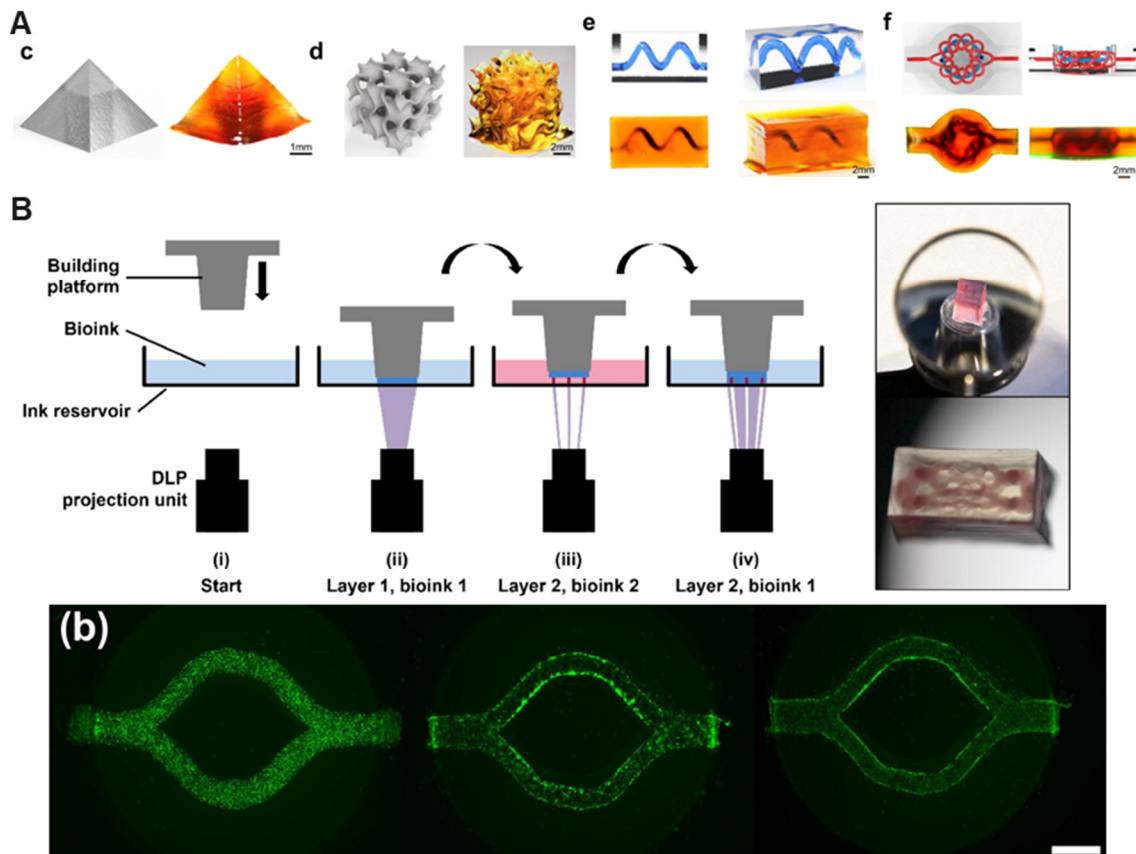


Figure 18. DLP printing of HA-MA. **A.** CAD models and images showing 3D-printed constructs with sharp features (pyramid, gyroid) and internal structures (cuboids with perfusable channels, torus knot) using a hybrid Gel-MA/HA-MA resin. Adapted from Wang et al., 2022, licensed under CC BY 4.0.^[117] **B.** Multi-material DLP printing of Gel-MA and HA-MA resins. Internal channels printed with HA-MA can be used to prepare a vascularized bioprinted scaffold after degradation of the sacrificial material with hyaluronidase. Adapted from Thomas et al., 2020, licensed under CC BY-NC-ND 4.0.^[99] Modifications permitted.

Few reports use thiol-ene based HA resins in DLP printing. Dhand et al. combined 0.6% w/w HA-MA (88 kDa, $F_{\text{ene}} \sim 100\%$) with 4.5% w/w HA-NB (88 kDa, $F_{\text{ene}} = 45\%$) and a dithiol crosslinker which led to the formation of interpenetrated hydrogel networks in a one-pot crosslinking step by simultaneous and independent polymerization of the two functionalities. Using DLP, 20 layer scaffolds with a layer thickness of 100 μm and a xy resolution of approx. 300 μm were printed in combination with 17 mM LAP as photoinitiator and 1 mM tartrazine as photoabsorber at exposure times of 2.5 s per layer (**Figure 19A**).^[83] Cell adhesion was enabled by including 2 mM thiolated RGD peptide which readily crosslinked with the pendant NB groups on HA-NB-based on the radical thiol-ene reaction during the printing process. Galarraga and coworkers used a 5% w/w HA-NB (88 kDa, $F_{\text{ene}} = 40\%$) formulation with DTT as crosslinker at thiol:ene ratio of 1. With

1.7 mM LAP as photoinitiator and 1 mM tartrazine as photoabsorber, 20-layer scaffolds with 100 μm layer thickness and a xy resolution of 500 μm were printed using 6 s exposure time per layer. DLP printing in the presence of 2 mM thiolated RGD peptide and bovine bone marrow-derived mesenchymal stromal cells fabricated scaffolds with >80% cell viability after 3 days, which demonstrates the cytocompatibility of HA-NB hydrogels (**Figure 19B**).^[84]

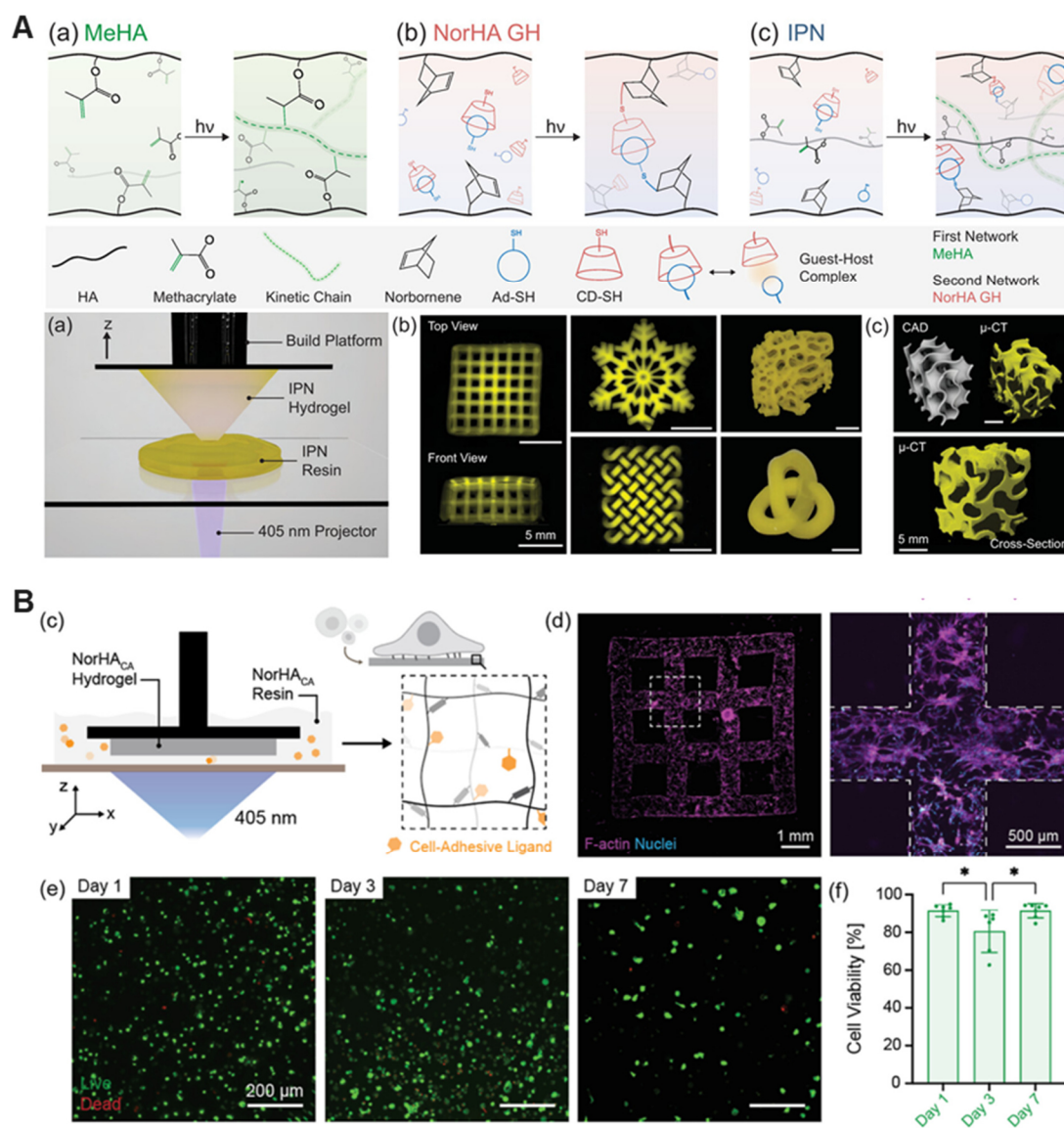


Figure 19. Thiol-ene based HA resins for DLP printing. **A.** A resin combination of methacrylated HA (MeHA), NB-modified HA (NorHA) and a thiolated Adamantane-Cyclodextrin crosslinker which forms a guest-host complex was used to print interpenetrating hydrogel networks using DLP. Various structures were printed using tartrazine as photoabsorber. Adapted with permission from Dhand et al.^[83] Copyright © 2022 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. **B.** Inclusion of a cell-adhesive ligand in a (degradable) HA-NB resin enabled printing of cell-laden scaffolds with sharp

edges. Encapsulated cells showed typical morphology and high viability over 7d. Adapted with permission from Galarraga et al.^[84] Copyright © 2023 American Chemical Society.

1.5.3. Volume-at-once: 3D printing using 3D processes

In volumetric additive manufacturing or printing technologies, the 3D object is fabricated in one single operation without repetitive lower-dimensional operations like point-by-point or layer-by-layer material deposition.^[109] In contrast to conventional vat polymerization techniques where photocrosslinking occurs at the surface of the photopolymer resin, in volumetric printing methodologies the light propagates through the resin to crosslinking the material inside the resin volume.^[134] The techniques take advantage of optical or chemical non-linearities of the photopolymerization processes to enable spatial control of the solidification at arbitrary positions inside the volume (**Figure 20**).^[135] This leads to curing of voxels (instead of pixels) without material replenishment. The techniques can be distinguished by the method of light projection used to trigger gelation in 3D voxels inside the resin and will be shortly presented in the following paragraphs.

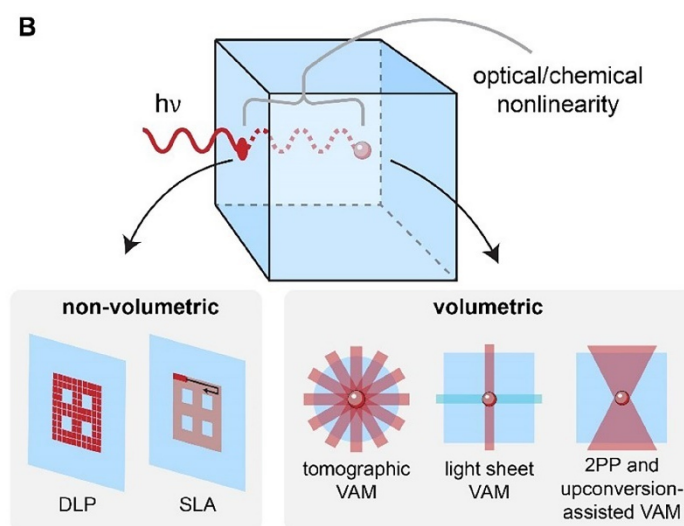


Figure 20. Volumetric printing relies on optical or chemical non-linearity to shift (photo)polymerization from the surface of the photocrosslinkable resin to an arbitrary point within the volume. Adapted with permission from Thijssen et al.^[135] Copyright © 2023 Elsevier Ltd. All rights reserved.

Two-photon polymerization (2PP) based volumetric printing relies on an optical non-linearity to crosslink the photopolymer resin.^[135] Focusing of two lasers in the resin leads

to simultaneous two-photon absorption which induces localized photocrosslinking in the volume where laser focal spots overlap. Curing of voxels occurs without material replenishment. However, the lasers move point-by-point through the volume to fabricate the object, instead of leading to simultaneous solidification of the whole object. Notably, this technique offers the highest resolution (~ 100 nm) but requires expensive equipment and is limited to small volumes along with slow scanning speeds.^[135, 136]

Light-sheet volumetric printing uses stepwise multi-photon absorption to induce a chemical non-linearity in the resin. The technique combines a light-sheet that moves through the resin with orthogonal projection of the photomask.^[137] A prominent example is the method Xolography which uses a dual-color photoinitiator system based on photo switches (**Figure 21**). A light-sheet with UV or blue light (375 or 405 nm) moves through the resin to switch the photoinitiator from an initial, dormant to an excited state. Upon illumination with a second, patterning light beam using visible light (570–585 nm), the polymerization and crosslinking are initiated only at voxels where the photoinitiator was simultaneously activated.^[136] Solidification solely occurs at the intersection of light of the two wavelengths.^[136] Using hydrogel-based resins, minimum positive feature sizes down to ~ 20 μm and negative channels with ~ 100 to 400 μm (after swelling) have been reported.^[138, 139] Similar to 2PP, no external movement for relocating the photopolymer solution is required. Although no layering artifacts occur, the object is still fabricated layer wise so that this volumetric printing technique cannot be defined as true volume-at-once process.^[109] Printing speeds range from 0.5 mm min^{-1} to 5 mm min^{-1} .^[136, 138, 139] Up to now, only chain-growth mediated (meth)acrylate systems have been reported for Xolography, radical thiol-ene resins have not yet been explored.

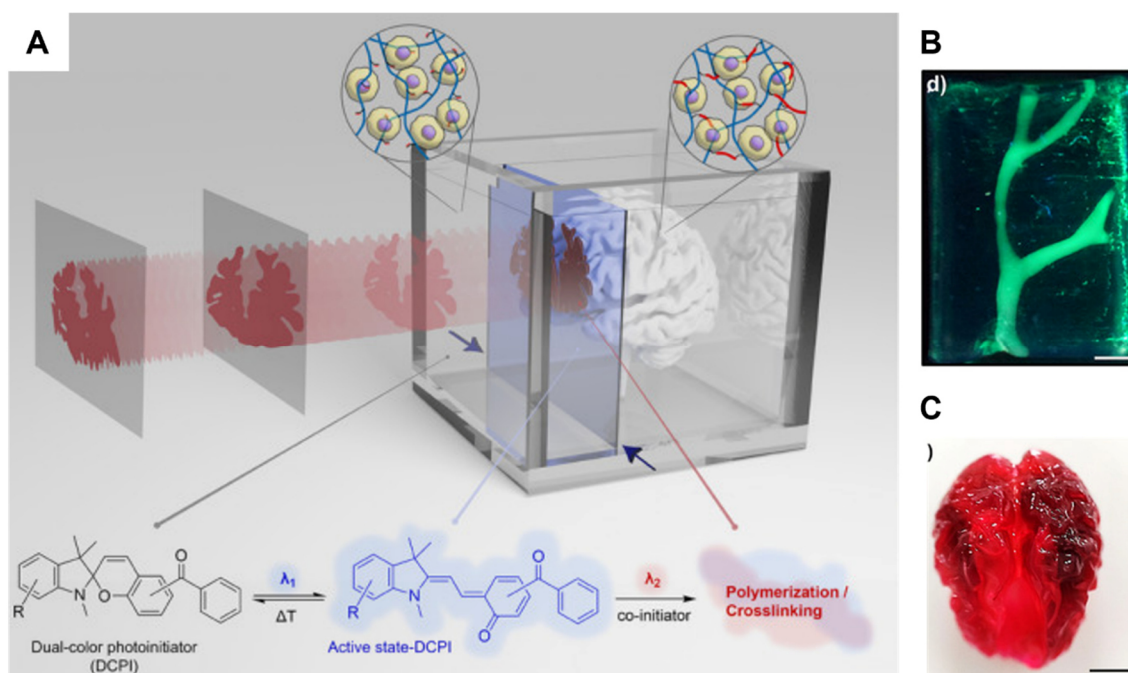


Figure 21. Xolography printing as on light-sheet volumetric printing methodology. **A.** Schematic illustration of light-sheet based printing in Xolography. Crosslinking of the resin occurs upon simultaneous exposure of two distinct light beams with different wavelengths. A first light-sheet activates the dual-color photoinitiator by transition from spiropyran to merocyanine. A 2D photomask is projected onto the light-sheet with a second wavelength to induce photocrosslinking at the intersection. **B, C.** Printed hydrogel structures. Scale bars represent 2 mm. Adapted from Wolfel et al., 2025, licensed under CC BY 4.0.^[138]

Tomographic volumetric printing (VP), also referred to as computed axial lithography, was inspired by computed tomography, a conventional imaging technique used in medicine.^[109] It is the most popular volumetric additive manufacturing technique and was introduced 2019 by Kelly and coworkers.^[140] This volumetric printing technique is based on a chemical non-linearity in photocrosslinking processes. A 3D light dose superposition is achieved by the projection of 2D light patterns from different angles into a rotating resin cylinder (**Figure 22**).^[135] To induce the transition from a liquid to a more solid structure, a certain threshold needs to be overcome which is usually defined as the previously mentioned t_{gel} or gelation threshold.^[44] At this point, the accumulated light dose triggers gelation, because sufficient crosslinks have been formed to produce a 3D network. After exceeding the gelation threshold, the whole object materializes at once inside of the resin volume. Thus, this VP methodology can be classified as 3D process with a volume-at-once formation of the scaffold. It enables printing speeds in the range from seconds to minutes independent from the object size reaching resolutions down to $\sim 40\text{--}50\text{ }\mu\text{m}$ for positive and $\sim 100\text{ }\mu\text{m}$ for negative features.^[109, 141, 142] Multi-material

constructs can be realized either by sequential VP processes with exchanging of the resin in the vial or by combination with different 3D printing techniques like embedded extrusion printing.^[143-146]

If not mentioned otherwise, this thesis will use the term “volumetric printing” (VP) for volumetric additive manufacturing based on tomographic pattern projection.

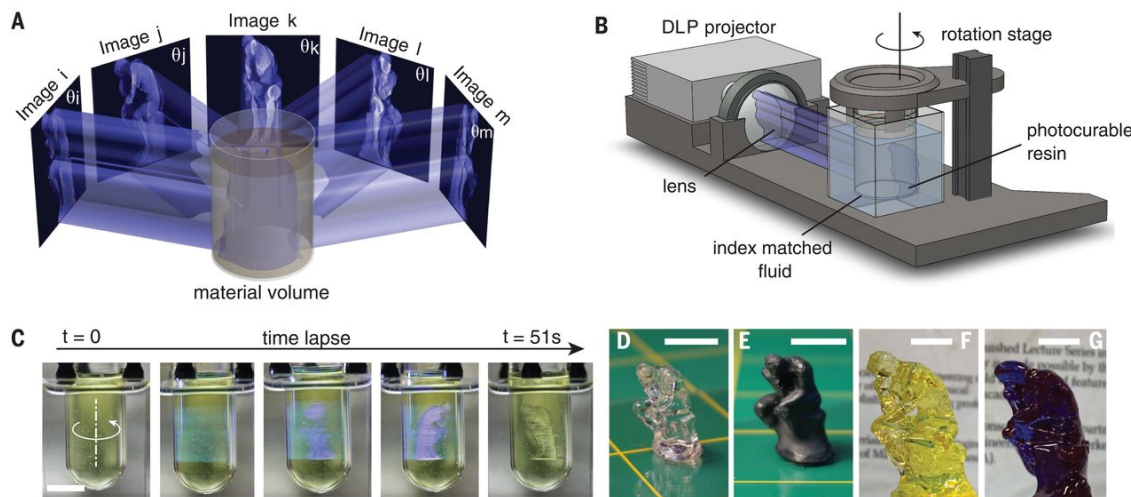


Figure 22. Tomographic volumetric printing. **A.** Patterned exposure from multiple angles delivers a computed 3D light dose to a photocrosslinkable resin. **B.** Schematic depiction of tomographic volumetric printer with DLP projector and rotating resin container. **C.** Time laps of illumination and scaffold materialization during volumetric printing process. **D.** Printed object after removal of the uncured resin and washing. **E.** Printed object after coloring. **F.** Larger object (40 mm height) using the same geometry. **G.** Larger object after coloring. Scale bars represent 10 mm. From Kelly et al., *Science* 2019, 363, 1075. Reprinted with permission from AAAS.^[140]

1.5.3.1. Workflow of the tomographic VP process

Like the workflow described for the DLP printing process in section 1.5.2.1, the tomographic VP process consists of three main steps including (i) setting of the print parameters for the preparation of the light dose distribution, (ii) the printing process itself and (iii) post-processing steps.

Preparation of the light dose distribution. Like in DLP process, the CAD model needs to be transferred into an appropriate light pattern which will be used for illumination of the resin to induce crosslinking. For each object loaded into the printing software, the light dose can be set individually. The light dose needed for successful printing with high

accuracy and without under- or overcuring is usually optimized in an iterative process and found around the light dose needed to induce gelation, the so-called gelation threshold.^[147] The refractive index of the resin is used to account for bending of the light paths that occurs at the air-resin interface according to Snell's law and to correct light ray trajectories for the light dose distribution.^[148] In addition, the absorption coefficient of the resin is used to compensate for light attenuation inside of the resin and lead to a theoretically higher light dose in the center of the printing vial ensuring homogeneous light dose accumulation at the illuminated voxels.^[148] The algorithm to calculate the light dose distribution and projection patterns is based on the Radon transformation.^[148, 149] Similar to the slicing process in DLP-based printing, the 3D model is converted into 2D images resembling the object from different perspectives. A second, inverse Radon transformation is used to calculate the light dose at specific positions from various angles leading to a 3D light dose distribution.^[140] A recent review explains the overall concept of tomographic VP as transferring a 3D model into 2D images, which will be used to reconstruct a final 3D object via the superposition of light patterns (**Figure 23**).^[150] A detailed description of the algorithm calculations is beyond the scope of this thesis.

Printing. The photopolymer resin is filled into cylindrical vials, the volume covering the height of the structure to be printed. After start of the print, rotation of the vial and simultaneous projection of the 2D patterns continues until the set light dose has been reached and the object solidifies.

Post-Processing. After the printing process, the printed object is retrieved from the printing vial and uncured resin is removed by washing. Depending on the resin formulation, uncured material can be re-liquefied through gentle heating. Post-curing steps can be added if needed.

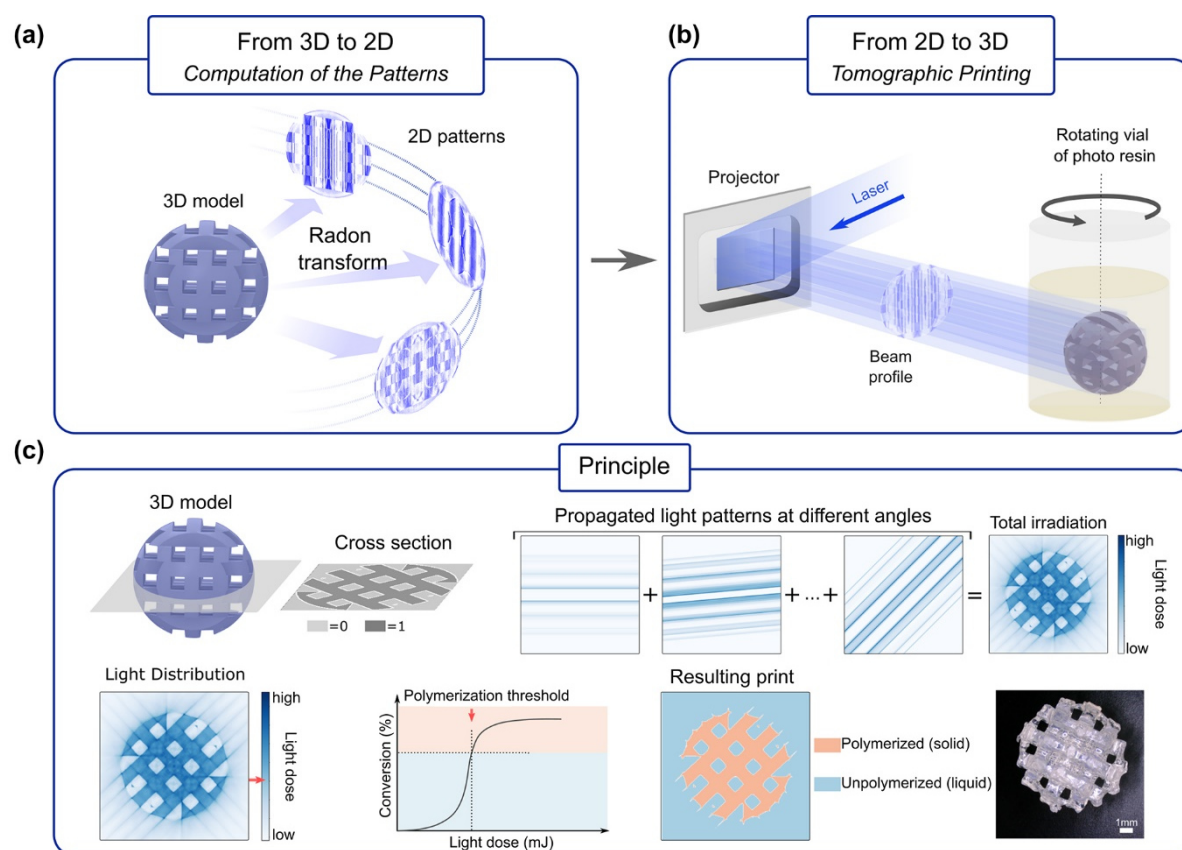


Figure 23. Volumetric 3D printing using tomographic back projection. (a) The 3D model is transformed into a set of 2D tomographic patterns using the Radon transformation. (b) The 2D patterns are back projected into a rotating vial containing the resin to induce polymerization of a 3D object. (c) Principle of tomographic volumetric printing with non-linear threshold behavior of the resin. Cross-section of the 3D model is regenerated in the resin by projection of light patterns from different angles to overpass a polymerization threshold leading to spatial-selective curing at illuminated volumes. Adapted from Dinc et al., 2023, licensed under CC BY 4.0.^[150]

VP is commonly used to print cell-laden hydrogels based on Gel-MA.^[134, 143, 144, 151-155] Thiol-ene based gelatin resins have been reported using NB or AE groups as ene moieties along with DTT, PEG2SH, PEG4SH or Gel-SH as thiolated crosslinkers.^[72, 76, 145, 156, 157] Also other thiol-ene crosslinkable resins based on PVA or collagen have been described.^[85, 87] A few reports have used HA-MA for VP.^[144, 158, 159] To render HA-MA printable, the resin included thermogelating polymers like Pluronic^[158] or gelatin^[144] or used a high MW HA^[159] to increase the viscosity. VP of hydrogels for cell encapsulation will be discussed in more detail in section 1.5.3.3.

1.5.3.2. Resin requirements for tomographic VP

Physicochemical properties of the resin such as viscosity, absorption and reactivity which were critical for the applicability in DLP, also must be considered for optimizing the printability in VP processes.

Viscosity. In contrast to DLP-based printing processes, no repetitive stage-movement is involved in the object fabrication in VP, thereby not relying on low-viscosity resins. A challenge in VP is to avoid part movement or sedimentation during the light exposure.^[149] To prevent sedimentation, high-viscosity resins (>10 Pa s) were until recently considered compulsory for VP.^[72, 76, 77, 144] This is achieved primarily by exploiting the thermoreversible physical network formation in gelatin, which is applied either as modified derivative with photosensitive moieties^[72, 76, 77, 87, 134, 143, 151-154, 156, 157, 160] or as a sacrificial additive^[85, 144, 154]. As alternative, Pluronic has been included for thermoreversible gel formation.^[158] For bioprinting, higher viscosities are beneficial to prevent cell sedimentation during the pre-gelation required to form the physical gel.^[134, 152] Riffe and coworkers explored the printability of resins based on acrylamide/bisacrylamide, HA-MA or PEGDA.^[144] For all three resins, the addition of sacrificial gelatin was required to prevent part sedimentation and loss of resolution (**Figure 24**). More recently, researchers have explored the potential to overcome the gelatin constraint and to expand the resin formulations to print hydrogels using VP. Hall and coworkers were able to print resins with a viscosity of 50 mPa s using a combination of PEGDA (<1 kDa) as photocrosslinkable component and alginate as viscosity modifier.^[161] Successful VP was further demonstrated using a high-viscosity resin based on high molecular weight HA-MA.^[159] Both reports challenge the established paradigm that physically gelled resins are uniquely required and essential for VP of hydrogels.

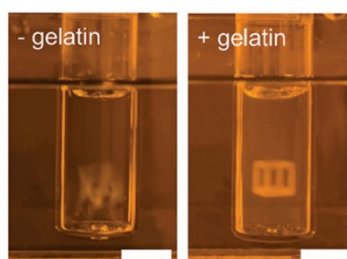


Figure 24. Volumetric printing of a resin containing acrylamide and bisacrylamide with or without the addition of gelatin. The formation of physically crosslinked gelatin hydrogel

was required to render the resin printable. Scale bar represents 8 mm. Adapted with permission from Riffe et al.^[144] Copyright © 2024 Wiley-VCH GmbH.

Absorbance. While DLP benefits from resins with higher absorbance to prevent photocuring in previous layers, resin transparency is an essential feature for VP.^[72, 76] For example, Oh et al. employ 0.5% w/w LAP in DLP printing, but reduce the photoinitiator concentration to 0.025% w/w when the resin was applied in VP.^[162] The resin absorptivity is usually dominated by the photoinitiator concentration.^[72] In VP, light needs to penetrate throughout the resin to induce photoinitiation and lead to crosslinking above the gelation threshold. If the absorbance of the resin is too high, too much light will be absorbed and crosslinking beyond the center of the vial will be inefficient. The decay of light intensity along the volume is explained by the Lambert-Beer law according to the following equation:^[134]

$$I = I_0 e^{-\epsilon_{\lambda} c d}$$

Where I is the light intensity at a specific position, I_0 is the initial light intensity, ϵ_{λ} is the molar extinction coefficient, c is the concentration of photoabsorbing species and d is the distance between I and I_0 . Assuming an LAP concentration ($\epsilon_{405\text{nm}} = 50 \text{ M}^{-1} \text{ cm}^{-1}$ ^[45]) of 0.5% w/v, the light intensity at the end of a 20 mm cuvette will be less than 20% of the initial value (**Figure 25**). In contrast, the light intensity decreases by less than 10% for a resin containing 0.05% w/v LAP. According to Bernal and coworkers, the light intensity should decrease by less than 37% throughout the resin volume.^[134] Reported hydrogel-based resins contain 0.01–0.1% LAP.^[72, 77, 85, 87, 134, 143, 144, 151-154, 156-158, 160]

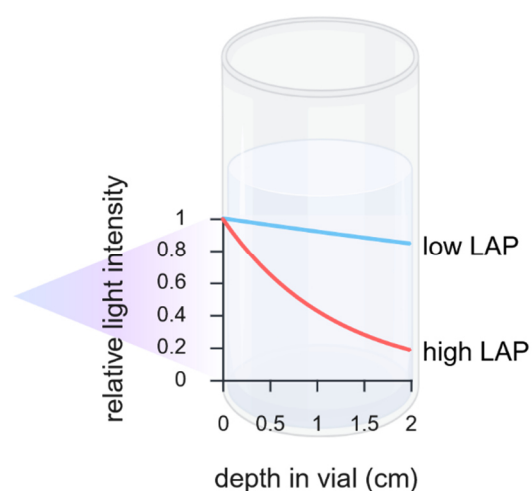


Figure 25. Decrease in relative light intensity through resin-filled vials with low (0.05% w/v) or high (0.5% w/v) LAP photoinitiator concentration according to Lambert-Beer law. *Created with BioRender.com.*

The addition of cells can alter the optical properties of bioresins by additional scattering and impact the light dose and overall printability.^[119, 151] When printing with higher cell densities ($>10^7$ cells mL⁻¹) the addition of iodixanol (20–30% w/w) can reduce scattering and improve the homogeneity of the bioresin.^[87, 151] Instead of optimizing the printing parameters or resin composition, Sgarminato and co-authors developed a numerical correction of the light dose to improve the shape fidelity and print resolution without the addition of contrast agents.^[153]

Reactivity. Like in DLP, a higher LAP or polymer concentration will reduce the gelation threshold of the resin leading to a lower light dose for VP. Gehlen and coworkers systematically evaluated the optimal light dose needed for different Gel-MA and LAP concentrations.^[152] Increasing the LAP concentration from 0.03 to 0.08% w/w led to a decrease of the optimal light dose by 220–250 mJ cm⁻² while increasing the Gel-MA concentration from 2.5 to 10% w/w only decreased the light dose by 100–150 mJ cm⁻². While the high LAP concentration led to a lower cell viability and overcuring of negative features, the low LAP concentration led to blurring and mechanically unstable features. As demonstrated by Ribezzi et al., a higher molecular weight or a higher degree of modification in the studied Gel-MA resin led to a decrease in the printing light dose by 37.5 or 25.5 mJ cm⁻², respectively.^[143] As mentioned before, the radical polymerization of MA groups is inhibited by oxygen. To reduce the dissolved oxygen content in the resin, Hall et al. added sodium sulfite as radical scavenger which reduced the light dose for

polymerization.^[161] Increasing the PEGDA concentration further reduced the gelation point and light dose and decreased the minimum positive feature size.

As mentioned before, resin reactivity is influenced by the crosslinking mechanism. Although publications have reported and emphasized the faster printing and lower light dose needed to print thiol-ene based resins containing Gel-NB^[72, 157], Gel-AE^[77] or PVA-NB^[85] as photocrosslinkable components, only Duquesne et al. directly compare the printability of Gel-NB and Gel-MA resins for VP. The thiol-ene resin required a three-times lower LAP concentration and over 50% lower light dose for printing with high shape fidelity.^[76] Oxygen inhibition and diffusion lead to undercuring in smaller, but overcuring on larger features in Gel-MA resins while the thiol-ene scaffolds were printed more uniformly thereby improving resolution.

1.5.3.3. (Bio)printing using tomographic VP

VP offers several advantages over conventional 3D printing techniques. Cell-laden hydrogel scaffolds can be fabricated with tremendous speeds within 10 to 60 s. This mitigates cellular stress and allows higher throughput of consecutive prints. The layerless 3D printing process allows printing of true free-form 3D geometries with overhangs, internal cavities without the need for support structures.^[150] Especially for bioprinting or tissue engineering, internal channels or networks can be included in the structure which is crucial for vascularization.^[150] The generated surfaces can be expected to be smoother without layering effects. The print time is independent from the object size allowing facile scalability.^[134] The overprinting capacity allows to include pre-defined objects like e.g. melt-electrowritten meshes in the printed structure.^[163] Sequential VP processes further allow for multi-material printing.^[143, 144] In addition, photografting is especially interesting in thiol-ene resins, where post-printing illumination allows the selective functionalization of the printed structure with thiolated dyes, peptides or bioactive molecules.^[85, 156] **Table 1** gives an overview of the state of the art for hydrogels with or without encapsulated cells prepared by tomographic volumetric printing.

Table 1. Reports on tomographic volumetric (bio)printing of hydrogels.

Resin	Photoinitiator	Viscosity	Viscosity modifier	Light dose (mJ cm ⁻²) [print time]	Minimum feature sizes	Bioprinting: Light dose (mJ cm ⁻²) [print time]	Cell type or microorganism	Bioprinting: Minimum feature sizes	Reference
Gel-MA									
2% w/v Gel-MA	0.01% w/v LAP		gelatin						Riffe et al. (2024) ^[144]
2% w/v Gel-MA, 8% w/v NIPAM	0.1% w/v LAP		gelatin		spikes: 59.6 ± 2 µm (pre-shrinking), 45.9 ± 1.1 µm (post-shrinking) holes: 123.7 ± 2.5 µm (pre-shrinking), 39.1 ± 2.3 µm (post-shrinking)				Viola et al. (2024) ^[154]
5% Gel-MA	0.05% LAP		gelatin	350	channels: 0.9-1.2 mm (theoretical size)	[2.5 min]	hMSCs		Gehlen et al. (2023) ^[152]
5% w/v Gel-MA	0.1% Li-TPO-L		gelatin	464	channels: 780 µm (theoretical size) prism: 240.39 µm holes: 198.97 µm	500	human dental pulp-derived stem cells		Duquesne et al. (2025) ^[76]
5% w/v Gel-MA	0.1% LAP	>10 ² Pa s	gelatin	117–154.5			iβ cells		Ribezzi et al. (2025) ^[143]
5% w/v Gel-MA, 10 – 30% iodixanol	0.1% w/v LAP		gelatin		channels: 104.0 ± 5.5 µm spikes: 41.5 ± 2.8 µm	250–375	hepatic single cells, hepatic organoids	s-shaped strut: ~ 500 µm spikes: 49.2 ± 8.4 µm (cells) 50.5 ± 6.0 µm (organoids)	Bernal et al. (2022) ^[151]
5–7% Gel-MA	0.16–0.5 mg mL ⁻¹ LAP		gelatin				human fibroblasts		Sgarminato et al. (2024) ^[153]
6% w/v Gel-MA	0.1% w/v LAP		gelatin	180–215	channels: 500–1000 µm (theoretical size)		MSCs	channel: 1500 µm (theoretical size)	Buchholz et al. (2025) ^[155]
10% w/v Gel-MA	0.037% w/v LAP		gelatin		channels: 200 µm	[12.5 s]	articular cartilage-derived progenitor cells	wall thickness: 144.69 ± 13.55 µm	Bernal et al. (2019) ^[134]

Gel-NB or Gel-AE (thiol-ene)									
2.75 or 6% Gel-AE, PEG4SH or Gel-SH	0.1% LAP		gelatin	220 (low conc.) 320 (high conc.)	s-shaped strut: ~100 μm	420–480 (low conc.) 320–380 (high conc.)	adipose-derived stromal cells		Cianciosi et al. (2023) ^[77]
2.5% w/v Gel-NB, PEG4SH or Gel-SH	0.05% w/v LAP		gelatin	[11–38 s]	hollow cone: 200 μm		mouse myoblasts, normal human dermal fibroblasts		Rizzo et al. (2021) ^[72]
5% w/v Gel-NB, DTT	0.1% w/v LAP		gelatin	120–192	spikes: $23.68 \pm 10.75 \mu\text{m}$ channels: $176.01 \pm 36.34 \mu\text{m}$				Falandt et al. (2023) ^[156]
5% w/v Gel-NB/Gel-SH	0.025% Li-TPO-L		gelatin	137	channels: 780 μm (theoretical size) prism: 157.46 μm holes: 138.83 μm	184.95–205.5	human dental pulp-derived stem cells		Duquesne et al. (2025) ^[76]
10% w/v Gel-NB/ Gel-SH, iodixanol (bioprinting)	0.05% w/v LAP		gelatin	56	pillars: 340 μm , steps: 210 μm , walls: 120 μm , hollow crosses: 120 μm	71	MSCs		Pien et al. (2025) ^[157]
Other proteins (silk, collagen)									
0.3% w/v Collagen-NB, 2.5% Gel-SH, 22% iodixanol	0.05% w/v LAP		gelatin		cone: ~275 μm hollow cone: ~400 μm		murine myoblasts, murine fibroblasts	mesh thickness: ~ 500 μm	Winkelbauer et al. (2025) ^[87]
0.5% w/v Collagen-NB, 0.5% w/v PEG4SH, 1% w/v gelatin, 22% iodixanol	0.05% w/v LAP		gelatin		cone: ~220 μm hollow cone: ~220 μm				Winkelbauer et al. (2025) ^[87]
2% w/v Silk-MA, 8% w/v NIPAM, 1% w/v gelatin	0.1% w/v LAP		gelatin		spikes: $36.7 \pm 1.8 \mu\text{m}$ (pre-shrinking), $27.0 \pm 0.9 \mu\text{m}$ (post-shrinking) holes: $220.3 \pm 2.4 \mu\text{m}$ (pre-shrinking), $71.4 \pm 3.1 \mu\text{m}$ (post-shrinking)				Viola et al. (2024) ^[154]
2.5 or 5% Silk sericin (SS) or silk fibroin (SF)	0.25 mM/ 2.5 mM or 0.5 mM/5 mM Ru/SPS			150–270	threads: 45.9 μm (SS), tubular mesh diameter: 26 μm (SS), 110 μm (SF) array of cubes: 108 μm (SS), 124 μm (SF)	135–171	Myoblasts (C2C12)		Xie et al. (2023) ^[164] *

HA									
2% w/v HA-MA, 2% w/v gelatin	0.035% w/v LAP		gelatin				chondrocytes, mesenchymal fibrochondrocytes, MSCs		Riffe et al. (2024) ^[144]
1% w/w HA-MA	0.1% w/v LAP			625	spikes: ~450 μ m channels: 770 $\pm$ 61 μ m				Iudin et al. (2025) ^[159]
Synthetic polymers									
1.5 or 3% PVA- NB, PEG2SH, 5% sacrificial gelatin	2 mm LAP	>100 Pa s	gelatin	140 (low conc.) 100 (high conc.)	channels: 1.02 $\pm$ 0.14 mm (low conc.), 0.94 $\pm$ 0.02 mm (high conc.)		hMSCs		Qiu et al. (2023) ^[85]
5% v/v PEGDA, 2% w/v gelatin	0.01% w/v LAP		gelatin						Riffe et al. (2024) ^[144]
5-15% w/w PEGDA, 0.5% w/w alginate, 10 mM Na ₂ SO ₃	5 mm LAP	~0.05 Pa s	alginate	150–350	channels (200 μ m theoretical size): 467 $\pm$ 33 μ m (5% PEGDA), 212 $\pm$ 21 μ m (10% PEGDA), 263 $\pm$ 50 μ m (15% PEGDA) spikes: 56 $\pm$ 9 μ m (5% PEGDA), 47 $\pm$ 12 μ m (10% PEGDA), 38 $\pm$ 7 μ m (15% PEGDA)				Hall et al. (2025) ^[161]
20% w/v Acrylamide, 0.5% w/v bisacrylamide, 2% w/v gelatin	0.025% w/v LAP		gelatin						Riffe et al. (2024) ^[144]
20% w/v Pluronic- Dimethacrylate, 1% w/v HA-MA	0.05% w/v LAP	>20 Pa s	Pluronic			120–140	<i>E. Coli</i> , <i>P. kishitanii</i>		Binelli et al. (2023) ^[158]

*All resins were photopolymerized at 400 or 405 nm with the exception of Xie et al., which used a 525 nm light source as indicated.^[164]

Volumetric bioprinting of cell-containing hydrogel scaffolds was first demonstrated by Bernal et al. using 10% w/v Gel-MA ($F_{\text{ene}} = 80\%$) with 0.037% w/v LAP as photoinitiator.^[134] Porous, cylindrical constructs resembling an anatomical trabecular bone model (~3.3 mm diameter, 4.4 mm height) were printed in the presence of mesenchymal stromal cells (MSCs) in 22.7 s with a smallest positive feature of $144.69 \pm 13.55 \mu\text{m}$ which was connecting the pores (**Figure 26A**). In addition, a rectangular scaffold was printed containing perfusable, straight channels with a channel diameter of $200 \mu\text{m}$. This resin was further used to bioprint articular cartilage-derived progenitor cells in meniscus shaped constructs which showed a cell viability $>85\%$ after 7 days.^[134] In a follow-up work, Bernal and co-authors used 5% w/v Gel-MA ($F_{\text{ene}} = 93.5\%$) with 0.1% w/v LAP as bioresin (**Figure 26B**).^[151] Perfusable channels with smallest negative features of $104.0 \pm 5.5 \mu\text{m}$ were printed. Cell-free, star-shaped constructs were printed with a minimal positive feature at the outer tip of the stars with $41.5 \pm 2.8 \mu\text{m}$. The authors printed a disc with 12 mm outer diameter and 8 mm empty core, where the inner part was connected with a curved strut with $500 \mu\text{m}$ thickness. The strut was resolved when printing in the presence of HepG2 cells ($1.0 \times 10^6 \text{ cells mL}^{-1}$) or organoids using a light dose of 312 mJ cm^{-2} . Increasing the cell density ($2.5 \times 10^6 \text{ cells mL}^{-1}$) required a light dose of 375 mJ cm^{-2} . Higher cell densities ($5 \times 10^6 \text{ cells mL}^{-1}$) needed 625 mJ cm^{-2} for printing, but the strut was overpolymerized with a thickness of approx. 8 mm. To improve printing performance in the presence of cells and organoids, they optimized the resin by including iodixanol (10–40%) as refractive index matching compound. With 30% iodixanol, the strut was printable with high cell densities using 375 mJ cm^{-2} for single cells, and 10% iodixanol was included to print organoids using 250 mJ cm^{-2} . The minimum feature size increased to $49.2 \pm 8.4 \mu\text{m}$ for single cells and to $50.5 \pm 6.0 \mu\text{m}$ for printing in the presence of organoids. Gel-MA has also been used as bioresin by others to print bone tissue^[152], a pancreatic model^[153], multicellular constructs^[152] and for multi-material printing where resins were exchanged after printing one structure^[144]. For example, Gehlen et al. used 5% w/v Gel-MA ($F_{\text{ene}} \sim 57\%$) along with LAP (0.05% w/v) as photoinitiator for the encapsulation of hMSC. The optimal light dose for cell-free constructs was 350 mJ cm^{-2} and perfusable channels with 0.9, 1.0 and 1.2 mm theoretical size were resolved precisely.^[152] Bioprinted scaffolds with encapsulated hMSCs showed high cell viability ($>90\%$). The bioresin was further used to prepare a multicellular construct. hMSCs were

encapsulated during the VP, and HUVECs were seeded in the hollow channels. Sgarminato et al. used Gel-MA (5–7% w/v) and LAP (0.16–0.5 mg mL⁻¹) as bioresin to print cylinders (5–6 mm, 5–7 mm height) containing cavities of approx. 1 mm in the presence of human fibroblasts.^[153] The structures required a print time of 2.5 min. Human pancreatic ductal epithelial cells were seeded in the cavities to model a exocrine pancreatic unit.

Volumetric printed hydrogels using the faster, thiol-ene reaction was first reported by Rizzo et al. using 2.5% w/v Gel-NB ($F_{\text{ene}} = 50\%$) crosslinked in the presence of PEG4SH or Gel-SH.^[72] Using 0.05% w/v LAP as photoinitiator, and a print time of 11 – 38 s, scaffolds with 8.5 x 9.3 mm² and a minimal distance between to features without overlap in a hollow cone structure of approx. 200 μm were printed. Encapsulated myoblasts and fibroblasts showed a high cell viability (>90%) for up to 7 days. Another Gel-based thiol-ene resin using 2.75 or 6% w/v Gel-AE was printed using PEG4SH as crosslinker and 0.1% w/v LAP as photoinitiator with a light dose of 320 or 220 mJ cm⁻², respectively, for cylinders (6 mm diameter, 1 mm height).^[77] The addition of cells increased the required light dose to 420–480 mJ cm⁻² for the lower Gel-AE resin, and to 320–380 mJ cm⁻² for the higher Gel-AE concentration. Without embedded cells it was possible to achieve features around 100 μm of a s-shaped filament in a hollow disc (**Figure 26C**). ASCs were successfully encapsulated in discs during the VP process and showed high cell viability (>90%). Further investigation was performed by Pien et al. using a resin composed of 10% w/v Gel-NB ($F_{\text{ene}} \sim 60\%$) and Gel-SH, and 0.05% w/v LAP. At a light dose of 56 mJ cm⁻², a 6.75 x 18.25 x 4.24 mm³ scaffold with pillars of 340 μm , steps of 210 μm , and hollow crosses and walls of 120 μm was printed.^[157] For bioprinting, the Gel-NB resin was supplemented with iodixanol and MSCs were encapsulated in discs (5.28 mm diameter, 0.88 mm height) or a package structure using an optimized light dose of 71 mJ cm⁻². The VP constructs showed enhanced osteogenesis. Falandt et al. used a resin composed of 5% w/v Gel-NB ($F_{\text{ene}} = 80\%$) and a small dithiol crosslinker for VP and selective photografting of fluorescently labeled thiols.^[156] Printing of the resin was characterized by a minimum feature size of $23.68 \pm 10.75 \mu\text{m}$ for positive spikes in a star-shaped structure, and $176.01 \pm 36.34 \mu\text{m}$ for internal channels. Using the photografting approach, it was possible to covalently bind vascular endothelial growth factor and allow selective cell

adhesion of HUVECs to the region of interest. Duquesne et al. compared the printability of Gel-MA ($F_{\text{ene}} = 95\%$) and Gel-NB ($F_{\text{ene}} = 176\%$) resins using Li-TPO-L (0.01–0.1% w/v) as photoinitiator.^[76] The reference light dose was determined by printing a rectangular construct with perfusable channels with 0.78 mm diameter. The light dose for Gel-NB was 50% lower (137 vs. 464 mJ cm⁻²) using a 4-times lower photoinitiator concentration in comparison to a Gel-MA resin and led to a 2-times higher conversion of functional groups after printing and a 3-times higher stiffness. The minimal positive feature was determined as the edge of a prism with 157.46 µm for Gel-NB and 240.39 µm for Gel-MA. A disc containing holes was printed to identify the negative resolution. The diameter of the smallest hole was 138.83 µm for Gel-NB and 198.97 µm for Gel-MA. To print the rectangular scaffold in the presence of cells, the light dose increased by 10–50%, to 500 mJ cm⁻² for Gel-MA and to 184.95–205.5 mJ cm⁻² for the Gel-NB bioresin.

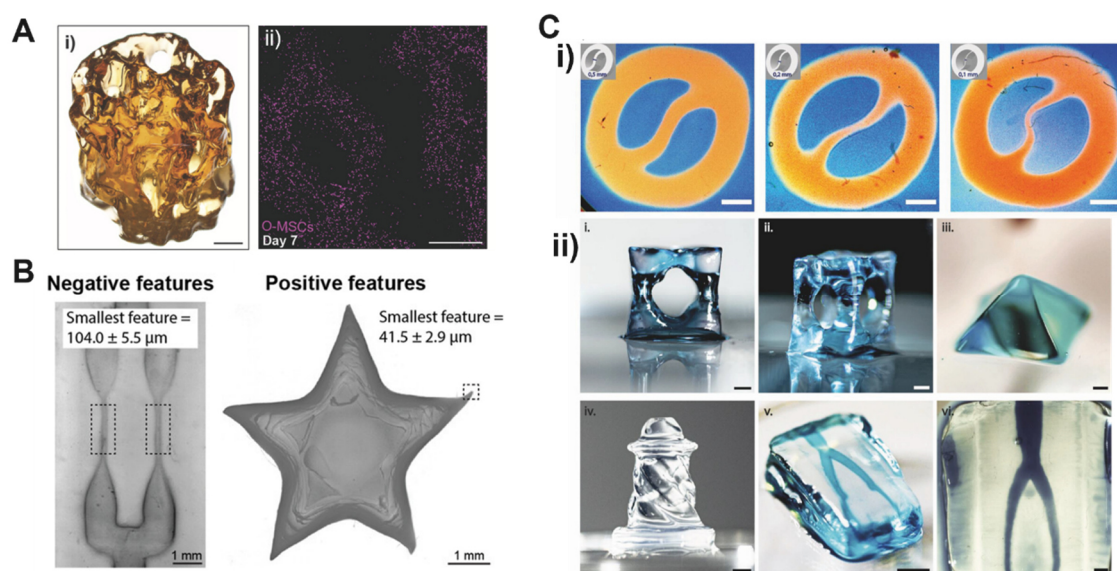


Figure 26. Characterization of print resolution in gelatin-based resins prepared by volumetric (bio)printing. **A.** A porous trabecular bone model was printed in the presence of articular cartilage-derived progenitor cells using Gel-MA. The wall thickness of the connected pores was found to be $144.69 \pm 13.55 \mu\text{m}$. Scale bars represent 2 mm (i) and 1 mm (ii). Reproduced from Bernal et al., 2019, licensed under CC BY-NC-ND 4.0.^[134] **B.** Negative and positive features printed with Gel-MA without cells or organoids. Scale bars represent 1 mm. Adapted from Bernal et al., 2022, licensed under CC BY 4.0.^[151] **C.** Volumetric printed constructs using Gel-AE resin. (i) Printing resolution was characterized using a s-shaped disc scaffold with different inner filament diameters (500, 200, 100 µm). Scale bar represents 1 mm. (ii) Images of different printed structures (hollow cage, pyramid, chess-pawn, and branched geometry). Scale bars represent 1 mm (i, ii, iii, vi) and 5 mm (iv, v). Adapted from Cianciosi et al., 2023, licensed under CC BY-NC 4.0.^[77]

While the previous studies focus on the investigation and demonstration of (bio)resins based on photocrosslinkable gelatin, Qiu et al. developed a bioresin based on 1.5–3% w/v PVA-NB, PEG2SH and 2 mM LAP supplemented with 5% w/v sacrificial gelatin (**Figure 27**).^[85] For the lower PVA-NB concentration, a scaffold containing perfusable channels with a diameter of 1.02 ± 0.14 mm in swollen state were printed using an optimal light dose of $\sim 140 \text{ mJ cm}^{-2}$. The higher PVA-NB concentration reduced the optimal light dose to $\sim 100 \text{ mJ cm}^{-2}$ and led to slightly smaller channels with a diameter of 0.94 ± 0.02 mm. The resin was further used for bioprinting in the presence of hMSCs which showed a high cell viability ($\sim 90\%$). In addition, HUVECs were seeded in hollow channels to prepare a multicellular scaffold.

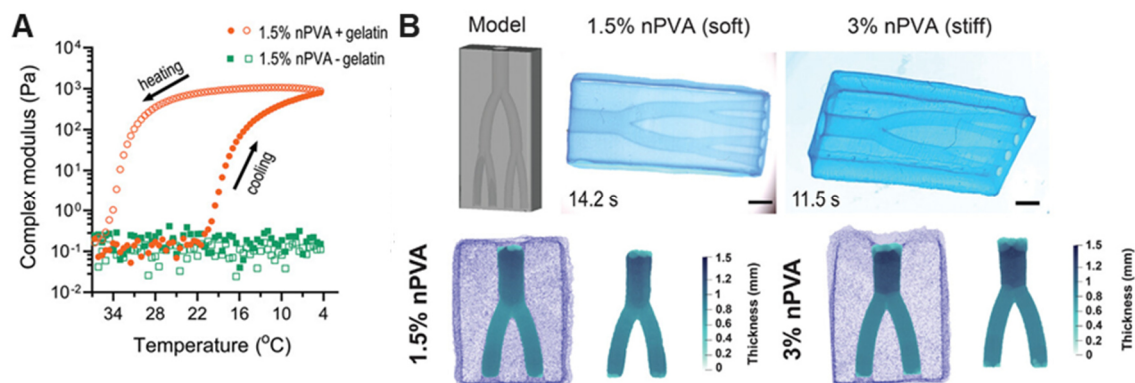


Figure 27. Volumetric Printing of PVA-NB (nPVA). **A.** PVA-NB was supplemented with gelatin to prepare a resin which can undergo thermoresponsive physical gelation. **B.** A branched model was printed using different PVA-NB concentrations. Scale bars represent 2 mm. Adapted from Qiu et al., 2023, licensed under CC BY-NC 4.0.^[85]

So far, little research on HA-based resins for VP can be found in literature. Riffe et al. used 2% w/v HA-MA (70 kDa, $F_{\text{ene}} \sim 35\%$) resin with 2% w/v sacrificial gelatin to print cone-shaped objects (~ 1.4 mm diameter, ~ 1.1 mm height) and discs with a compressive modulus of approx. 1 kPa (**Figure 28A**).^[144] Discs were printed in the presence of bovine musculoskeletal cells which showed a high cell viability after 1 and 7 days. Binelli et al. optimized a 1% w/v HA-MA (1500–2200 kDa, F_{ene} n.d.) resin for the encapsulation of bacteria using VP.^[158] By adding 20% w/v methacrylated Pluronic, a resin with viscosity $>20 \text{ Pa s}$ and hydrogels with a storage modulus of 57 kPa were achieved. Centimeter-scaled scaffolds containing luminescent bacteria into vase-shaped geometries with ~ 12 mm diameter and ~ 6 mm height and an empty inner core of ~ 7 mm diameter and ~ 5 mm height were printed in 10 s (**Figure 28B**). A recent study employed 1% w/w HA-MA

(1500 kDa, $F_{\text{ene}} \sim 23\%$) without viscosity modifiers for VP.^[159] The high viscosity of the resin prevented sedimentation during printing times >79 s. The authors report star-shaped objects with a minimum printable distance of ~ 450 μm and discs with internal channels with a diameter of 770 ± 61 μm (**Figure 28C**). A post-curing step was needed to stabilize the scaffolds.

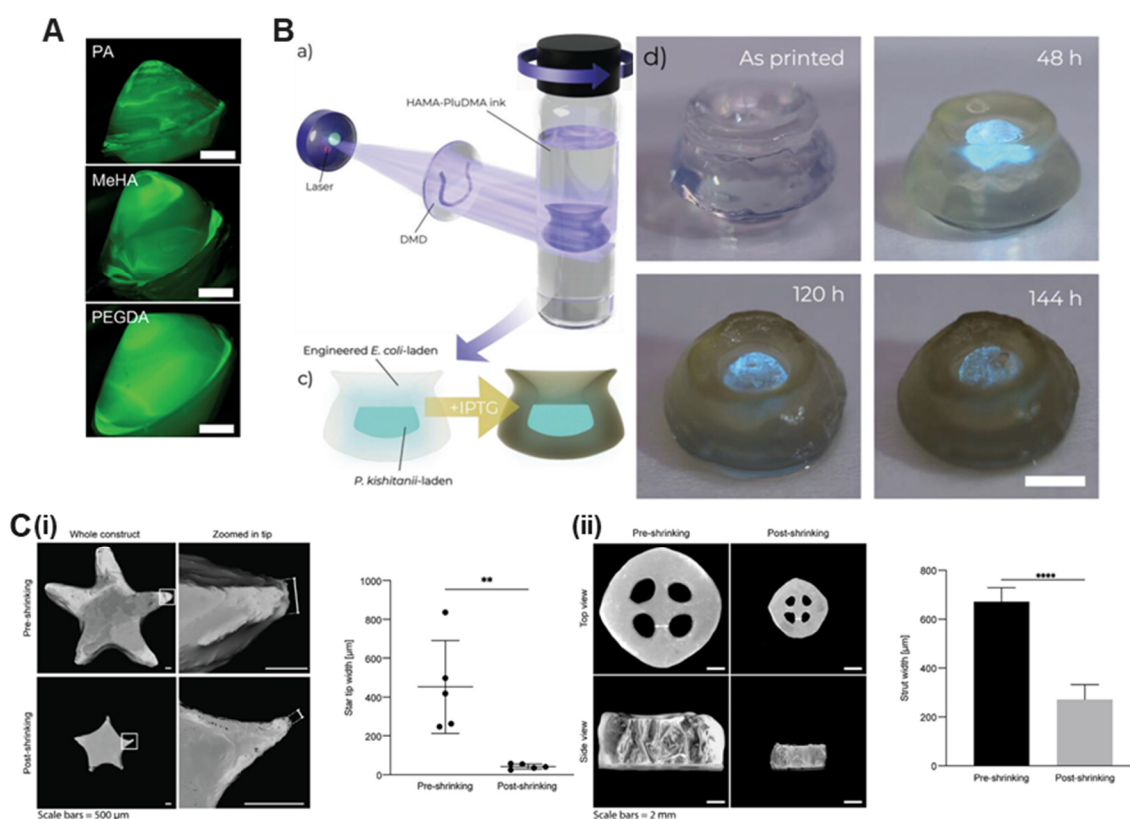


Figure 28. Volumetric Printing of HA-based resins. **A.** Fluorescent images of cone-shaped structures fabricated using polyacrylamide (PA), PEGDA and HA-MA (MeHA) resins by volumetric printing. The resins contained 2% w/v sacrificial gelatin. Scale bar represents 1 mm. Adapted with permission from Riffe et al.^[144] Copyright © 2024 Wiley-VCH GmbH. **B.** A resin composed of HA-MA and Pluronic dimethacrylate was printed in the presence of bacteria into a vase-shaped geometry. The core was filled with genetically programmed bacteria which exhibit a fluorescent signal after induction. Scale bar represents 5 mm. Adapted from Binelli et al., 2023, licensed under CC BY-NC 4.0.^[158] **C.** Characterization of print resolution of a high MW HA-MA resin before and after shrinking. Positive (i) and negative (ii) features were analyzed. Adapted from Iudin et al., 2025, licensed under CC BY 4.0.^[159]

1.6. Light-based fabrication of hydrogel-based contact lenses

Contact lenses (CLs) are hemispherical transparent medical devices that are placed on the tear film of the eye.^[165] Since Leonardo da Vinci first described the concept of a CL for vision correction in 1508, geometries, employed materials and manufacturing methods have undergone continuous development. The vision of CLs is no longer limited to refractive vision error corrections or cosmetic use, as smart CLs with advanced functionalities like sensors, microfluidic channels or with drug-eluting properties have gained increased interest as well as the development of personalized CLs with modular shapes.^[106, 166-173]

In the following section, common materials and fabrication methods for CLs will be described and printing will be introduced as alternative CL fabrication approach. Reports on DLP printing of CLs will be presented. As the state of the art for tomographic volumetric printing of CLs is very limited, literature demonstrating fabrication of optical elements using volumetric printing technologies will be reviewed.

1.6.1. Common materials for CL fabrication

CLs can be classified as soft or hard based on the materials employed in their fabrication. Hard CLs are rigid lenses fabricated from copolymers containing polymethylmethacrylate, fluoro silicones or siloxane acrylates along with some hydrophilic monomers.^[12] Their prevalence is low due a typically lower wearing comfort and patient acceptance than soft CLs.^[12, 165] These are based on hydrogels materials and show higher flexibility and comfort. Soft CLs can be classified by the CL industry based on their chemical nature. Silicon hydrogel CLs are the most applied ones.^[169] They consist of crosslinked hydrophobic silicone monomers like 3-[tris(trimethylsiloxy)silyl]propyl methacrylate copolymerized with more hydrophilic monomers.^[12, 169] Another class are CLs based on hydroxy ethyl methacrylate (HEMA) copolymerized with monomers such as ethylene glycol dimethacrylate, methacrylic acid or *N*-vinyl pyrrolidone.^[169] Soft CLs can also be based on PVA hydrogels.^[12] While silicone and HEMA-based soft CLs are typically manufactured as solids by thermoinitiated radical polymerization of (meth)acrylated precursors in a disposable mold followed by a swelling step, PVA hydrogels are manufactured in the final, swollen CL geometry by photo-initiated polymerization of

water-based (meth)acrylated PVA precursor solutions using reusable, transparent molds (LightStream® process).

1.6.2. Conventional CL manufacturing methods

The industrial manufacturing of CLs uses three main technologies: cast molding, spin casting and lathe cutting. Cast molding and spin casting allow large scale manufacturing and are applied to off-the-shelf CLs. Lathe cutting is applied to personalized CLs for patients with special cornea pathologies or needs.

In cast molding (**Figure 29A**), the crosslinkable precursor solution is processed by compression molding using a two-part (female and male) mold with hemispherical geometry and crosslinked by thermal or photo curing. In spin casting (**Figure 29B**), the liquid polymer solution is spin-coated in a hemispherical mold and the geometry is fixed by photocuring. Lathe cutting is a subtractive process where the final CL geometry is cut out of a solid material block (**Figure 29C**). Lathe cutting is a time-intensive manufacturing process, but it offers the capability to adapt the CLs for personalized and patient-specific geometries.

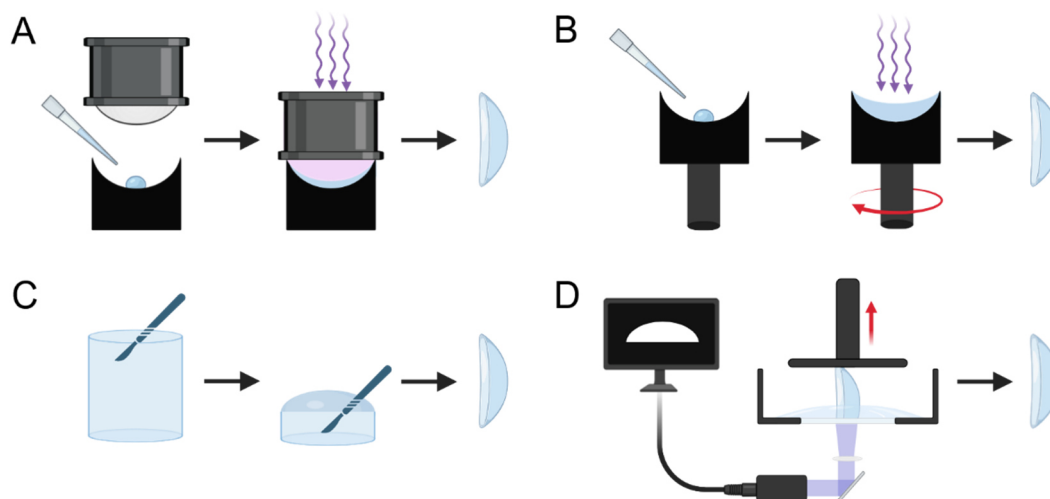


Figure 29. Technologies to fabricate contact lenses. **A.** Cast molding: The liquid prepolymer solution is placed in a curved mold. A second mold is placed to shape the material during crosslinking by light or temperature. **B.** Spin casting: The liquid precursor solution is placed in a curved mold which is rotated to shape the material during photocrosslinking. **C.** Lathe cutting: The CL is cut out of a solid polymer block. **D.** 3D printing: A digital model of the CL is transferred to a 3D printer where the CL is fabricated out of a photopolymerizable resin by spatially controlled illumination. Redesigned from Alam et al. (2021).^[165] Created with BioRender.com.

1.6.3. 3D printing as alternative for the fabrication of CLs

3D printing offers an interesting alternative to lathe cutting for the production of CLs with customized geometries (**Figure 29D**). As 3D printing relies on digital models and the printed structures are fabricated without additional tools out of the (photo)crosslinkable polymer solution, the design flexibility is high. A main challenge of 3D printing for CL fabrication is the optical surface quality, i.e. the need for surfaces with very low roughness.

A few literature reports demonstrated the feasibility of 3D printing CLs. One of the first 3D printed CLs was reported in 2021 by Alam and co-authors.^[174] Using a commercial acrylate-based resin (Asiga DentaClear) and DLP, CLs in a commercial-like geometry were fabricated. To demonstrate the possibility of integrating advanced functionalities, the CAD models were modified to print CLs with microchannels at the edges which are suitable for sensing applications. Alternatively, various colored dyes were included in the liquid resin to obtain tinted CLs. Using DLP further allowed the fabrication of multiple constructs on one build plate. To minimize defects introduced by the build platform, scaffolds were printed with support structures or on a hydrophobic plastic film. The layer-by-layer fabrication of the DLP-based printing technology created several challenges. The 35 μm thick layers were visible on the surface of printed CLs in scanning electron micrographs (**Figure 30A**). This so-called “staircase effect” arises from the slicing of curved structures. Additional coating and curing with resin of the printed structures as post-processing smoothed the surface and reduced roughness. The mechanical properties of swollen structures decreased which was associated with the weak interfacial adhesion of the layers. Hisham et al. employed DLP to print a soft, HEMA/PEGDA-based CL.^[113] Exchanging material in the vat in between different layers allowed the introduction of multi-material elements (**Figure 30B**). The developed CAD model for the CL had a diameter of 14 mm, a height of 2.5 mm, a thickness of 200 μm and was printed with a layer thickness of 35 μm . The multi-material printing procedure was optimized to maintain sharp material interfaces and good interfacial adhesion between consecutive layers and materials. The observed staircase effect was reduced by post-processing steps. The incorporation of different dyes in spatially defined areas

modulated the absorption spectra of the CLs showing promising application as multiband optical filters for color blindness correction.

Kothari and co-authors used extrusion to print drug-eluting CL-like patches with a diameter of 15 mm, a height of 4 mm and a layer or filament thickness of 300 μm .^[175] The resin contained 15% w/v PVA-MA (180 kDa, $F_{\text{ene}} = 98.9\%$) and 1–2 % w/v HA-MA (47 kDa, $F_{\text{ene}} = 98.5\%$) along with 2% w/v Irgacure 2959 as photoinitiator. After extrusion on a cooled printing bed, the structure was post-cured by UV illumination and freeze thawed. For the drug-eluting patches, tobramycin was included in the resin. The CL patches were highly transparent and flexible. The drug-release was confirmed over 24 h and antibacterial properties were demonstrated in vitro and in vivo.

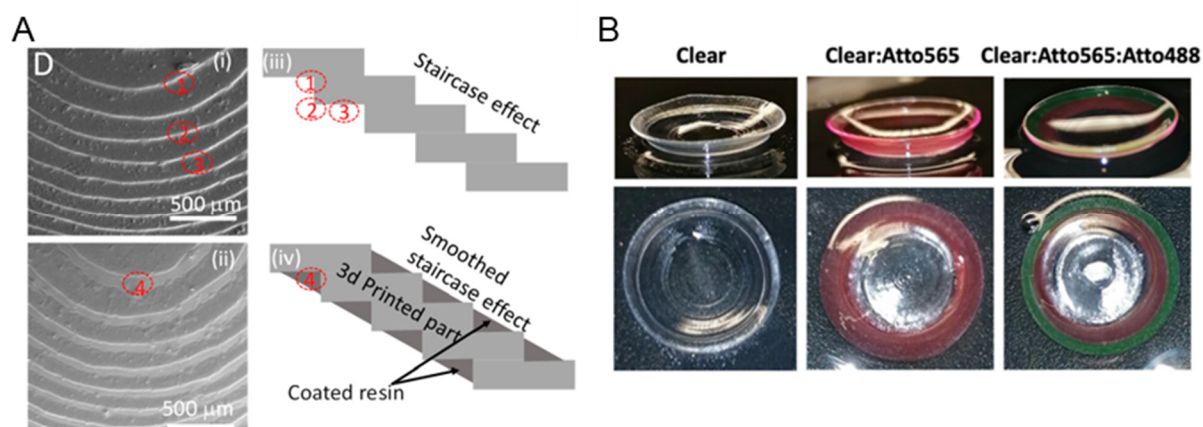


Figure 30. 3D printing of contact lenses. **A.** SEM images and observed staircase effect in DLP printed CLs due to layer-by-layer fabrication. Additional coating of the printed structure smoothens the surface. Adapted from Alam et al. (2021), licensed under CC BY 4.0.^[174] **B.** Multi-material CLs for multiband color blindness correction printed by DLP. Adapted from Hisham et al. (2023), licensed under CC BY 4.0.^[113]

Besides extrusion and DLP printing technologies which rely on point-by-point or layer-by-layer scaffold fabrication (section 1.5.1 and 1.5.2), layerless volumetric printing technologies have been investigated for the fabrication of optical elements. Using 2PP along with a commercial 2PP compatible acrylate-based resin, Gissibl and co-authors printed rigid singlet, doublet, triplet and multi-lens systems with a diameter of $\sim 120 \mu\text{m}$ and 120–200 μm height suitable for micro- and nano-optics.^[176] The precision of this VP technology fabrication yielded sub micrometer accuracy with surface roughness of $<15 \text{ nm}$ as characterized by atomic force microscopy. The overprinting capacity was applied to directly print the lenses on the end of optical fiber tips which would be suitable

for endoscopes or other fiber-imaging systems. Although 2PP does not require support structures or layers allowing the fabrication of complex free-form optics, the scaffold is printed point-by-point and voxels are cured consecutively which can result in distinct steps. Improvement of the surface quality of lenses printed with 2PP was carried out by Siegle and co-authors to obtain step-free optical elements.^[177] With optimized grayscale mapping of the laser power, aspherical singlet (100–150 μm diameter) and air-spaced doublet (300 μm diameter) lenses with approx. 70–200 μm heights were printed with a surface roughness of 4–15 nm. The rigid micro-optics were printed out of a commercial acrylate base resin suitable for 2PP. The lens designs and grayscale mapping were optimized in an iterative process to minimize form errors to <100 nm. Up to today, 2PP technologies give the highest resolution of structures printed using VP.^[178] However, the serial writing process prolongs printing times and limits throughput. Siegle et al. stated print times of 7–15 min for singlet or doublet lenses.^[177] The scalability is further compromised by the working distance of the applied lasers usually limiting the object sizes to the mm-scale.^[178]

In tomographic VP, the printed object materialized simultaneously after a light dose threshold is exceeded resulting in short print times. Toombs et al. built a microscale computed axial lithography printer using tomographic image projection for crosslinking.^[179] Printing of a photopolymerizable silica glass nanocomposite resin in a layer-free manner enabled the fabrication of different aspherical and spherical lenses (1.6 mm diameter) as well as micro lens arrays after binding and sintering. As the silica nanoparticles in the resin led to increased scattering of the resin, addition of (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) could significantly improve the printing resolution by reducing out-of-voxel curing. The silica glass resin was printable with minimum positive feature size of 50 μm . The shape deviation of the lenses varied between 10–60 μm and exhibited a surface roughness of approx. 6 nm after sintering. The printing time for the silica glass objects was 30–90 s. Despite tomographic VP being a layer- and step-free process, the writing beam produces striations as artifacts in the printed scaffolds. Webber et al. introduced blurred tomography to smooth the surface of printed micro-optics.^[180] The employed high-viscosity photoresin was based on diurethane dimethacrylate and PEGDA. Singlet plano-convex lenses with 3 mm diameter and

1.5 mm height as well as microlens arrays were printed with a surface roughness of 0.53 nm and surface deviation of 1.4 μm . The tomographic projections and light dose distribution was optimized in an iterative process to minimize the dimensional error of the printed structures. The overprinting capacity of the VP methodology was used to print ball lenses onto an optical fiber. All printed objects were fabricated in <1 min. Notably, the optical blurring of the laser beam led to a loss of spatial resolution in comparison to conventional tomographic VP. The minimum theoretically achievable resolution with this setup was 172.8 μm . By measuring the refractive index as indication for degree of polymerization along the printed parts, the authors identified the degree of homogenization. The found form errors (1–10%) were attributed to deviations in the optical path lengths during printing as well as shrinkage-induced local changes.

So far, VP has not been demonstrated for fabrication of soft CL. Existing reports are largely restricted to micro-optical elements or solid lens-like structures using non-hydrogel photopolymer resins. The VP methodologies employed allowed printing of optical elements with high surface quality and low form error, it remains unclear the absence of soft CL demonstrations primarily reflects (i) material limitations (lack of VP-compatible hydrogel or silicone hydrogel resins) or (ii) process constraints which compromise printability of thin, curved shell geometries as they are especially sensitive to projection blur or scattering. This work explores tomographic VP methodology for the fabrication of soft CL using PVA-based resins.

2. Comparison of Thiol-ene and Methacrylate Photocrosslinkable Hyaluronic Acid Hydrogel Resins for Digital Light Processing

This chapter compares the performance of HA-based hydrogels with different photocrosslinking chemistries for DLP. It describes the syntheses of HA functionalized with norbornene, allyl ether and methacrylate groups at comparable degrees of alkene functionalization. Based on rheological studies, it quantifies the differences in kinetics and control of the photocrosslinking process between thiol-ene and methacrylated crosslinked resins. Finally, it evaluates the printability of the resins using DLP.

This chapter is based on the following publication and includes reused and adapted text and figures:

Steudter, T.; Lam, T.; Pirmahboub, H.; Stoppel, C.; Kloke, L.; Pearson, S.; del Campo, A.: *A comparative Study between Thiol-Ene and Acrylate Photocrosslinkable Hyaluronic Acid Hydrogel Inks for Digital Light Processing. Macromolecular Bioscience* (Wiley-VCH), 2025, 25, e2400535. <https://doi.org/10.1002/mabi.202400535>

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The included DLP experiments in this chapter were performed during several research stays at the company Cellbricks in Berlin together with Dr. Tobias Lam, Dr. Hamidreza Pirmahboub and Christian Stoppel who are (or were) employees of the company. Some of the final prints depicted in **Figure 41** and **Figure 42** were performed after those stays by Christian Stoppel and Dr. Tobias Lam.

2.1. Introduction

Digital light processing (DLP) of photocrosslinkable resins and hydrogels typically uses (meth)acrylated formulations crosslinked via the chain-growth process of photo-initiated radical polymerization (**Figure 31A**). A limitation of radical chain-growth polymerization for DLP printing is its oxygen sensitivity.^[49, 50] To counteract oxygen inhibition, higher photoinitiator concentrations and higher light doses are typically applied, which can lead to phototoxicity in bioprinting contexts.^[1] Photocrosslinkable precursors based on radical thiol-ene photocrosslinking are interesting alternatives to radical (meth)acrylate polymers, as described in Chapter 1, Section 1.2. The radical thiol-ene reaction involves the addition of a thiyl radical to a vinyl group to form a thioether (**Figure 31B**). The reaction proceeds via a step-growth mechanism and the resulting networks show higher homogeneity than (meth)acrylate networks crosslinked by radical polymerization (**Figure 1C**).^[3, 49, 52] The radical thiol-ene reaction is not sensitive to oxygen as the generated peroxy radical readily abstracts a hydrogen atom from a thiol which can then proceed in the thiol-ene reaction. Moreover, the kinetics of the addition of the thiyl radical can be tuned by the chemical design of the ene: faster with electron-rich or strained vinyl groups like vinyl ether, AE or NB groups and slower for electron-poor vinyl groups or vinyl groups where the intermediate radical is stabilized as in MA.^[49, 52] The thiol-ene reaction offers higher conversions than the radical polymerization of (meth)acrylates^[62-64], leading to lower generation of reactive oxygen species and higher cell viability in bioprinting applications.^[65-67]

As presented in section introduction 1.2.1.3 a few reports have compared the photocrosslinking kinetics and properties of thiol-ene vs MA hydrogel systems. The introduction of this chapter elaborates differences between thiol-ene and MA hydrogel-based resins studied for different 3D printing technologies including printability, printing parameters (i.e. print time, irradiance) and resolution which were reported in literature. In addition, reports of HA-based resins are presented.

Apart from photorheology and analysis of the physicochemical properties, Van Hoorick et al. compared the processability of 5 or 10% w/v Gel-NB and Gel-MA hydrogels using 2PP.^[64] Printing of stable hydrogel structures required a 3-fold lower laser power for Gel-

NB than for Gel-MA at the same concentration upon 2PP excitation. With Gel-NB, scaffolds of $200 \times 200 \times 200 \mu\text{m}^3$ with pores of $30 \mu\text{m}$ could be laser written. In a follow-up study, the required laser power to obtain reproducible structures using Gel-NB (10% w/v) was 20-fold lower than for Gel-MA using 2PP processing.^[59] Self-supportive scaffolds with a minimum feature size of $50 \mu\text{m}$ were fabricated using Gel-NB, while Gel-MA scaffolds written at the same writing speed and laser power collapsed. Using DLP photomask projection, Ortiz-Cardenas et al. compared a Gel-SH/PEG-NB-based resin with Gel-MA.^[125] The Gel-MA required a 2.6-times longer exposure time and led to structural deviations of 13-32% while Gel-NB was printable with feature sizes down to $200 \mu\text{m}$ with <10% deviation from the theoretical structure. In a different study, the printability of NB or MA-functionalized gelatin was studied using a volumetric printer.^[76] Printing of Gel-NB was achieved using a 4-times lower photoinitiator and 2-times lower light dose in comparison to Gel-MA. The authors further report a positive resolution of $157.46 \mu\text{m}$ for Gel-NB and $240.39 \mu\text{m}$ for Gel-MA. A negative resolution of $138.83 \mu\text{m}$ was achieved printing Gel-NB and $198.97 \mu\text{m}$ for Gel-MA. Qui et al. used 1.5–3% w/v PVA-NB to print structures with a negative resolution down to $900 \mu\text{m}$ by volumetric printing using PEG2SH as crosslinker.^[85] The PVA-NB precursor solution was compared to 10% w/v Gel-MA regarding their physicochemical properties and their printability. To print structures of PVA-NB, an approx. 2-fold lower light dose was required which reduced the printing time from 20.0 to 11.5–14.2 s. Thiol-ene and methacrylated gelatin hydrogels have also been tested in extrusion printing (see section introduction 1.5.1) but no concrete improved printability except for a broader printable window was reported for Gel-NB vs Gel-MA.^[65, 66, 73]

HA is a non-sulphated glycosaminoglycan present in the extracellular matrix and widely used in biomedical applications. It can be chemically functionalized at the carboxylic acid and hydroxyl groups on the disaccharide units with photocrosslinkable groups for hydrogel formation.^[33, 89, 181] HA of different molecular weights is commercially available and solutions with different viscosities can be formulated by adjusting the molecular weight and the polymer concentration. This makes photocrosslinkable HA solutions suitable for 3D printing techniques requiring both high-viscosity resins like extrusion printing and low-viscosity resins like DLP printing and is interesting as alternative to

conventionally used gelatin-based resins.^[120] Successful DLP printing has been reported using 88 kDa HA-NB at polymer concentration of 5% w/v,^[84] and 1000 kDa HA-MA at a concentration of 1–2% w/v^[99]. Higher viscosity can be problematic for DLP printing by restricting flow away from the cured layers and solution refill in the resin reservoir when the printing platform is raised.^[120] Two reports have demonstrated the applicability of the thiol-ene resin HA-NB for DLP-based (bio)printing. Dhand et al. printed 20 layer scaffolds with a layer thickness of 100 μm using 2.5 s exposure time per layer achieving a xy resolution of approx. 300 μm .^[83] Using the same scaffold height and layer thickness, the HA-NB resin developed by Galarraga et al. required a exposure time per layer of 6 s rendering stable structures with approx. 500 μm xy resolution.^[84] Although both studies provide insights on thiol-ene HA resins and hydrogels for DLP, no comparison to HA-MA is pursued to quantify the advantages.

In this work, the precursor stability and reaction kinetics of thiol-ene photocrosslinkable HA functionalized with NB or AE groups and radical photocrosslinkable HA-MA precursors with similar functionalization degree F_{ene} are compared. The advantages that can be expected from thiol-ene-functionalized HA precursors and corresponding thiol-ene HA-based photocurable hydrogels are quantified and show the resolution that can be achieved in DLP with the HA-NB resin.

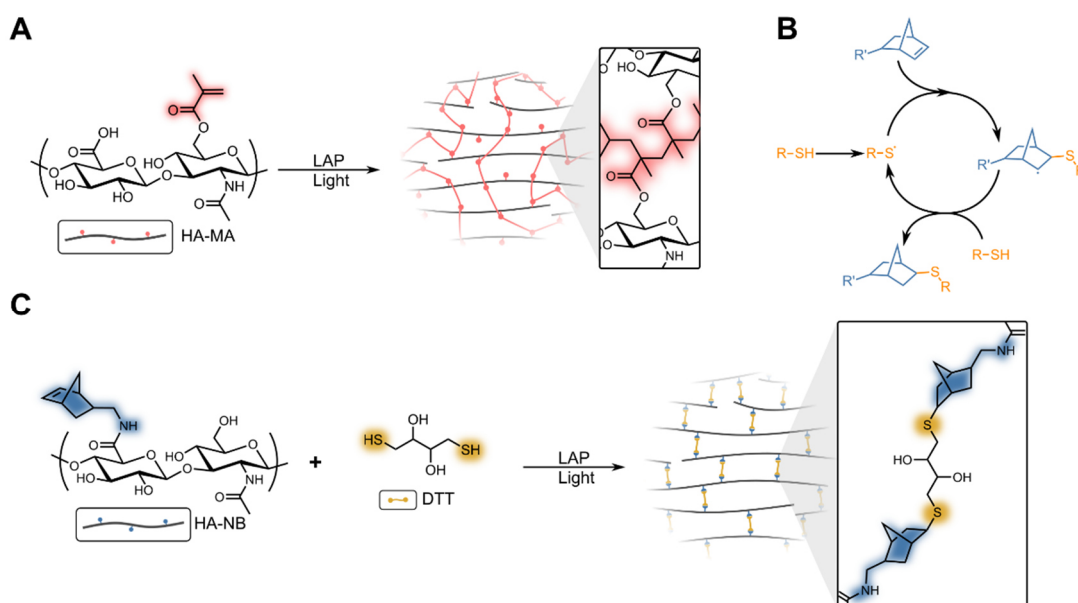


Figure 31. A. Schematic representation of thiol-ene hydrogel formation: upon irradiation with light and in the presence of photoinitiator, the dithiol crosslinker DTT reacts with the pendant NB groups on HA to create a crosslinked hydrogel network containing thioethers.

B. Step-growth mechanism of the radical thiol-ene reaction. **C.** Schematic representation of hydrogel formation with MA groups: crosslinking of MA groups proceeds via radical chain-growth mechanism leading to multiple crosslinks per group and heterogeneous networks. Reproduced from Ref.^[182]. © 2024 The Author(s). *Macromolecular Bioscience* published by Wiley-VCH GmbH. All rights reserved.

2.2. Results

2.2.1. Formulation of HA-based photocrosslinkable solutions

For this study, HA with a molecular weight of 40–50 kDa (as stated by the manufacturer) and a polymer concentration of 4% w/v was selected. This composition is in the range of HA-based resins relevant for bioprinting.^[22, 83, 84, 183] The molecular weight and concentration are lower than those used for DLP bioprinting in other reports.^[84] Therefore, viscosity in the solutions was expected to be low enough for DLP.

The reactivity of enes in the radical thiol addition follows the sequence NB > VE > alkene > AE > acrylate > maleimide > MA.^[52] This trend largely corresponds to decreasing electron density, with NB particularly reactive due to the release of ring strain and MA particularly unreactive due to intermediate radical stabilization.^[52] For the experiments in this chapter the NB and AE groups as enes with high and intermediate reactivity were selected. In contrast to (meth)acrylate groups, NB and AE groups cannot undergo thiol-Michael addition which ensures that crosslinking proceeds only in the presence of radicals to give high temporal control over the network formation in photo-initiated systems. It also prevents side reactions with other nucleophiles e.g. on the cell surface leading to higher cell viability.^[22, 60, 66, 184, 185] In addition, NB and AE groups are not prone to autopolymerization^[55, 56] which should increase the polymers' storage stability.

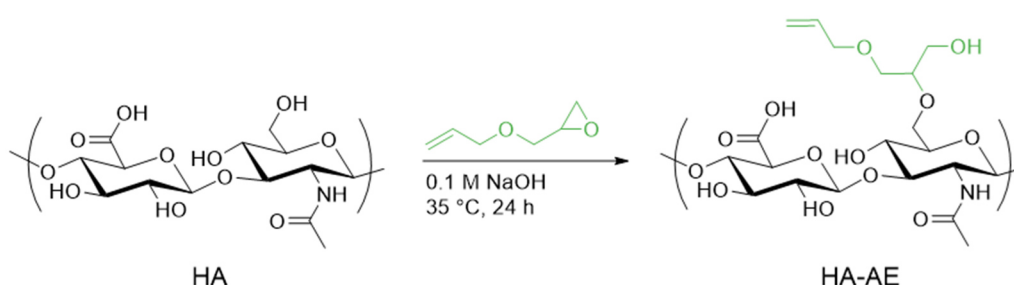
In the following, the HA derivatives will be named HA-X where X stands for the pendant, crosslinkable functional group NB, AE or MA. This work aimed for HA-X with a number of functional groups per HA disaccharide repeating unit, F_{ene} , of 30–50% to obtain stable hydrogel networks. To obtain hydrolytically stable hydrogels, the alkene functionalities were introduced onto the HA backbone via an amide in HA-NB and an ether bond in HA-AE. These linking groups are expected to have higher hydrolytic stability than the ester

group in HA-MA so that degradation in the HA hydrogel in a cell culture scenario could only occur by the addition of hyaluronidases.

2.2.2. Synthesis of photocrosslinkable HA-X

2.2.2.1. Synthesis and chemical characterization of HA-X

The synthesis pathways to obtain HA-NB and HA-AE were refined from reported one-step processes in non-toxic solvents.^[29, 186] To synthesize HA-AE, the primary alcohol group on the C-6 of the *N*-acetyl-D-glucosamine unit in HA was deprotonated in basic medium and reacted with allyl glycidyl ether through a nucleophilic ring-opening reaction (**Scheme 1**).^[186] The functionalized polymer was purified by precipitation and dialysis and lyophilized. The derivatization of the HA was corroborated by ¹H NMR spectroscopy (**Figure 32**). The vinyl protons of AE appeared between 5.15 and 5.36 ppm and confirmed the presence of the allyl group. The F_{ene} was determined by the integral ratio of the vinyl protons relative to the acetyl group of HA ($\delta = 2.03$ ppm) to give $F_{\text{ene}} = 0.42 \pm 0.03$. Gel permeation chromatography (GPC) analysis of HA-AE showed no significant differences between the elugrams of HA and HA-AE, indicating that no degradation or crosslinking of the polymeric chains occurred during the synthesis and purification steps (**Figure 33A**). By decreasing the equivalents of allyl glycidyl ether in the reaction mixture from 3 to 1.5, HA with a F_{ene} of 0.2 was obtained. HA-AE polymers were soluble in water at tested concentrations up to 5% w/v. For the following studies, HA-AE with $F_{\text{ene}} = 0.40$ – 0.47 were used.



Scheme 1. Synthesis of HA-AE through nucleophilic ring-opening reaction of allyl glycidyl ether under basic conditions.

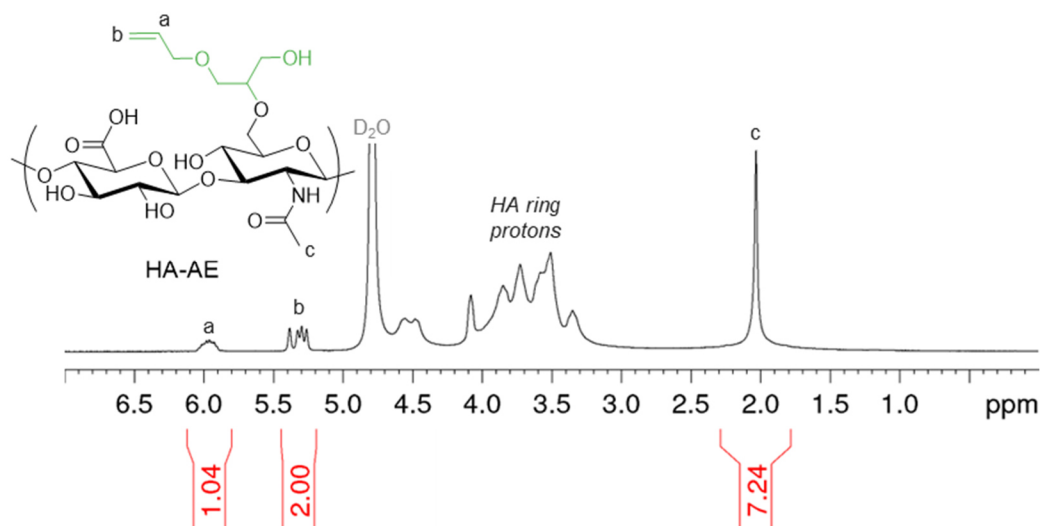


Figure 32. ^1H NMR spectrum of HA-AE in D_2O . The integral of vinyl protons signal ‘b’ from the allyl ether group (5.38 – 5.26 ppm) was set as reference to $I_b = 2.00$ which corresponds to $n = I_b / 2 = 2.00 / 2 = 1.00$ allyl ether groups. The integral protons ‘c’ from the acetyl group in *N*-acetyl-D-glucosamine (2.03 ppm) were used to calculate the number of HA repeating units m , i.e. $m = I_c / 3 = 7.27 / 3 = 2.42$. The F_{ene} was then calculated by calculating the ratio between allyl ether groups n and HA repeating units m , i.e. $F_{\text{ene}} = n / m = 1.00 / 2.42 = 0.41$.

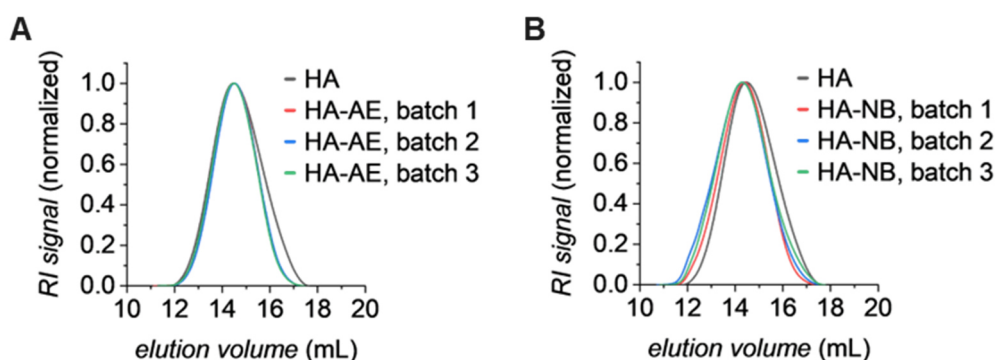
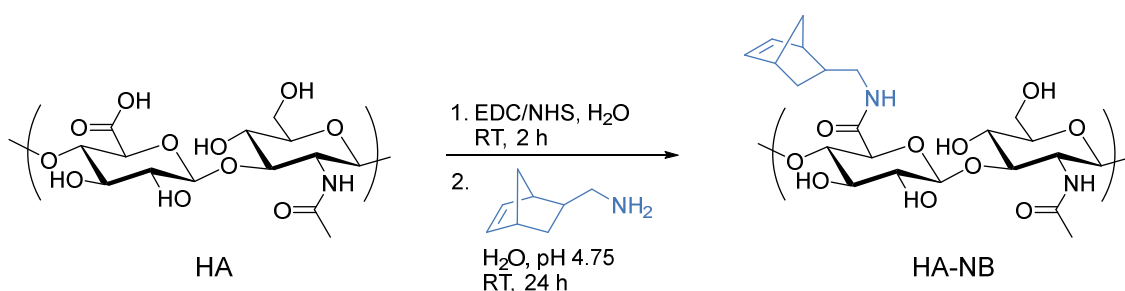


Figure 33. Representative GPC elugrams of different HA-AE (A) and HA-NB (B) batches after purification and lyophilization.

HA-NB was prepared by activating the carboxylic acid group of HA with EDC and NHS in H_2O followed by reaction with 5-norbornene-2-methylamine in one step at $\text{pH} = 4.75$. For purification of batches > 1 g, an intermediate precipitation step in ethanol was added to remove the excess EDC and reduce dialysis time. For the precipitation in ethanol, the pH and salt concentration in the reaction mixture were adjusted to reported concentrations used for the purification of native HA out of broth ($\text{pH} = 7.0$, 2M NaCl).^[187] This step allowed precipitation of the HA even from solutions at concentrations below 1% w/v, thereby decreasing the dialysis time. The appearance of three peaks in the ^1H -NMR spectrum between 5.88 and 6.28 ppm confirmed the successful coupling of NB to the

polymer backbone (**Figure 34**). F_{ene} was calculated from the integration of the vinyl protons of NB relative to the acetyl protons of HA to be $F_{\text{ene}} = 0.41 \pm 0.03$. HA-NB with a lower F_{ene} of 0.12 ± 0.03 could be obtained using lower EDC and 5-norbornene-2-methylamine concentrations in the reaction mixture. The GPC traces of the HA-NB polymer was similar to that of the original HA, indicating that the reaction and purification conditions did not lead to coupling or degradation of the HA chains (**Figure 33B**). For further experiments, HA-NB with $F_{\text{ene}} = 0.36\text{--}0.42$ were used. The polymer was soluble in water at all used concentrations.



Scheme 2. Synthesis of HA-NB with EDC/NHS coupling and 5-norbornene-2-methylamine.

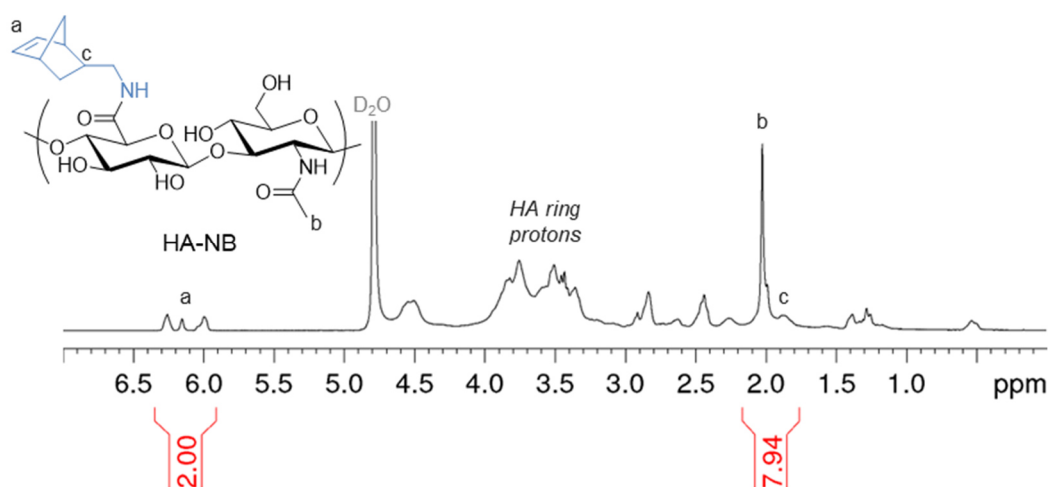
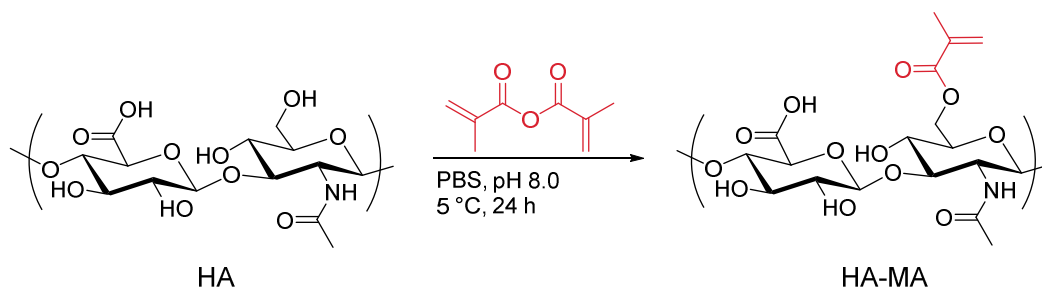


Figure 34. ^1H NMR spectrum of HA-NB in D_2O . The vinyl protons ‘a’ from the norbornene group (6.26 – 6.00 ppm) were set as reference to $I_a = 2.00$ meaning that the number of norbornene groups was $n = 2.00 / 2 = 1.00$. The number of HA protons was determined by integrating the signal of the acetyl protons ‘b’ and the quaternary proton of norbornene ‘c’ (2.03 – 1.87 ppm) by subtracting the integral of the latter one found in the starting material 5-norbornene-2-methylamine where $I_c = 0.84$. This value is smaller than 1 because the used 5-norbornene-2-methylamine contains both, endo- and exo isomers. This results in a number of HA disaccharide units of $m = (I_{b+c} - I_c) / 3 = (7.94 - 0.84) / 3 = 2.37$. Comparing the ratio of norbornene groups versus the number of HA repeating units gives a functionality of $F_{\text{ene}} = n / m = 1.00 / 2.37 = 0.42$.

HA-MA was synthesized as described before.^[95] In short, HA was dissolved at 2% w/v in phosphate-buffered saline (PBS) and reacted with methacrylic anhydride for 24 h at 5 °C at pH 8.0 (**Scheme 3**). F_{ene} was calculated from the integration of the vinyl protons of the MA group relative to the acetyl protons of HA (**Figure 35**). For the following studies HA-MA with $F_{\text{ene}} = 0.39\text{--}0.42$ was used.



Scheme 3. Synthesis of HA-MA with methacrylic anhydride under basic conditions.

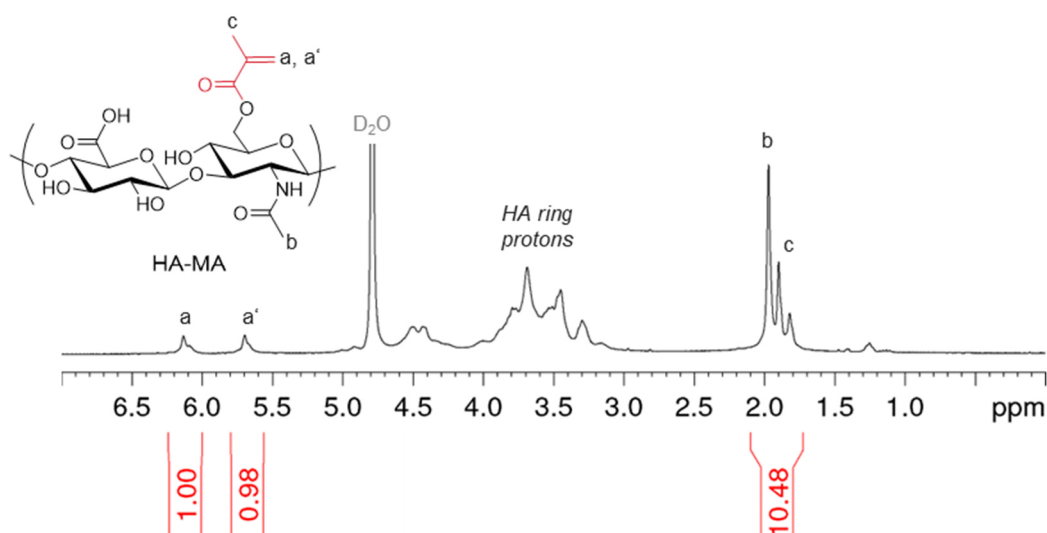


Figure 35. ¹H NMR spectrum of HA-MA in D₂O. The integral of one of the two vinyl protons 'a' from the methacrylate group (6.12 ppm) was used as reference with $I_a = 1.00$ corresponding to $n = I_a / 1 = 1.00 / 1 = 1.00$ methacrylate groups grafted onto to the polymer backbone. The integral of the proton signals 'b' and 'c' (1.97–1.82 ppm) is used to calculate the number of the HA repeating units m by subtracting the methyl protons e of the methacrylate group, i.e. $m = I_b / 3 = (I_{b+c} - 3) / 3 = (10.48 - 3) / 3 = 2.49$. The ratio of methacrylate groups n and HA repeating units determined the functionality to be $F_{\text{ene}} = n / m = 1.00 / 2.49 = 0.40$.

2.2.2.2. Storage stability of photocrosslinkable HA-X prepolymers

The stability of the synthesized HA derivatives was studied by ¹H NMR spectroscopy and GPC analysis. The polymers were stored as solids at room temperature in the dark for

15 months. At different time points, samples were dissolved at 15 mg mL⁻¹ in D₂O and ¹H NMR spectra were recorded (**Figure 36**). The NMR spectra of HA-AE and HA-NB showed no changes in the vinyl proton signals in the 15 months, whereas HA-MA showed additional peaks in the spectrum already after 4 months of storage. The stability studies confirm the higher shelf-life of ene- precursors, presumably as consequence of insusceptibility to autopolymerization of AE and NB groups. A higher stability of the amide and ether groups in HA-AE and HA-NB in comparison to the ester group in HA-MA is also expected.

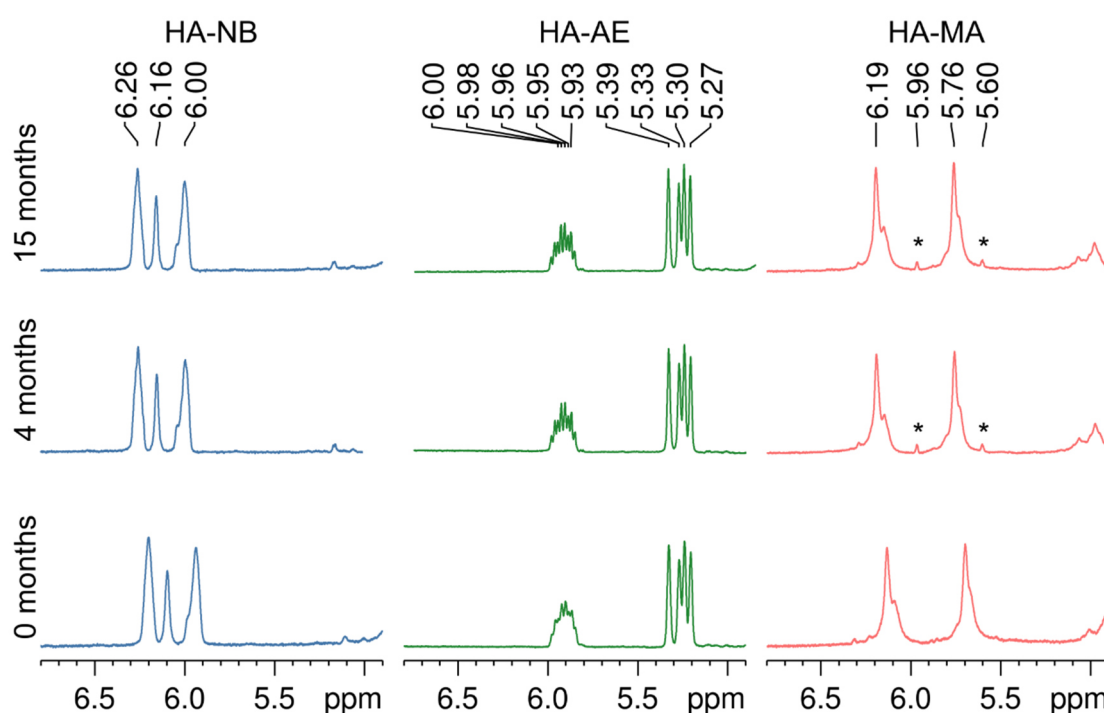


Figure 36. Stability study of HA-X prepolymers over 15 months storage at room temperature in the dark, as measured by ¹H NMR. Asterisks indicate the changes in the vinyl protons signals during storage. Reproduced from Ref.^[182]. © 2024 The Author(s). *Macromolecular Bioscience* published by Wiley-VCH GmbH. All rights reserved.

2.2.2.3. Viscosity of the hydrogel precursor solutions

DLP requires low-viscosity resins to ensure the fluid can flow away from the cured material and hollow parts while the printing stage is lifted, and to allow refill of the resin under the raised scaffold for recoating. While literature reports show DLP of resins with viscosities between 4 and 2000 mPa s, a viscosity <100 mPa s is considered more advantageous for good printability.^[82, 84, 99, 120, 128, 188] The HA-X solutions at 4% w/v in PBS

showed viscosities well below 100 mPa s (**Figure 37**) and, therefore, were considered suitable for DLP. Within the tested conditions, the hydrogel precursor solutions mostly exhibited a Newtonian behavior i.e. constant viscosity with varying shear rate which is characteristic of diluted polymer solutions.^[22, 92, 98] While HA-AE possess an overall lower viscosity than native HA, the viscosity of HA-NB and HA-MA is higher. Introduction of functional groups can change the ionic character and the conformation of the polymeric chains in solution and lead to differences in the viscosity of solutions containing different modified HA derivatives.^[189, 190] For example, Genc et al. found lower viscosity for Gel-AE solutions and a different rheological and thermogelating behavior in comparison to solutions of Gel-MA.^[191]

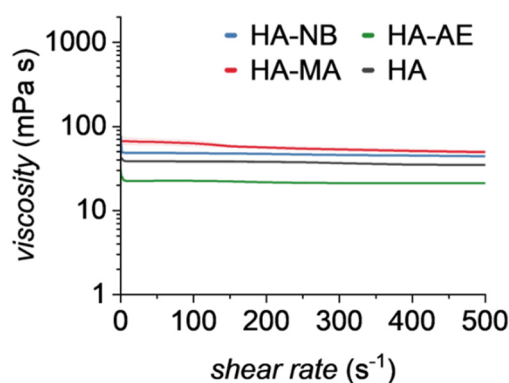


Figure 37. Viscosity measurement of HA-X derivatives as function of the shear rate. HA-X was dissolved at 4% w/v in PBS. Data are shown as mean $\pm$ SD of $N = 3$ samples.

2.2.3. Reactivity and conversion of hydrogel formation

2.2.3.1. Photocrosslinking kinetics and mechanical properties of HA-X hydrogels studied by photorheology

To investigate the photocrosslinking kinetics of the different HA-X hydrogels, oscillatory shear rheology with in situ illumination at 400–500 nm was used. **Figure 38** shows the evolution of the shear storage and loss moduli upon illumination. **Table 2** shows the comparative values of the gelation time t_{gel} and the value of the shear modulus at full crosslinking ($G'_{20\text{min}}$) extracted from the curves. t_{gel} is the time needed to reach the crossover point of storage and loss modulus, $G' = G''$, which is usually called the gel point.^[192] At t_{gel} , the polymer solution transitions from a liquid-like (viscous) behavior to a

solid-like (elastic) state corresponding to a three-dimensional network. $G'_{20\text{min}}$ reflects the stiffness reached by the hydrogels after crosslinking and 20 min light exposure, illustrating the plateau value of the shear modulus after full exposure.

The HA-NB resin showed a shorter gelation time ($t_{\text{gel}} = 9.0 \pm 0.7$ s) than HA-AE ($t_{\text{gel}} = 21.4 \pm 1.2$ s). The rapid reaction kinetics of NB is a consequence of the ring strain which is released upon the addition of a thiyl radical.^[52] HA-MA showed a longer gelation time ($t_{\text{gel}} = 42.2 \pm 2.4$ s) than thiol-ene resins. This is in agreement with the radical chain-growth mechanism in HA-MA where oxygen inhibition needs to be overcome introducing an additional lag phase before the onset of gelation is reached. Both HA-NB and HA-AE hydrogels reached a similar value of $G'_{20\text{min}} = 8$ kPa, suggesting a similar internal structure and crosslinking degree in the final network. HA-MA which crosslinks via a chain-growth mediated mechanism achieved a three-fold higher stiffness ($G'_{20\text{min}} = 26$ kPa).

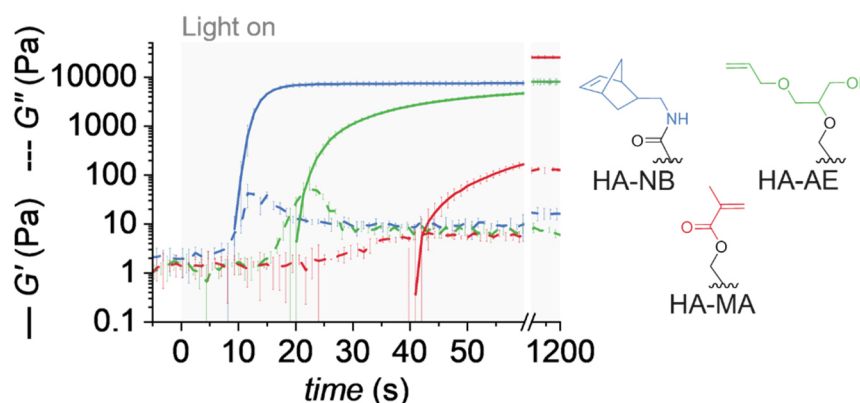


Figure 38. Rheological characterization of the photocrosslinking of HA-X precursor solutions. Evolution of storage modulus G' (continuous line) and loss modulus G'' (dashed line) of HA-X precursor solutions during illumination (400–500 nm, 15 mW cm^{-2}). Exposure started at $t = 0$ s. The shaded box indicates the period where the light was switched on. Polymers were dissolved at 4% w/v in PBS. Solutions contained 0.1% w/v LAP as photoinitiator. The thiol-ene solutions HA-AE and HA-NB contained DTT as crosslinker at thiol:ene ratio of 1. Data are shown as mean $\pm$ SD of $N = 3$ samples. Reproduced from Ref.^[182]. © 2024 The Author(s). *Macromolecular Bioscience* published by Wiley-VCH GmbH. All rights reserved.

Table 2. The composition, gelation time t_{gel} and storage modulus $G'_{20 \text{ min}}$ of different HA-X precursor solutions determined by oscillatory shear rheology and in situ illumination. All samples contained 0.1% w/v LAP as photoinitiator. Reproduced from Ref.^[182]. © 2024 The Author(s). *Macromolecular Bioscience* published by Wiley-VCH GmbH. All rights reserved.

HA-X	F_{ene}	C _{HA-X} (% w/v)	C _{DTT} (% w/v)	t_{gel} (s)	$G'_{20 \text{ min}}$ (kPa)
HA-NB	0.36	4.0	0.27	9.0 ± 0.7	8.1 ± 0.9
HA-AE	0.46	4.0	0.35	21.4 ± 1.2	8.0 ± 1.2
HA-MA	0.39	4.0	-	42.2 ± 2.4	25.5 ± 2.0

In a photo-initiated radical reaction, tuning the light intensity and illumination profile should allow control over the kinetics of the crosslinking progress. This was tested by monitoring gelation of HA-NB at different irradiance values between 1 and 6 mW cm⁻². At 6 mW cm⁻² the t_{gel} was reached in less than 1 s (**Figure 39**). Decreasing the irradiance to 1 mW cm⁻² increased t_{gel} to 5 s. The time for G' to reach the final stiffness increased from 20 to approx. 190 s.

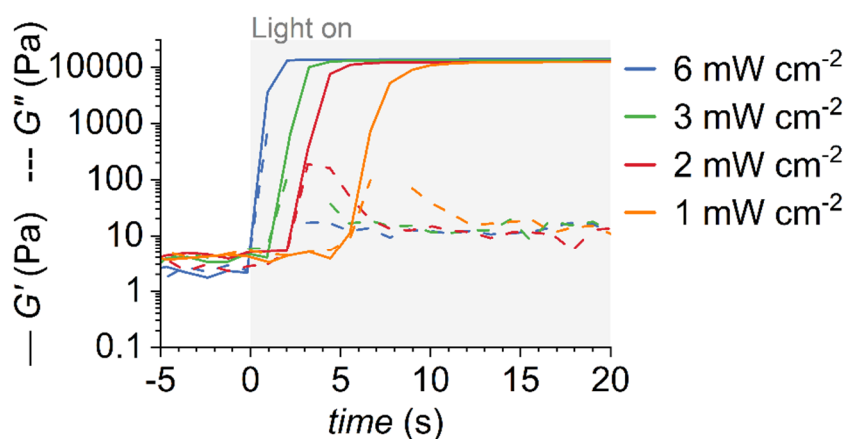


Figure 39. Evolution of storage modulus G' (continuous line) and loss modulus G'' (dashed line) of HA-NB precursor solutions during photocrosslinking with light (365 nm) with light intensities between 1–6 mW cm⁻² ($N = 1$). Exposure started at $t = 0$ s.

2.2.3.2. Conversion of the thiol-ene crosslinking reaction by quantification of the free thiol end groups

The degree of crosslinking in a thiol-ene crosslinked hydrogel can be quantified by reacting the free thiol groups with the Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).^[81, 83, 193] The reaction leads to a disulfide and a free TNB anion that absorbs at 412 nm. A thiol conversion of 77 and 78% was found in HA-AE and HA-NB hydrogels prepared with stoichiometric concentrations of thiol and ene functions after full exposure. The incompleteness of the crosslinking reaction is attributed to the limited mobility of the dithiol crosslinkers once attached to a HA chain by one end, which reduces their probability to meet remaining alkene groups at more distant positions in the network. The remaining functional groups could be exploited for post-crosslinking functionalization of the hydrogels with e.g. peptides or fluorescent dyes.^[156, 193]

2.2.3.3. Post-exposure reactivity

In a chain-growth radical propagation reaction, as in the photocrosslinking of HA-MA, the chains mobility is hindered as gelation proceeds and termination is slowed down. As a result, the lifetime of the propagating radicals exceeds the illumination time and the crosslinking reaction can continue after exposure. This has previously been demonstrated in 30% w/v Pluronic/Pluronic diacrylate hydrogels where the shear storage modulus increases after UV exposure demonstrating post-exposure polymerization of the polymer chains.^[194] In a photo-initiated step-growth radical reaction, the situation is different. To quantify post-exposure reactivity of HA-X derivatives, photocrosslinking during stepwise exposure with blue light (400–500 nm, 15 mW cm⁻²) was monitored (**Figure 40**). Thiol-ene crosslinked hydrogels showed a rapid increase of G' during the illumination periods and did not show any further variation of G' during the dark periods, indicating that the crosslinking starts and stops with the light exposure. This shows that step-growth mediated crosslinking mechanism require a continuous radical flux and no significant homopolymerization of the employed alkenes NB and AE occur. In contrast, the storage modulus in the HA-MA system continued increasing after the light was switched off throughout the whole 40-s dark phase. The post-exposure crosslinking in HA-MA limits the possibility to control the crosslinking process in time and space. In a

DLP context, this could lead to lower resolution of printed features and could explain the dark curing which is often observed for methacrylated resins.

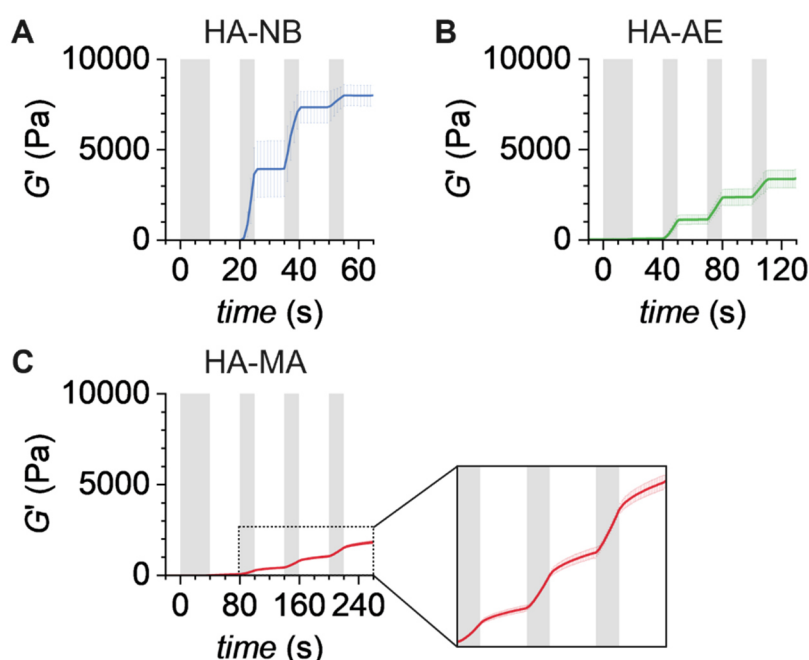


Figure 40. Evolution of storage modulus hydrogel precursor solutions HA-NB (A), HA-AE (B) and HA-MA (C) upon sequential irradiation at 400–500 nm, 15 mW cm⁻². Shaded boxes indicate the periods where the light was switched on. Polymers were dissolved at 4% w/v in PBS. Solutions contained 0.1% w/v LAP as photoinitiator. The thiol-ene solutions HA-AE and HA-NB contained DTT as crosslinker at thiol:ene ratio of 1. Data are shown as mean ± SD of *N* = 3 samples. Adapted from Ref.^[182]. © 2024 The Author(s). *Macromolecular Bioscience* published by Wiley-VCH GmbH. All rights reserved.

2.2.4. Digital light processing of HA-X

In DLP-based 3D printing, the printed scaffolds are fabricated layer-by-layer by projecting a specific pattern onto a liquid photocrosslinkable material which leads to spatially controlled crosslinking of the polymeric chains and formation of a hydrogel structure (**Figure 41**). The printability and the printing resolution achieved with a given photocrosslinkable prepolymer depend on the crosslinking mechanism, the concentration of the functional groups, the viscosity, crosslinking kinetics, and the polymer content.^[33] To test the printability of the HA-X resins by DLP, the following formulation was used: 4.0% w/v HA-X, 0.1% w/v LAP, and for the thiol-ene resins, DTT at

a thiol:ene ratio of 1. *The following experiments were performed in collaboration with Christian Stoppel, Hamidreza Pirmahboub and Tobias Lam at the company Cellbricks.*

2.2.4.1. Determination of printing speed

To determine the minimum exposure time for each resin, discs with 3 layers with a diameter of 6–9 mm and a layer thickness of 1 mm were printed with the HA-X resins. The exposure time per layer (t_{layer}) was varied between 1 and 50 s. The resulting discs were imaged and their dimensions and the contours were compared. **Figure 41B** shows representative images of HA-X scaffolds printed with different exposure times.

Undercured prints showed smaller-than-targeted dimensions while overcured prints showed larger-than-targeted dimensions and oblique walls. Using the HA-NB resin, a minimum t_{layer} of 2 s gave a printed scaffold with the targeted dimensions, while HA-MA required t_{layer} of 10 s. Surprisingly, the minimum t_{layer} for HA-AE was also 10 s which did not reflect its faster curing vs HA-MA found in photorheology experiments. The reason for this difference is not known at this point. HA-NB allowed 5-fold faster printing compared to HA-MA, which is comparable to the fold-difference obtained for t_{gel} of the two resins in photorheology experiments. The significantly faster printing of HA-NB is particularly interesting in bioprinting, where shorter exposure times minimize cellular photodamage and the time cells are exposed to an environment outside of ideal growth and incubation conditions.

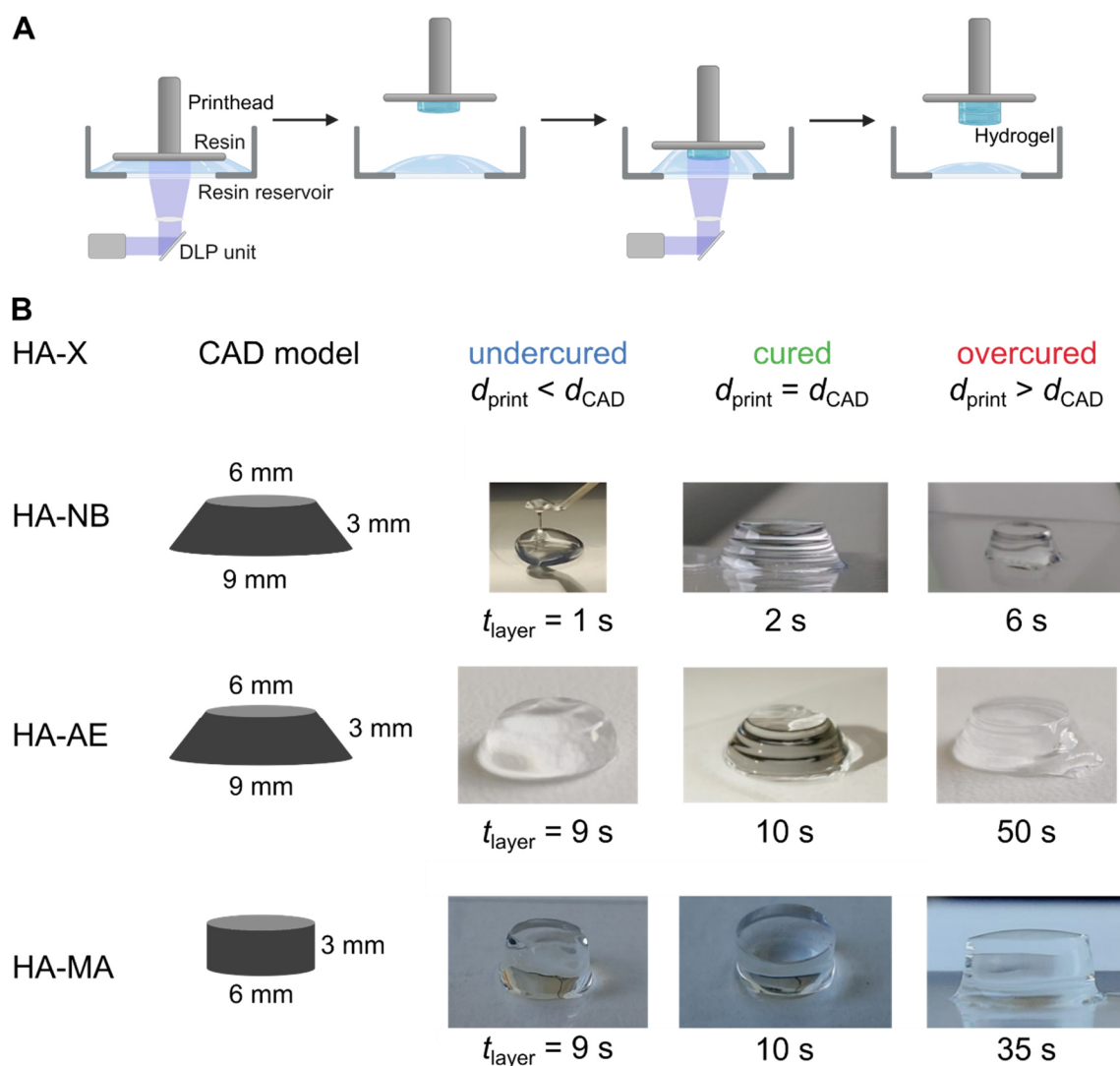


Figure 41. A. Schematic working principle of a DLP printer where the scaffold is printed layer-by-layer. Projection of a photomask through the transparent bottom of the resin reservoir onto the liquid precursor solution (resin) leads to spatially controlled photocrosslinking of one layer. Raising the printing stage and illuminating again photocrosslinks the next layer. *Created with BioRender.com* **B.** Determination of minimum and maximum exposure time t_{layer} for HA-X hydrogels. Comparison of the printed structure to the CAD model allowed qualitative assessment of undercured, cured and overcured prints. Reproduced from Ref.^[182]. © 2024 The Author(s). *Macromolecular Bioscience* published by Wiley-VCH GmbH. All rights reserved.

2.2.4.2. Determination of xy resolution for HA-NB

Printing resolution defines the minimum feature size that can be printed. In DLP-based 3D printing, resolution depends on the diffusion rate of the reactive species inside the resin, the kinetic of the crosslinking reaction and on the magnification of the light source (i.e. pixel size).^[33] To achieve high resolution in the printed structure, the crosslinking

reaction ideally occurs fast with temporal control to limit diffusion of the reactive species outside of the illuminated zone.^[129]

To test the spatial resolution of printed scaffolds obtained from the thiol-ene based resin HA-NB, single 1000- μm thick layers containing rectangular positive and negative features which are referred to as protrusions and channels were printed. Protrusions were 1 mm long and channels were 2 mm long, and their widths decreased from 500 to 100 μm (**Figure 42A**). To avoid damage of the scaffold during handling, the structures were printed directly on coverslips attached to the printing platform. The printing time per layer was $t_{\text{layer}} = 2.7$ s. The microscopy image of the printed structure shows well-defined features down to 100 μm width with good dimensional agreement with the theoretical size of the CAD model (**Figure 42B and C**). So far, this is the smallest printed feature size demonstrated with HA-based resins in DLP printing. HA-NB therefore allows printing of structures with high resolution and high shape fidelity.

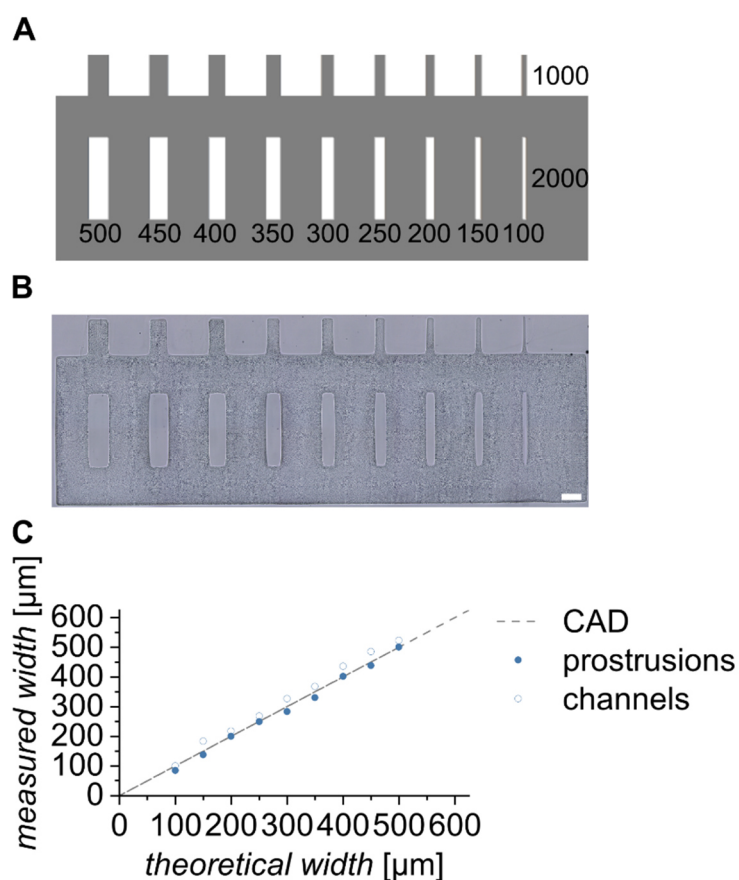


Figure 42. Printing resolution experiments. **A.** CAD model used to print scaffolds for determination of xy resolution. The widths of the positive and negative features are stated in μm . **B.** Microscopy images of printed HA-NB scaffold consisting of 1 layer with 1000 μm

height. Scale bar represents 500 μm . **C.** Measured width for protrusions (closed symbols) and channels (open symbols) for the HA-NB scaffolds printed with 1000 μm layer height compared to the theoretical width of the CAD model (dashed line). Reproduced from Ref.^[182]. © 2024 The Author(s). *Macromolecular Bioscience* published by Wiley-VCH GmbH. All rights reserved.

2.3. Discussion

HA-AE, HA-MA and HA-NB were synthesized with comparable degree of functionality in aqueous media. The syntheses of HA-AE and HA-NB were re-defined from published protocols. In contrast to most reported HA-NB syntheses,^[81, 83, 86, 101] the EDC/NHS synthesis route used in this work is considerably less labor intensive and does not require the use of organic solvents like DMSO or dimethylformamide. In one popular method, HA is first converted into a tetrabutylammonium salt and then reacted with either 5-norbornene-2-methylamine or 5-norbornene-2-carboxylic acid in dimethyl sulfoxide, requiring up to 14 days of dialysis for purification.^[83, 86, 101] In a different approach, the NB is introduced in two steps by first attaching adipic acid dihydrazide to the carboxylic acid of HA, followed by purification and reaction with *cis*-5-norbornene-*endo*-2,3-dicarboxylic acid.^[81] The synthesis method in this work only requires one synthesis step and uses water as solvent. A recently published protocol by Plaster et al. uses a similar approach to synthesize HA-NB using an aqueous buffer system and the water-soluble triazine derivative 4-(4,6-dimethoxy-1,3,5-triazin-2-yl) 4-methylmorpholinium chloride as coupling agent.^[93] Both, HA-AE and HA-NB showed a higher storage stability in comparison to HA-MA derivative when stored under oxygen atmosphere at room temperature.

As expected from literature, the thiol-ene based resins showed a faster gelation than the photoinitiated chain-growth mediated crosslinking of HA-MA. Especially the release of ring strain in the NB group upon the thiol radical addition in HA-NB led to an approx. 2.4-times faster t_{gel} than for HA-AE and approx. 4.7-times faster than for HA-MA. The approx. 3-times higher stiffness obtained in the HA-MA hydrogels suggested a different network structure in comparison to the thiol-ene hydrogels HA-AE and HA-NB. The different network structure could be expected from the different crosslinking mechanisms. In the chain-growth mechanism of HA-MA, MA units from the same or different chains can add

to the growing macroradical and form a “cluster” of intra- and intermolecular crosslinks (**Figure 31A**). In contrast, in the radical thiol-ene reaction each DTT dithiol crosslinker molecule can only connect to two -ene functions of the same or different chains (**Figure 31B**). Intramolecular thiol-ene reactions form loops and do not contribute to mechanical strength. Intermolecular thiol-ene reactions connect only two chains. This molecular scenario would lead to a more regular but looser thiol-ene network and, therefore a hydrogel with a lower G' . Similar trends have been observed in other comparative reports of NB vs MA-functionalized resins.^[59, 64] Examples in literature which study the influence of different thiol crosslinkers demonstrate that higher storage moduli could be obtained in NB hydrogels using a multivalent thiol crosslinker which can connect multiple polymer chains in contrast to DTT.^[59, 67] The photorheology experiments with sequential illumination profile highlight another difference. While the photocrosslinking via chain-growth mechanism in HA-MA continuous after the end of the exposure and shows enhanced dark curing, the thiol-ene resins shows a higher temporal control of the photocrosslinking and no increase in the stiffness is observed during dark phases.

HA-NB resins in literature required an exposure time per layer of 2.5 or 6 s to print layers with 100 μm thickness.^[83, 84] The HA-NB resins developed in this work needed only 2 s to crosslink layers with 1 mm. While others incorporated photoabsorbers, no photoabsorber was used in the HA-NB resin in this thesis which potentially led to a higher reactivity and higher cure depth. t_{layer} of HA-NB was approx. 5-times faster than for the corresponding HA-MA resin which aligned with the results observed in the photorheology experiments. Notably, the HA-AE resin showed a different behavior than in the photorheology experiments and did not lead to an improvement in the printability i.e. exposure time per layer. With the HA-NB resin, a xy resolution, i.e. a minimum printable positive and negative feature size of approx. 100 μm was achieved. In addition, the observed dimensions were in good agreement with the theoretical sizes of the CAD model demonstrating the high spatial control of radical thiol-ene crosslinking. Previous studies reported a xy resolution in the range of 250–400 μm using HA-based resins and DLP printing (**Table S1**).^[83, 84, 99]

2.4. Conclusion

In this chapter, HA precursors carrying AE or NB moieties were synthesized, and their photocrosslinking behavior was compared with the commonly used methacrylated derivative HA-MA. Syntheses routes to obtain HA-AE and HA-NB of the same degree of functionalization in one step under aqueous conditions were developed, and the protocols were optimized to minimize the time and solvent required for dialysis. In photorheology and DLP printing studies, HA-NB crosslinked over 4-times faster than HA-MA. Both thiol-ene resins showed higher temporal control over the network formation, with storage moduli only increasing during illumination while the storage modulus of the HA-MA network continued to rise in the dark state between illumination periods. Because of the different network structures, the stiffness of the thiol-ene hydrogels was lower than in the methacrylated ones after extended photocrosslinking. Scaffolds with feature sizes down to 100 μm were obtained with the HA-NB resin. The results quantify the advantages of thiol-ene vs. methacrylate-based photocrosslinking mechanisms for DLP of HA hydrogels at typical concentrations used in bioprinting (4% w/v), where the concentration of photocrosslinkable groups is very low.

2.5. Experimental section

2.5.1. Materials

Hyaluronic acid (purity 97%) with 40–50 kDa molecular weight was purchased from Biosynth s.r.o. (Slovakia). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, purity $\geq 99\%$) was bought from Carbolution Chemicals GmbH (Germany). Allyl glycidyl ether (purity $>99.0\%$) and 5-norbornene-2-methylamine (purity $>98.0\%$) were purchased from TCI Chemicals (Belgium), dithiothreitol (purity 98%) from abcr GmbH (Germany). D₂O for ¹H NMR spectroscopy was purchased from Deutero (Germany). All other reagents were obtained from Carl Roth GmbH (Germany) and Sigma-Aldrich (Germany).

2.5.2. Methods

2.5.2.1. Synthesis of HA-NB

HA-NB was synthesized using EDC/NHS coupling according to a previously reported protocol by Bian et al.^[29] HA (5.00 g, 12.47 mmol disaccharide repeating units) and NHS (2.87 g, 2 eq.) were dissolved at 1% w/v of Milli-Q H₂O. After complete dissolution, EDC (9.56 g, 4 eq.) was added as solid and the solution stirred for 2 h at room temperature. 5-Norbornene-2-methylamine was added (6.14 g, 4 eq.) to the reaction mixture and stirred at pH = 4.75 for 24 h. During the reaction, pH was adjusted several times to 4.75 using 1.0 M NaOH and 1.0 M HCl. Subsequently, pH was adjusted to 7.0, NaCl concentration increased to 2 M and the solution precipitated into 1000 mL ice-cold ethanol. After filtration under reduced pressure, the precipitate was washed with ethanol several times and redissolved in 100 mM NaCl at 1% w/v. The solution was transferred in a dialysis bag (MWCO 3.5 kDa, SpectraPor) and dialyzed against 100 mM NaCl solution for 24 h and against Milli-Q H₂O for 48 h. At last, the solution was lyophilized to obtain HA-NB as solid.

2.5.2.2. Synthesis of HA-AE

AE modification of HA was obtained by basic ring opening of allyl glycidyl ether following a previously reported protocol for the synthesis of AE modified dextran.^[186] HA (2.5 g, 6.23 mmol disaccharide repeating units) was dissolved at 5% w/v in 0.1 M NaOH and stirred for 30 min. After addition of allyl glycidyl ether (2.1 g, 3 eq.), the solution was

stirred at 35 °C for 24 h. The reaction mixture was precipitated in 250 mL ice-cold ethanol and subsequently filtrated and washed as described before. The solid was redissolved in Milli-Q H₂O and dialyzed against Milli-Q H₂O for 48 h. Lyophilization yielded HA-AE as white solid.

2.5.2.3. Synthesis of HA-MA

HA-MA was synthesized as described previously using methacrylic anhydride under basic conditions.^[95] After precipitation and dialysis, HA-MA was obtained as white solid by lyophilization.

2.5.2.4. ¹H NMR analysis

¹H NMR spectra were recorded using a Bruker Avance 300 spectrometer in D₂O at 298 K with a number of scans of 256 and a relaxation delay of 10 s. Chemical shifts (δ) are reported in parts per million (ppm) with the ¹H NMR solvent peak (D₂O: δ = 4.79 ppm) as reference. Analysis of spectra was conducted using Bruker's TopSpin software.

2.5.2.5. Determination of degree of functionality

To determine the degree of alkene functionalization (F_{ene}) of HA-X, ¹H NMR spectrum of the polymer dissolved at 15 mg mL⁻¹ in D₂O was recorded. Peak area of the acetyl proton signal of HA at 1.97 ppm was compared to the corresponding alkene protons of the attached AE-, NB- or MA groups in the vinyl region (6.30–6.20 ppm).

2.5.2.6. Gel permeation chromatography

GPC was performed with a Polymer Standards Service (PSS) system using a combination of a PSS SUPREMA LUX pre-column and a PSS SUPREMA LUX analytical column equipped with refractive index detector and with 0.07 M Na₂HPO₄ and 20% acetonitrile, pH = 9 as mobile phase.

2.5.2.7. Precursor stability in storage

To assess storage stability of different HA derivatives, solid polymers were stored at room temperature in the dark. After different time points (t = 0, 4, 8, 12, and 15 months), each derivative was dissolved at 15 mg mL⁻¹ in D₂O and ¹H NMR spectrum was recorded.

2.5.2.8. Calculation of molecular weight per alkene unit

First, the apparent average molar mass of the repeating units in the functionalized HA derivative was calculated by the ratio of the molecular weight, MW_{average} , of the native HA repeating unit MW_{HA} and the functionalized repeating unit HA-X, $MW_{\text{HA-X}}$, where X can be either AE, MA or NB depending on the derivative:

$$MW_{\text{average}} = MW_{\text{HA}} * (1 - F_{\text{ene}}) + MW_{\text{HA}} * F_{\text{ene}} \quad (1)$$

F_{ene} was the functionality determined for the respective batch. From the average molecular weight per repeating unit, the molecular weight per alkene unit MW_{ene} can be calculated as followed:

$$MW_{\text{ene}} = \frac{MW_{\text{average}}}{F_{\text{ene}}} \quad (2)$$

2.5.2.9. Calculation of thiol concentration for hydrogel precursor solution

In all experiments involving thiol-ene hydrogel precursor solutions or resins, equal moles (n) of alkene (ene) and thiol (SH) functional groups were used:

$$n_{\text{ene}} = n_{\text{SH}} \quad (3)$$

With a final polymer concentration of $c_{\text{HA-X}} = 4\%$ w/v, the volume V of the solution and the previously calculated molecular weight per alkene unit MW_{ene} , n_{ene} can be re-expressed as

$$n_{\text{ene}} = \frac{c_{\text{HA-X}} * V}{MW_{\text{ene}}} = n_{\text{SH}} \quad (4)$$

Considering that the used crosslinker is bifunctional and therefore $n_{\text{SH}} = 2 * n_{\text{DTT}}$, the necessary concentration of DTT in the final solution in % w/v can be calculated by

$$c_{\text{DTT}} = \frac{n_{\text{SH}} * MW_{\text{DTT}}}{2 * V} \quad (5)$$

Substitution of eq. (4) for n_{SH} , c_{DTT} can be expressed as

$$c_{\text{DTT}} = \frac{c_{\text{HA-X}} * MW_{\text{DTT}}}{2 * MW_{\text{ene}}} \quad (6)$$

2.5.2.10. Preparation of hydrogel precursor solution

First, the required amounts of polymer, DTT, LAP and PBS to obtain a 4% w/v HA-X solution were calculated using the functionality F_{ene} and the resulting MW per alkene unit of the specific batch to ensure that the final thiol:ene ratio is 1.00. HA-AE, HA-MA or HA-NB were dissolved in PBS, pH = 7.3 on a shaker at 350 rpm for 60 min, excluded from light. Stock solutions of LAP (1.0% w/v) and DTT (5.0% w/v) were prepared in PBS and added right before the addition the polymer solution to prevent disulfide formation in DTT. To thiol-ene resins, DTT was added as dithiol crosslinker in an equimolar ratio of $n(\text{SH})$ and $n(\text{ene})$. As last step, the photoinitiator was added to obtain a final concentration of 0.1% w/v.

2.5.2.11. Crosslinking kinetics and mechanical properties of hydrogels by rheology

Rheological properties and curing kinetics of different HA-based hydrogels were determined using a rotation rheometer (DHR3, TA Instruments, USA) equipped with a 12 mm parallel plate geometry as upper plate and a transparent lower plate connected to a UV/Vis illumination source (Omnicure, Series 1500) with a 400–500 nm filter to allow in situ curing of hydrogels at room temperature. For each polymer HA-NB, HA-MA and HA-AE, hydrogel precursor solutions were prepared as above. An aliquot was loaded onto the transparent lower plate of the rheometer using a micropipette and the upper parallel plate was lowered to a gap size of 300 μm . The sample was surrounded with silicone oil to prevent evaporation and covered with a metal cover to avoid disturbance during the measurements. Strain sweeps (0.1 to 100% strain at frequency = 2 Hz) and frequency sweeps (0.01 to 100 Hz at strain = 1%) were performed to determine the linear viscoelastic region. Time sweeps were carried out in the determined parameters. Storage and loss moduli were recorded over time using 1% applied strain and 2 Hz frequency. For continuous curing measurements, the samples were irradiated with 400–500 nm light (15 mW cm^{-2}) from the 60-s time point onwards using TRIOS software to automatically start the illumination. For sequential curing measurements, the same light source was switched on and off manually. To compare crosslinking kinetics of the different HA

derivatives, the gelation time t_{gel} where $G' = G''$ corresponding to the gel point was compared. Stiffness of the hydrogels were determined by comparing G' after 20 min of irradiation.

2.5.2.12. Viscosity measurements

The rheological properties of the uncured precursor solutions were characterized with a modular compact rheometer (Anton Paar, Austria) equipped with a 50 mm, 1° cone plate geometry and Peltier stage temperature control system. The temperature was set to 25 °C. First, HA-AE, HA-NB and HA-MA were dissolved at 4% w/v in PBS without photoinitiator and dithiol crosslinker. The solution was placed on the lower plate and the gap lowered to 105 μm . The temperature was held for 1 min and then viscosity was measured in rotational mode at shear rates from 0.1 to 500 s^{-1} at 25 °C.

2.5.2.13. Determination of degree of thiol conversion (Ellman's assay)

To quantify the thiol conversion in photocrosslinked thiol-ene hydrogels, Ellman's assay was used to detect free thiols.^[193] The protocol was adapted from the manufacturer's instruction. In short, crosslinked hydrogels (20 μL) of HA-AE and HA-NB were incubated with an excess of a 0.2 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) solution (5 mL) in Ellman's buffer (0.1 M sodium phosphate, 1 mM EDTA, pH = 8.0). Thiol containing standards were prepared by diluting a freshly prepared DTT stock solution (10 mg mL^{-1} , $c(\text{SH}) = 129.7 \text{ mM}$) with PBS to final concentrations ranging from 0 to 3 mM SH. 20 μL of the standards or hydrogel precursor solutions were incubated along with the hydrogels in 0.2 mM DTNB (1 mL) for 90 min in the dark. 100 μL of supernatant were transferred into a new well plate and the absorbance was measured at 412 nm using a Tecan infinite 200Pro plate reader. The thiol content in the samples was calculated using the calibration curve prepared by the DTT standards. Finally, the conversion of thiols was determined by comparison of the initial thiol concentration in the hydrogel precursor solution and the thiol content found in the crosslinked hydrogel.

2.5.2.14. Determination of minimum exposure time per layer in DLP printing

Printing of hydrogel scaffolds was conducted using a DLP printer developed and manufactured by Cellbricks GmbH. The 3D model of the printed scaffold was created

using a CAD software and processed and sliced into different layers by the printer itself. Before each experiment, the printing platform was calibrated to set $z = 0$. Resins for printing experiments were prepared freshly as described before. In addition, the solution was filtered through a $0.45\ \mu\text{m}$ syringe filter and centrifuged to remove any particles or bubbles. Approx. 1 mL resin was loaded into the resin reservoir consisting of a transparent, hydrophobic foil which allows illumination from the bottom. The constructs were fabricated layer-by-layer: the first layer was photocrosslinked directly on the printing platform and the subsequent layers on top of the previous one by projection of a specific mask with blue light illumination ($385\ \text{nm}$, $20\ \text{mW cm}^{-2}$). After printing one layer, the printing platform was lifted by 1 mm with a speed of $20\ \text{mm s}^{-1}$ to then lower in the liquid resin to print the next layer. Each model consisted of three layers (1 mm layer height) resulting in a final height of 3 mm with a diameter of 6–9 mm. The printing time per layer t_{layer} was varied to determine the minimum and maximum exposure time per layer. After printing, the scaffold was detached from the printing platform and inspected by eye to determine whether the used exposure time per layer resulted in an undercured, cured or overcured scaffold. The time where the scaffold was no longer undercured but cured i.e. had the desired dimensions of the CAD model, was defined as minimum t_{layer} . The maximum t_{layer} designated the exposure time per layer where the scaffold was cured but higher t_{layer} resulted in overcuring.

2.5.2.15. Determination of printing resolution

To determine the xy resolution of the HA-NB resin, a CAD model with positive (1000 μm length) and negative (2000 μm length) features with decreasing width (500 to 100 μm) was designed. The resin were prepared as described before. The CAD model was loaded into the software of the printer, sliced and the printing platform was calibrated. A coverslip with 24 mm diameter was semi-permanently fixed on the printing platform using a photocurable PEGDA resin. The resin was loaded into the resin reservoir, the printing platform lowered into the resin and the designated construct printed. The model consisted of one layer (1000 μm layer height) with a length of 13.5 mm and width of 4 mm. After printing, the scaffold was carefully washed with PBS to remove uncured resin, detached from the printing platform, and imaged with a microscope (Leica DMI1,

Keyence, Osaka, Japan) equipped with a 4x objective. Feature sizes were measured using the microscope software.

3. Volumetric Printing of Low-Viscosity Hyaluronic Acid-based Resins Through Fast Crosslinking Reactions

This chapter uses the HA-NB and HA-MA polymers to develop resins suitable for volumetric printing. The syntheses of the polymers were further optimized using an automatic titration system and the polymer functionalization confirmed using Raman spectroscopy. The relevant optical and rheological properties of the resins for VP were characterized. The printing conditions and printability window of the resins are evaluated and compared. Printability in the presence of biological species is tested in bioprinting experiments.

A publication based on this chapter is currently in revision at the journal *Biofabrication* (IOP Publishing).

The measurement of Raman spectra was performed in collaboration with Dr. Petra Herbeck-Engel. The acquisition of printed scaffolds by fluorescence microscopy imaging was performed in collaboration with Dr. Cao Nguyen Dong. Cell experiments and bioprinting were performed in collaboration with Dr. Aleeza Farrukh.

3.1. Introduction

Tomographic volumetric printing (VP) has emerged as a new light-based 3D bioprinting process (**Figure 43A**). In VP, a light dose distribution projected inside a vial leads to crosslinking of the contained resin above a certain light dose threshold and simultaneous solidification of the desired object geometry inside of the vial. This enables 3D printing of complex structures in seconds to minutes, without the need of support structures with printing times which are independent from object size.^[135, 141] VP is different from digital light processing (DLP) methodologies, as used in Chapter 2 of this thesis, where a 2D image is projected on the surface of the vat and the object is fabricated by photocrosslinking consecutive layers. In VP, voxels (instead of pixels) are used to define the exposed volumes of the resin.

Both DLP and VP rely on photopolymerizable resins but require different resin properties for scaffold fabrication. In DLP, the curing depth depends on the light dose used to print one layer, defined by exposure time and the light intensity. To optimize the curing depth

at minimum exposure time and low layer height, the photoinitiator and photoabsorber concentration are balanced in the resin. In VP, tuning of the light dose is used to optimize printability and shape fidelity. As light needs to propagate through the entire volume, low absorbance of the resin at the printing wavelength is important, which requires the use of resins with low photoinitiator concentrations and without photoabsorbers. Crosslinking kinetics need to be fast to minimize exposure and prevent curing in out-of-part voxels. For printing consecutive layers in DLP, flow of the resin is required at each printing step. For this purpose, DLP relies on low-viscosity resins (<0.1 Pa s).^[182] In contrast, VP requires resins with higher viscosity (>4 – 10 Pa s) to avoid sedimentation of the crosslinked, densified parts, and maximize resolution and shape fidelity during prolonged printing times.^[141, 195]

Gelatin is the gold standard in VP of hydrogels for biomedical purposes, as it forms a physical gel at room temperature and can be liquefied at >35 °C. VP of Gel-MA^[76, 134, 143, 151-153, 163] has been reported, as well as VP of gelatin derivatives which can undergo a radical thiol-ene reaction in the presence of a thiolated crosslinker, like Gel-NB^[72, 76, 156, 157] or Gel-AE^[77]. The radical thiol-ene reaction proceeds via a step-growth mechanism where in the presence of light and photoinitiator, a thiyl radical is formed which can form a thioether with a specific alkene like e.g. norbornene (NB) (**Figure 43B**).^[52] Thiol-ene crosslinking has several advantages for light-based printing vs. crosslinking of MA groups. It is not prone to oxygen inhibition and allows faster crosslinking at lower doses with higher spatial and temporal control.^[182] This minimizes cell exposure to potential harmful radicals which can compromise cell viability.

Other hydrogels like HA-MA^[144, 158, 159], PVA-NB^[85], acrylamide^[144] or PEGDA^[161] have also been used for VP. In all these cases, the resins were supplemented with a viscosity modifier, mainly sacrificial gelatin, to obtain printable scaffolds with high structural fidelity. An alternative viscosity modifier employed in VP is methacrylated Pluronic which was used in combination with HA-MA.^[158] Similar to gelatin, Pluronic solutions show a thermoresponsive behavior and form physical hydrogels at room temperature. As alternative to thermoreversible hydrogels, ionic crosslinkable hydrogels like alginate have been used as viscosity modifiers.^[161] Besides the addition of viscosity modifiers,

high molecular weight HA-MA which provides a higher viscosity has been proven suitable for VP.^[159]

In this chapter, the suitability of 4% w/v NB-functionalized HA (HA-NB) based resins and bioresins for VP in the absence of thickening additives using the fast photocrosslinking thiol-ene system with DTT as crosslinking molecule and LAP as photoinitiator (**Figure 43C**) is evaluated. The printability of scaffolds with internal channels in the presence of fibroblasts is analyzed. The properties and performance of the thiol-ene resin in terms of printability are compared to HA-MA resins with equivalent composition (**Figure 43D**).

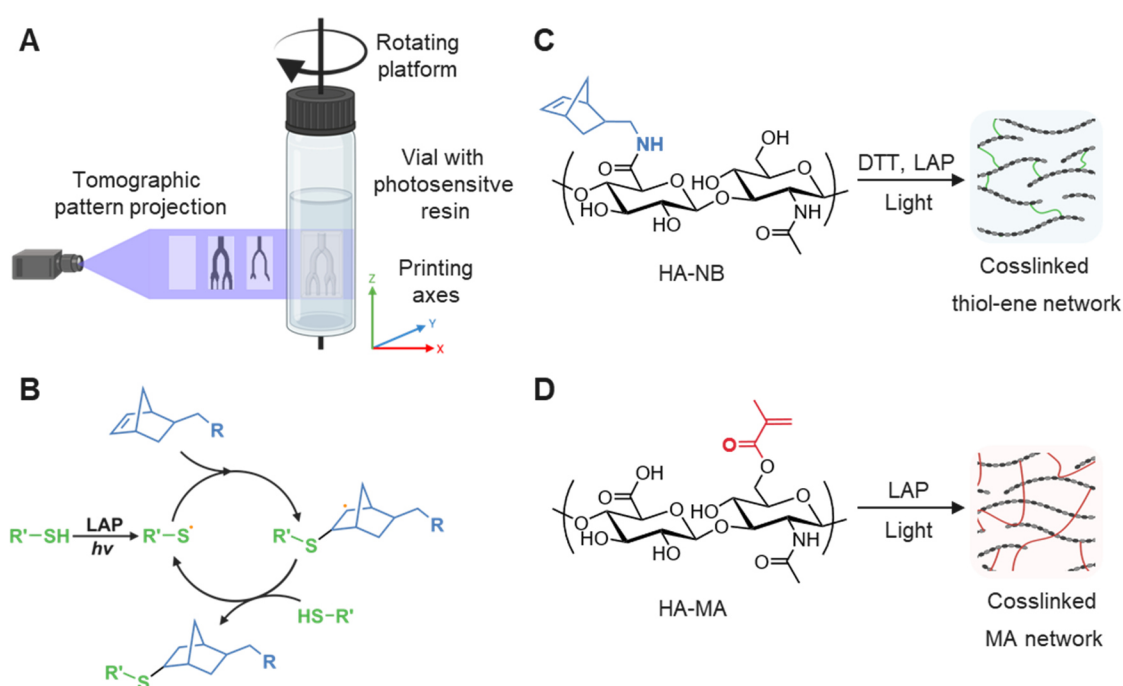


Figure 43. **A.** Tomographic pattern projection in a VP process inside a vial containing a photosensitive resin placed on a rotating platform. The scheme also shows the orientation of the printing axes. *Created with BioRender.com.* **B.** Reaction mechanisms of a photo-initiated radical thiol-ene reaction for crosslinked network via step-growth mechanism. **C.** Crosslinking of thiol-ene resin in the HA-NB hydrogel via step-growth mechanism. **D.** Crosslinking HA-MA resin in the final hydrogel via chain-growth mechanism.

3.2. Results

3.2.1. Synthesis of photocrosslinkable HA derivatives

HA-NB and HA-MA were synthesized following a reported protocol and optimized from the manual synthesis using an automatic titration system using the same commercial HA starting material as in Chapter 2.^[182] The incorporation of the NB and MA functional groups at the carboxylic acid of the disaccharide units was confirmed by ¹H NMR (**Figure 44A**). The degree of functionalization was quantified by comparing the intensity of the vinyl protons to the acetyl proton of the HA backbone as described in Chapter 2. HA-NB and HA-MA polymers with F_{ene} of ~40% were obtained and used in the following experiments which did not differ from the synthesized batches without automatic titration in Chapter 2. No change in the gel GPC elugrams was observed after derivatization, indicating that the reaction conditions did not lead to unwanted side reactions like the crosslinking or degradation of the HA chains (**Figure 44B**). The incorporation of the NB groups led to a slightly greater hydrodynamic radius in comparison to the native HA, while HA-MA showed a slightly lower MW as determined by multi-angle static light scattering (MALLS) analysis of the GPC traces.

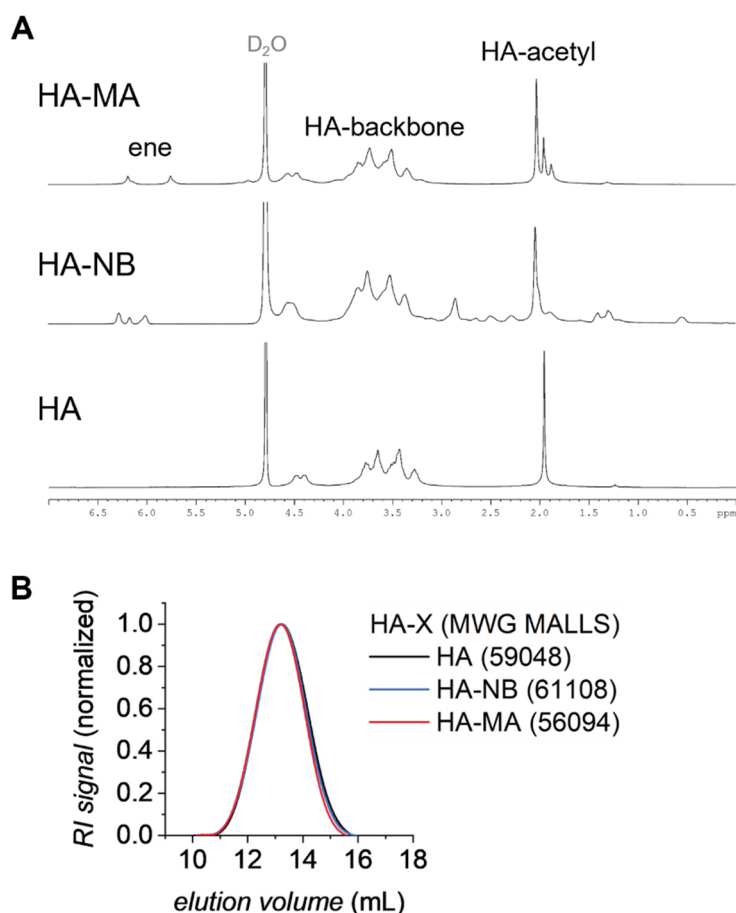


Figure 44. Polymer synthesis and characterization. **A.** Representative ^1H NMR spectra of HA-MA, HA-NB and starting material HA in D_2O . **B.** Representative GPC elugrams of HA-MA, HA-NB and starting material HA. The molecular weight indicated in brackets was calculated from the MALLS detector signal.

Raman spectroscopy confirmed the functionalization (**Figure 45**). For native HA, only the vibrations of the amide bonds were detected in the $1500\text{--}1800\text{ cm}^{-1}$ range: amide I at 1655 cm^{-1} and amide II at 1522 cm^{-1} . In the Raman spectrum of HA-MA, the stretching vibration of the ene bond at 1639 cm^{-1} and the vibration of the carbonyl bond of the ester at 1713 cm^{-1} verified the successful attachment of the MA group.^[196] In HA-NB, the ene bond of the NB group was visible at 1570 cm^{-1} and the presence of the second amide bond was reflected in the new band at 1640 and 1551 cm^{-1} .^[197, 198]

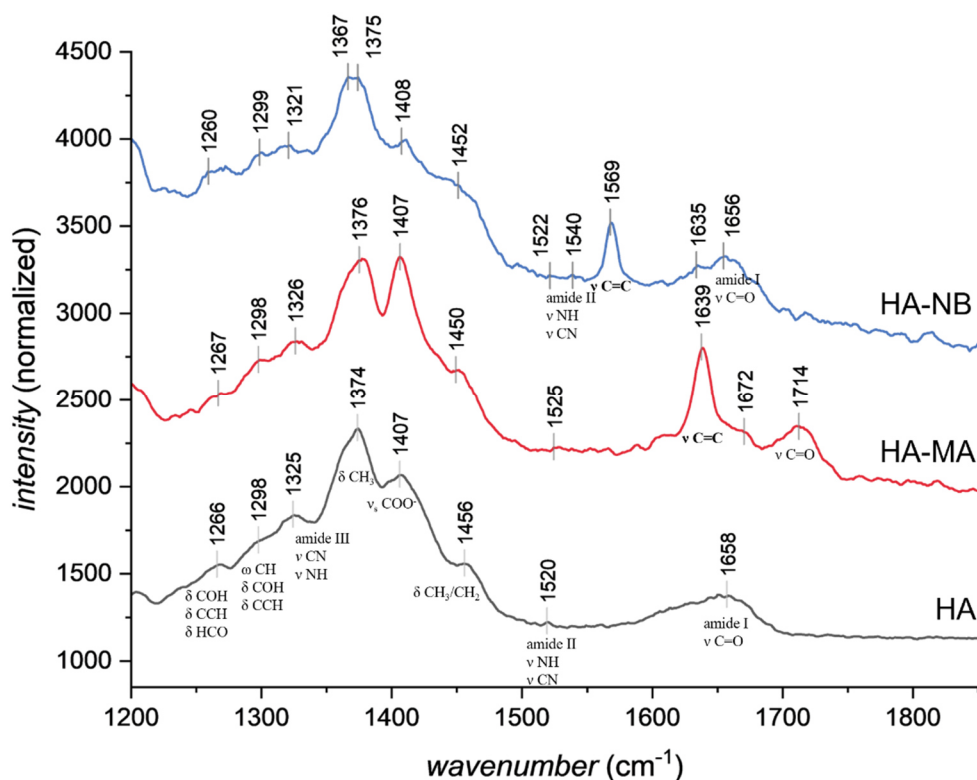


Figure 45. Raman spectra (vertically shifted for clarity) of native HA, HA-MA and HA-NB polymer powders. The HA-NB polymer shows the C=C vibration mode of the norbornene group at 1570 cm^{-1} . The HA-MA polymer shows the C=C vibration mode of the methacrylate group at 1639 cm^{-1} . Both modes are absent in the HA powder.

3.2.2. Optical properties of HA solutions and resins

In VP, the absorption coefficient a of the resin is a quantitative indicator of the expected decrease in light intensity per unit path length. During VP, this value is compensated by the software by increasing the exposure dose at the inner volume of the structures. The absorption in the UV/VIS spectrum of the resin was dominated by the absorbance of the 0.05% w/v LAP photoinitiator (**Figure 46A**). The values of a for the water- and medium-based HA resins were 0.3 and 0.4 cm^{-1} at 400 nm (the lasing wavelength of the volumetric printer). The refractive index (RI) of 4% w/v HA-NB and HA-MA polymer solutions (without LAP) at 400 nm was estimated to be 1.351 using the Cauchy model (**Figure 46B**).^[199]

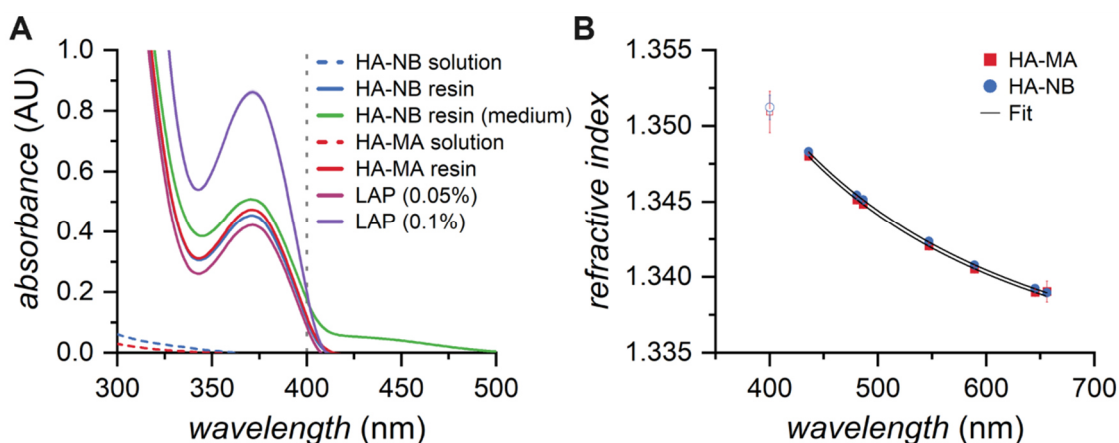


Figure 46. Optical properties of HA-X solutions and resins. **A.** UV/VIS spectra of HA-X resins, polymer and LAP solutions. The samples 0.05% w/v LAP solution, 0.1% w/v LAP solution, 4.0% w/v HA-NB solution and resin, 4.0% w/v HA-MA solution and resin and 4.0% w/v HA-NB resin with medium in DPBS measured with a UV/VIS spectrophotometer. All resins contain 0.05% w/v LAP, the thiol-ene resins HA-NB and HA-NB (medium) contain DTT in a thiol:ene ratio of 1. The polymer solutions HA-NB and HA-MA did not contain photoinitiator. The dotted line indicates the printing wavelength at 400 nm from which the absorbance values were used to calculate the absorption coefficient a . **B.** Refractive index (RI) of HA-NB and HA-MA solutions at 4% w/v in DPBS measured at different wavelengths (closed symbols). The experimental results were fitted to the Cauchy model and used to calculate the RI at 400 nm (open symbol). Data are shown as mean $\pm$ standard deviation for $N = 3$ measurements.

3.2.3. Viscosity of HA solutions

The viscosity of 4% w/v HA-NB and HA-MA solutions in DPBS buffer was measured by rheology in oscillatory temperature sweeps. At room temperature, a complex viscosity value of 0.07 ± 0 and 0.09 ± 0.02 Pa s was obtained for the NB and MA derivatives, respectively (**Figure 47A**). For VP, a counterplay between viscosity and photocrosslinking kinetics is needed to avoid part sedimentation during light exposure. Typically, viscosity values > 4 Pa s are used in resins for VP.^[141, 195] For the 4% w/v HA-X solutions, addition of gelatin at 2% w/v allows increasing the viscosity to 0.13 ± 0.01 Pa s at room temperature and 24.44 ± 1.22 Pa s at 4 °C (**Figure 47B**). These values are within the range found by other authors who printed HA-MA in combination with sacrificial gelatin^[144]. The following experiments will not use gelatin, but these values provide a framework to better understand the results of this thesis.

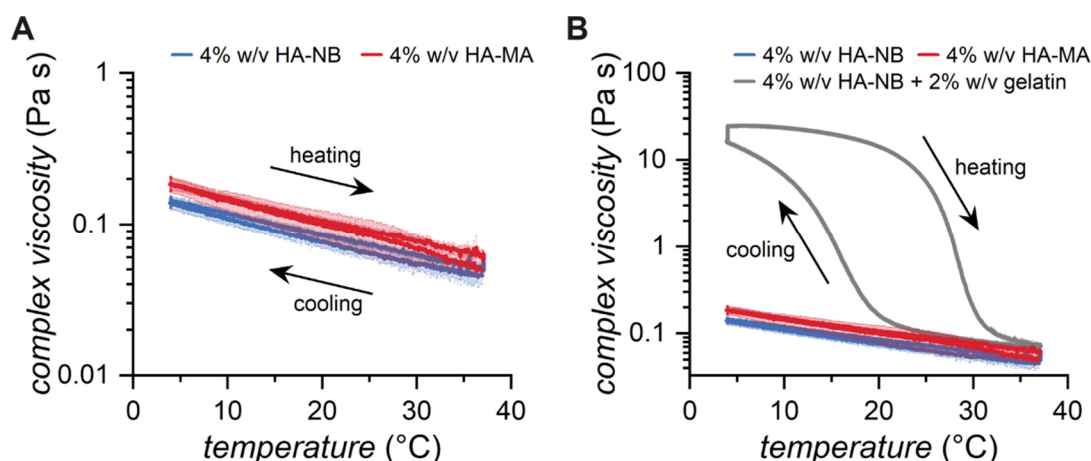


Figure 47. Rheological study of HA-X polymer solutions. **A.** Change of complex viscosity during cooling and re-heating cycle of HA-NB and HA-MA solutions at 4% w/v in DPBS. **B.** In addition, the thermoresponsive change of the complex viscosity of a HA-NB solution containing 2% w/v gelatin was measured. Data are shown as mean $\pm$ SD of $N = 3$ samples.

3.2.4. Photocrosslinking kinetics of HA solutions

To investigate the photocrosslinking kinetics of 4% w/v HA-MA and HA-NB resins, oscillatory shear rheology and in situ illumination at 400–500 nm was used. 0.05% w/v LAP was added to the resins as photoinitiator. This is lower than the photoinitiator concentration used for DLP-based printing of the resins to decrease the absorbance and minimize light attenuation during the VP process. DTT was added as dithiol crosslinker to the HA-NB resin in a thiol:ene ratio of 1. **Figure 48A** shows the increase of the shear storage and loss moduli during light exposure, confirming polymerization of both resins at these conditions. As before, the gelation time (t_{gel}) is defined as the time to reach the gel point at which the storage (G') and loss moduli cross (G''), $G' = G''$.^[192] **Table 3** shows the comparative values of t_{gel} for the different hydrogels, as extracted from the rheology curves, together with the values of G' after 20 min of light exposure. The HA-NB resin showed a $t_{\text{gel}} = 20.0 \pm 1.1$ s and reached a plateau value of G' after approx. 60 s of irradiation. The HA-MA resin showed a slower increase in G' with exposure, with $t_{\text{gel}} = 65.4 \pm 5.6$ s. The differences in the crosslinking rate agree with the different underlying crosslinking mechanisms. The HA-NB resin crosslinks via a radical thiol-ene reaction with step-growth mechanism and has a fast reaction kinetics as consequence of the strain release from the NB ring upon the addition of the thiyl radical.^[52] The HA-MA

resin crosslinks via a radical chain-growth mechanism. Despite their differences in crosslinking kinetics, both hydrogels reached a similar G' of 16.8 ± 1.2 kPa for HA-NB and 20.1 ± 2.6 kPa for HA-MA after 20 min of irradiation. Presumably, the slower crosslinking as consequence of lower light intensity and lower photoinitiator concentration in comparison to the results in Chapter 2 led to higher precision in the network formation with higher conversion. The conversion of crosslinked hydrogels will be discussed in section 3.2.7. Strain and frequency sweeps were conducted to determine the linear viscoelastic region and to validate the used measurements parameters (**Figure 48B, C**).

The gelation dose, which is the cumulated light dose at t_{gel} , meaning the light dose to induce gelation, was compared (**Table 3**). A gelation dose of 132.8 ± 7.4 mJ cm⁻² and 433.8 ± 37.0 mJ cm⁻² were observed for HA-NB and HA-MA respectively. Those values can be considered as first indication for reactivity of the resins in VP and indicate that a 3.3-times lower light dose is expected for printing HA-NB vs. HA-MA. These values will be compared with dose tests in VP studies in the following sections.

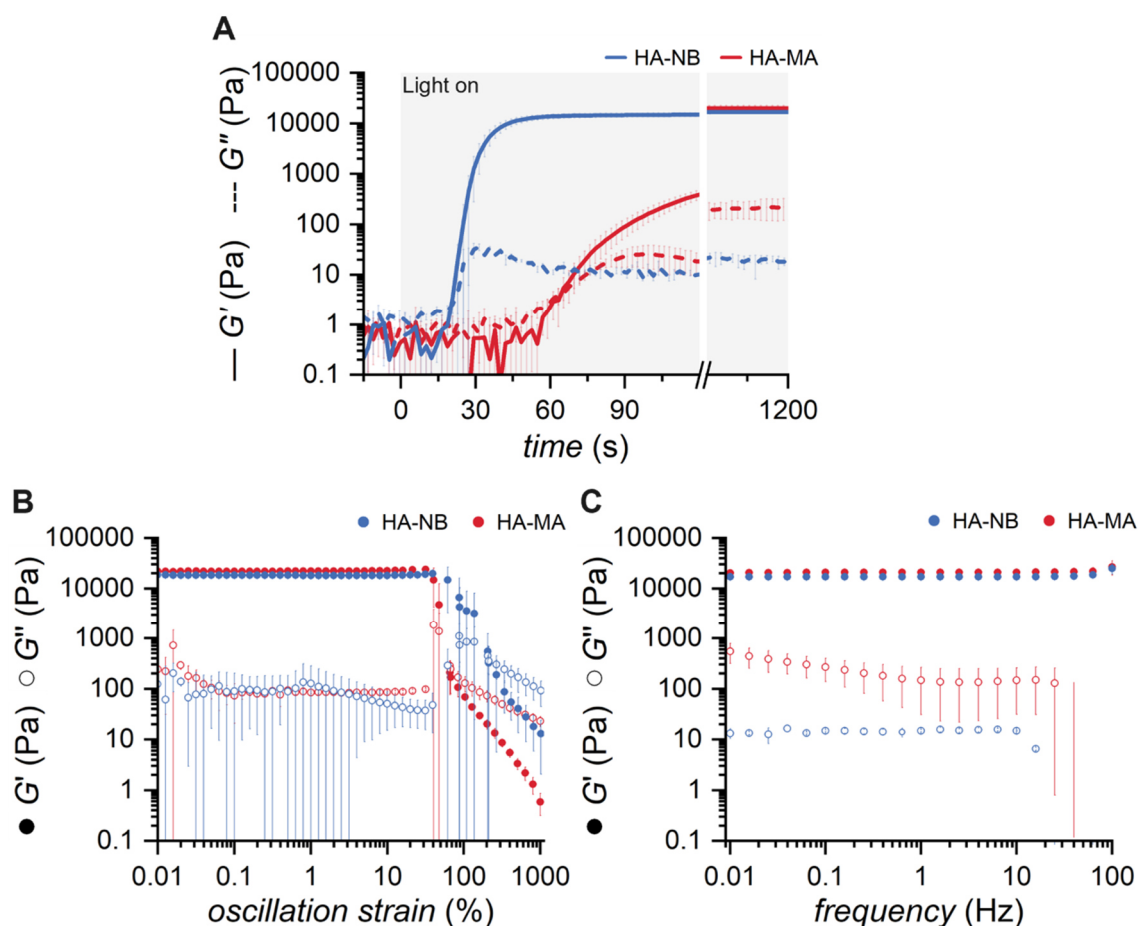


Figure 48. Rheological study of HA-X resins. **A.** Potorheology experiment. Evolution of shear storage modulus G' and loss modulus G'' of HA-NB and HA-MA resins in DPBS during illumination (400–500 nm, 6.6 mW cm⁻²). Exposure started at $t = 0$ s. The shaded box indicates the period when the light was switched on. The resins contained 4% w/v of HA polymer and 0.05% w/v LAP as photoinitiator. The HA-NB resin contained DTT as crosslinker at thiol:ene = 1. **B.** Strain sweep with increasing oscillation strain between 0.01 and 1000% at 2 Hz frequency. **C.** Frequency sweep with increasing frequency between 0.01 and 100 Hz at 1% strain. The parameters used for the time sweep photorheology measurements were set at 1% strain and 2 Hz frequency are within the linear viscoelastic region for HA-NB and HA-MA hydrogels were stress and strain are proportional and the storage modulus G' is independent from the set oscillation strain and frequency. For each rheology experiment, $N = 3$ independent measurements were performed. Data are shown as mean $\pm$ SD.

Table 3. Gelation time t_{gel} , gelation dose, and final shear storage and loss modulus of HA-NB and HA-MA resins determined from oscillatory shear rheology curves with in situ illumination. The resins contained 4% w/v HA-NB or HA-MA, respectively and 0.05% w/v LAP as photoinitiator. The HA-NB resin contained DTT in a final thiol:ene ratio of 1.0. Data are expressed as mean $\pm$ SD for $N = 3$.

Resin	Gelation time t_{gel} (s)	Gelation dose at t_{gel} (mJ cm ⁻²)	Storage Modulus G' (kPa) ^a	Loss Modulus G'' (kPa) ^a
HA-NB	20.0 $\pm$ 1.1	132.8 $\pm$ 7.4	16.8 $\pm$ 1.2	0.02 $\pm$ 0
HA-MA	65.4 $\pm$ 5.6	433.8 $\pm$ 37.0	20.1 $\pm$ 2.6	0.22 $\pm$ 0.11

^a Moduli correspond to hydrogels after 20 min of light exposure.

3.2.5. Setting of VP conditions by determination of the critical gelation threshold

To define the minimum exposure dose for crosslinking HA-NB and HA-MA resins in a VP process, a light dose test by projecting an array of dots with a diameter of 0.5 mm onto the resins using increasing light doses was performed (**Figure 49A**). The critical gelation threshold (CGT) defines the light dose at which solidification of the resin becomes visible.^[147] **Figure 49B** shows exemplary images of the light dose tests performed with the HA-NB and HA-MA resins. The CGT was 126 ± 7 mJ cm⁻² for HA-NB and 433 ± 29 mJ cm⁻² for HA-MA (**Figure 49C**). The 3.4-times lower CGT of HA-NB vs. HA-MA correlates with the 3.3-times lower gelation dose of HA-NB vs. HA-MA at t_{gel} observed in photorheology experiments. Similar results were observed by Rizzo et al., who found a CGT for a Gel-NB/PEG4SH resin to be 3.6 to 4.0-times lower than for the Gel-MA resin.^[72]

The diameters of the crosslinked HA-MA dots were < 0.5 mm whereas HA-NB dots were ~ 0.5 mm for the different exposure doses. The targeted diameter was 0.5 mm. This result suggests a higher spatial control in the photocrosslinking and network formation of thiol-ene hydrogels and aligns with the in Chapter 2 observed dark curing upon sequential exposure in photorheology experiments.

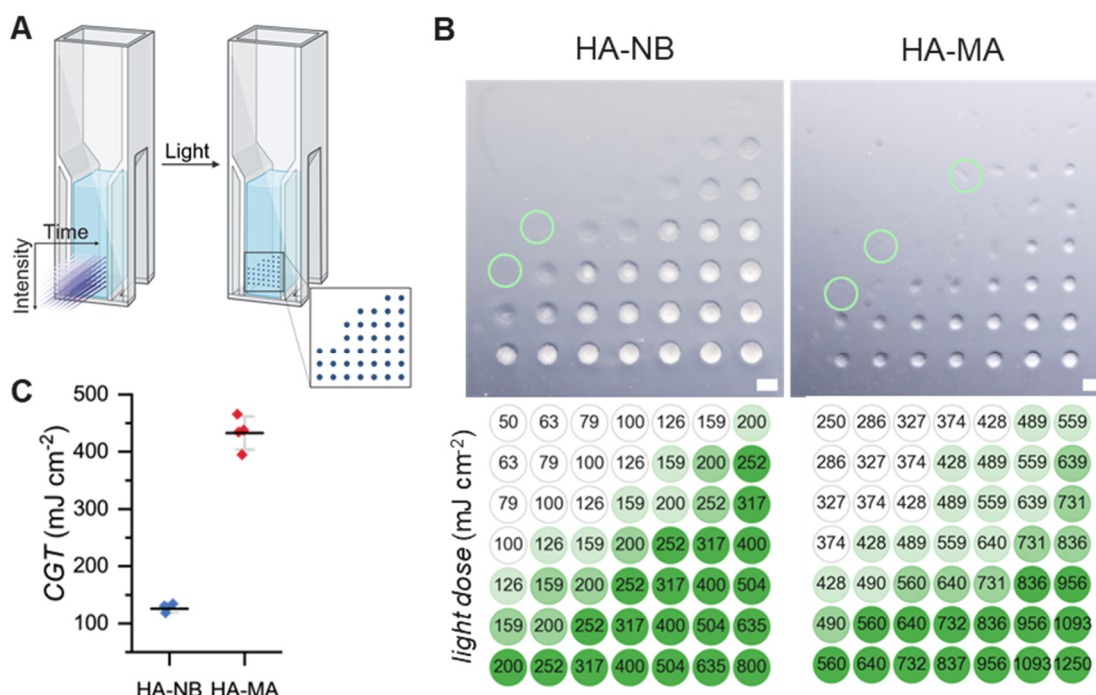


Figure 49. Light dose test to determine the CGT. **A.** Schematic representation of light dose test. An array of dots with different light doses is projected onto the resin filled in a cuvette. *Created with BioRender.com.* **B.** Images of dose tests for HA-NB and HA-MA resins with respective light doses. Dots crosslinked with the lowest light dose corresponding to CGT are marked. Scale bar represents 0.5 mm. **C.** CGT for 4.0% w/v HA-NB and 4% w/v HA-MA resins with 0.05% w/v LAP at 400 nm. The HA-NB resin contained DTT in a thiol:ene ratio of 1.0. Data are expressed as mean ± SD for N = 4 independent samples.

3.2.6. Printability and dimensional error

To evaluate and compare the printability of the HA-NB and HA-MA resins, a rectangular scaffold with 8 mm height, 5 mm width and 2 mm thickness (volume 80 mm³) containing channels with 800, 600, 400 and 200 μm diameter was printed. The position of the scaffold in relation to the light beam is shown in **Figure 43A**. For each resin, the light dose distribution was optimized by the printing equipment considering the RI and absorption coefficient *a* of the resin.

For assessing printability in the initial studies, the external dimensions of the scaffold were evaluated. Scaffolds were printed at the CGT suggested by the dose test, and at ±10% of the CGT stepwise until an under- or overcured printed structure was observed. The formation of the structure in the vial during polymerization was visible by the slight

change in the RI. The printing fidelity was quantified by imaging and quantification of the dimensional error (**Figure 50**). In this analysis, the width of the printed structure (along the x-axis of the vial) was compared with the theoretical width of the CAD model. The dimensional error was calculated as deviation from the theoretical width.^[200] Prints with dimensional error below -5% were classified as undercured and those with dimensional error above +5% as overcured.

HA-NB scaffolds printed at CGT (126.0 mJ cm^{-2} , 16.2 s) were undercured (**Figure 50A**, **Table 4**). By increasing the light dose by 10%, scaffolds with well-defined borders and targeted dimensions were obtained. At light doses between 138.6 and 176.4 mJ cm^{-2} , HA-NB scaffolds with dimensional error $<5\%$ were printed. At a light dose $>189.0 \text{ mJ cm}^{-2}$, scaffolds appeared overcured. Interestingly, a linear relationship was obtained between the light dose and the dimensional error. The scaffolds were mechanically stable and could be handled with spatulas without damage.

Printing of the HA-MA resin showed a different behavior. At the CGT (433.0 mJ cm^{-2} , 56.0 s) the printed structure was very soft and difficult to handle, but it appeared overcured according to our definition, with a dimensional error of $7.9 \pm 3.4\%$ (**Figure 50B**, **Table 5**). The dimensional error increased at light doses 10 or 20% higher (476.3 mJ cm^{-2} , 61.5 s and 519.6 mJ cm^{-2} , 67.5 s). This overcuring effect is attributed to the post-exposure reactivity typical of radical chain-growth crosslinking mechanisms.^[182] This is referred to as dark curing and is due to the retarded termination reaction of slow diffusing macroradicals which continue propagating once the light is switched off. The propagating macroradical diffuses out of the spatially illuminated areas and, therefore, spatial and temporal control over the photocrosslinking reaction is compromised. This is the reason for the apparent overcuring. In reality, the scaffold is undercured and the lower printing fidelity is due to the loss of spatial resolution as consequence of the radical chain-growth reaction. By lowering the light dose by -10% to 389.7 mJ cm^{-2} (50.4 s), scaffolds with an apparent dimensional error of $1.1 \pm 1.7\%$ were printed, but they were not mechanically stable, indicating that a network with very low crosslinking had been generated. Similar to the dimensional error of the HA-NB resin, a linear relationship in light dose and dimensional error of the HA-MA resin was found. The comparison between HA-NB vs. the

HA-MA resin demonstrates the superior printability of the thiol-ene resins in terms of (i) lower light dose, (ii) faster printing time, and (iii) the generation of cured, stable scaffolds that do not require post-printing, and showed (iv) higher printing fidelity.

To assess the performance of HA-NB resin in a bioprinting scenario, the printability of the HA-NB resins in medium was evaluated and compared to the results with the printability in buffer. Complete RPMI 1640 medium was used. The culture medium has a higher absorbance than buffer (**Figure 46A**) and contains biomolecules with thiol groups which could interfere with the crosslinking reaction. The amount of thiol groups in this medium is <0.04 mM, which is two orders of magnitude lower than the molar concentration of ene groups in the thiol-ene resin (35 mM) and therefore should not interfere with the photocrosslinking kinetics. While the HA-NB resin was printable at 176.4 mJ cm^{-2} dose with a $<5\%$ dimensional error, no scaffold was obtained at this exposure dose when printing in medium (**Figure 50C**, **Table 6**). The HA-NB (medium) resin became printable at light doses between 189.0 and 226.8 mJ cm^{-2} with a dimensional error $<5\%$. A linear relationship between light dose and dimensional error was observed, with lower slope than for the HA-NB resin.

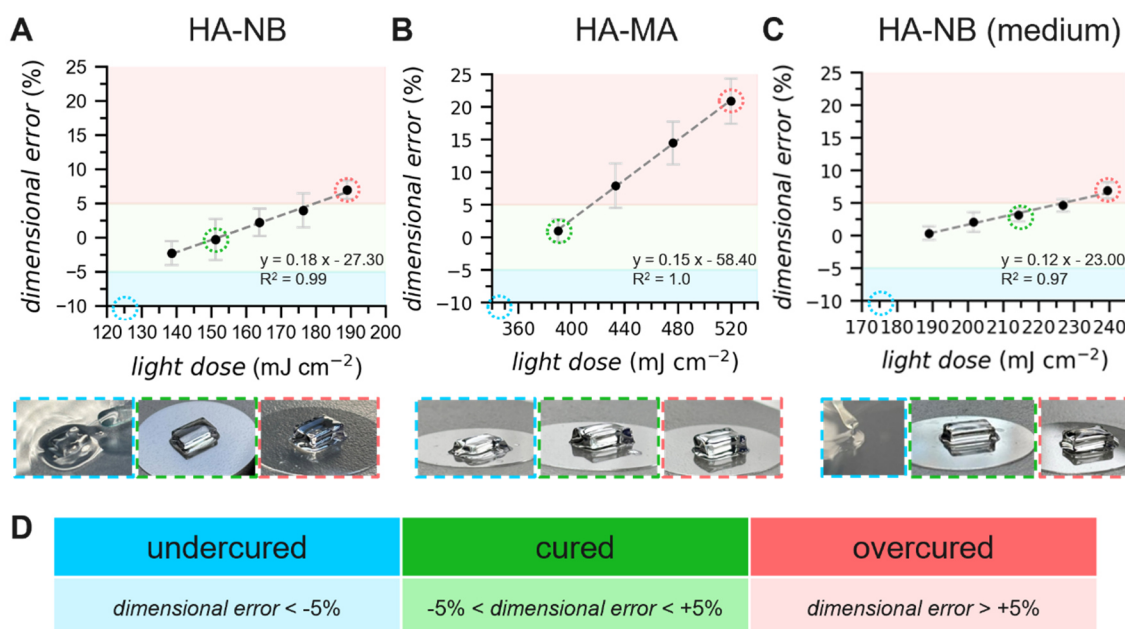


Figure 50. Volumetric printing of resins to define printability and dimensional error of the **A.** HA-NB, **B.** HA-MA and **C.** HA-NB (medium) resin. The dimensional error along the x-axis of the scaffold was determined at different light doses. For each resin, exemplary images of printed scaffolds placed on a 25 mm coverslip with different dimensional error are

depicted below the plots. A linear fit was performed demonstrating linear relationship between light dose and dimensional error. Data are shown as mean $\pm$ SD for $N \geq 3$ samples. **D.** A dimensional error $< -5\%$ was defined as undercured (blue), $> 5\%$ as overcured (red) and between $\pm 5\%$ as cured (green).

Table 4. Printability results and properties of the volumetric printed scaffold with the HA-NB resin. The light dose was varied stepwise from the CGT. For each light dose, the dimensional error, dimensional swelling and storage modulus were determined for $N \geq 3$ samples.

	Light dose (mJ cm ⁻²)	Print time (s)	Dimensional error (%)	Dimensional swelling (%)	Storage modulus (kPa)
CGT	126	16.2	not printable ^a		
CGT +10%	138.6	17.8	-2.2 ± 1.7	23.6 ± 6.7	9.4 ± 2.7
CGT +20%	151.2	19.5	-0.2 ± 3.0	20.9 ± 4.7	14.0 ± 0.8
CGT +30%	163.8	21.1	2.3 ± 2.0	13.6 ± 4.0	13.1 ± 0.5
CGT +40%	176.4	22.8	4.0 ± 2.5	14.3 ± 3.3	14.6 ± 0.4
CGT +50%	189.0	24.4	7.0 ± 1.3	10.0 ± 2.8	

^a no scaffold with the desired dimensions was obtained.

Table 5. Printability results and properties of the volumetric printed scaffold with the HA-MA resin. The light dose was varied stepwise from the CGT. For each light dose, the dimensional error, dimensional swelling and storage modulus were determined for $N \geq 3$ samples.

	Light dose (mJ cm ⁻²)	Print time (s)	Dimensional error (%)	Dimensional swelling (%)	Storage modulus (kPa)
CGT -20%	346.4	44.7	not printable ^a		
CGT -10%	389.7	50.4	1.1 ± 1.7	48.1 ± 1.9	1.0 ± 0.1
CGT	433.0	56.0	7.9 ± 3.4	43.3 ± 3.4	1.1 ± 0.1
CGT +10%	476.3	61.5	14.5 ± 3.3	41.6 ± 2.6	1.4 ± 0.1
CGT +20%	519.6	67.5	20.9 ± 3.4	40.9 ± 4.3	

^a no scaffold with the desired dimensions was obtained.

Table 6. Printability results and properties of the volumetric printed scaffold with the HA-NB (medium) resin. The light dose was varied stepwise from the CGT. For each light dose, the dimensional error, dimensional swelling and storage modulus were determined for $N \geq 3$ samples.

	Light dose (mJ cm ⁻²)	Print time (s)	Dimensional error (%)	Dimensional swelling (%)	Storage modulus (kPa)
CGT +40%	176.4	22.8	not printable ^a		
CGT +50%	189.0	24.4	0.4 ± 1.1	35.0 ± 4.2	7.8 ± 1.3
CGT +60%	201.6	26.0	2.1 ± 1.5	32.0 ± 3.5	8.7 ± 3.1
CGT +70%	214.2	27.7	3.2 ± 1.0	27.2 ± 3.5	8.6 ± 0.5
CGT +80%	226.8	29.3	4.7 ± 1.0	22.8 ± 3.2	9.7 ± 0.5
CGT +90%	239.4	30.9	6.9 ± 1.3	19.9 ± 1.9	9.2 ± 0.3

^a no scaffold with the desired dimensions was obtained.

The dimensional errors in the previous paragraphs refer to the dimensions of the scaffold within the xy plane of the VP vial. When analyzing the dimensional error in the z direction, values within the ±5% error range were obtained for all resins and light doses and no clear trend between light dose and z-axis dimensional error was observed (**Figure 51A**). This difference can be attributed to the disposition of the scaffold in relationship to the direction of the light beam, which is illustrated in **Figure 43A**. The width of the scaffold is aligned parallel to the direction of the light beam. During prolonged exposure, a higher exposure dose is expected to occur at the edge of the structure, with the received light dose potentially exceeding the CGT. Overexposure at this location would result in overcuring, i.e. $d_{\text{Print}} > d_{\text{CAD}}$. In contrast, the length of the scaffold is oriented perpendicularly to the irradiation direction. Regions above or below the structure are not exposed and overcuring cannot occur due to overexposure. Deviations from the projected structure in the z direction could be a consequence of sedimentation of the structure during printing as consequence of densification during crosslinking. Radical diffusion or scattering could also contribute to the loss of resolution in the z direction. The low dimensional error in the z direction of the structures reveals that the crosslinking of the HA-based resins does not lead to large density differences between the

unpolymerized resins and the polymerized structure and allows high resolution VP of low-viscosity resins. In addition, no sedimentation was visually apparent (**Figure 51B**).

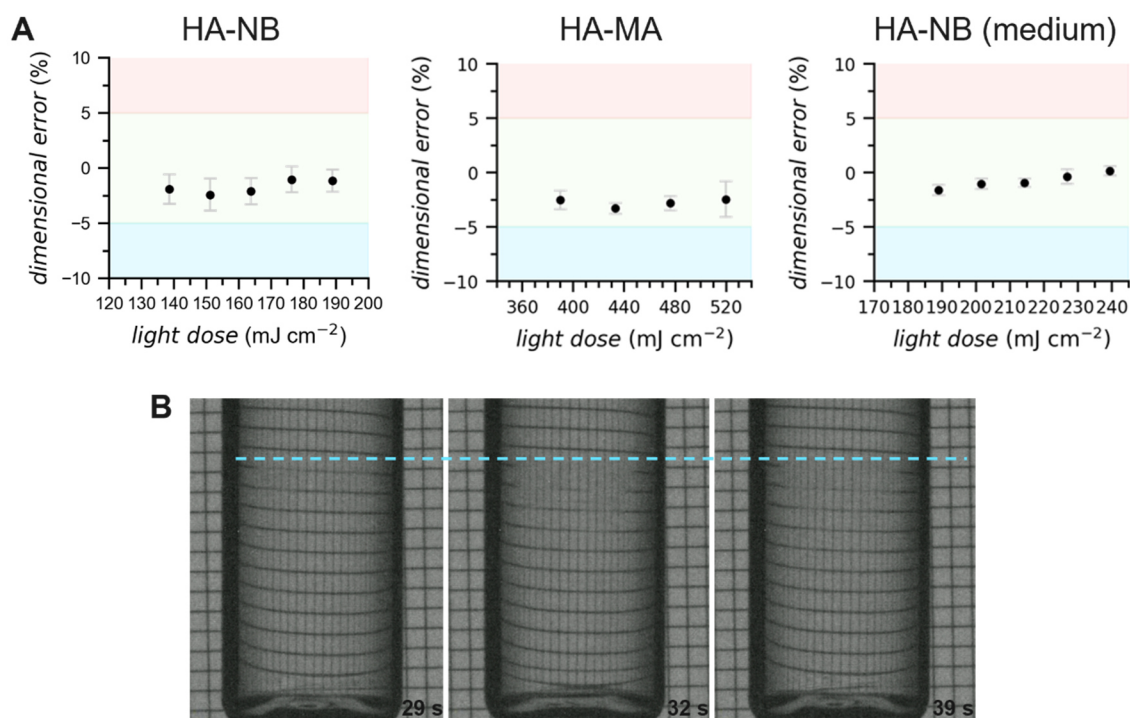


Figure 51. **A.** Dimensional error of the scaffold length (z-axis) printed at different light doses for the HA-NB, HA-MA and HA-NB (medium) resin. **B.** Images of video while printing the branched structure recorded by the Apparite software. Numbers indicate time stamps of the video. At 32 s, a change in RI under the blue line indicates the printed structure. At 39 s the height of the printed structure did not change, no sedimentation was visible.

3.2.7. Monitoring of alkene conversion during VP via Raman spectroscopy

The conversion of the crosslinking reaction in the HA-MA and HA-NB resins was analyzed by monitoring the decrease of the alkene stretching vibration band in the Raman spectrum. **Figure 52** shows the Raman spectra of resins before and after VP at different doses, and after a subsequent post-printing curing step. In HA-NB resins a gradual decrease of intensity was observed in the alkene stretching band as the exposure dose increased. The degree of conversion at the highest dose was <50%. This is in agreement with the rheology results since the light dose used in VP is lower than the dose required to reach the plateau value of G' according to the rheology curves (**Figure S1**). For comparison, Rizzo et al. found a NB conversion of 8–14.5% in volumetric printed 2.5 and

5.0% w/v Gel-NB/PEG4SH scaffolds using 0.05% w/v LAP as photoinitiator and 80 or 90 mJ cm⁻² light dose.^[72] VP of 10.0% w/v Gel-NB/Gel-SH led to a conversion of approx. 60% with 0.05% w/v LAP at a light dose of 56 mJ cm⁻².^[157] The dose provided at the post-printing curing step consumed the remaining crosslinking groups.

In HA-MA resins, no visible decrease of the alkene stretching band was observed after VP, and a conversion around 40% was observed at full exposure. These results are in agreement with the VP and rheology results, which showed a mechanically weak hydrogel print. A literature revision confirms that VP printing with MA-functionalized resins is typically followed by a post-printing curing step for stabilization of the printed structures.^[134, 151, 154, 163]

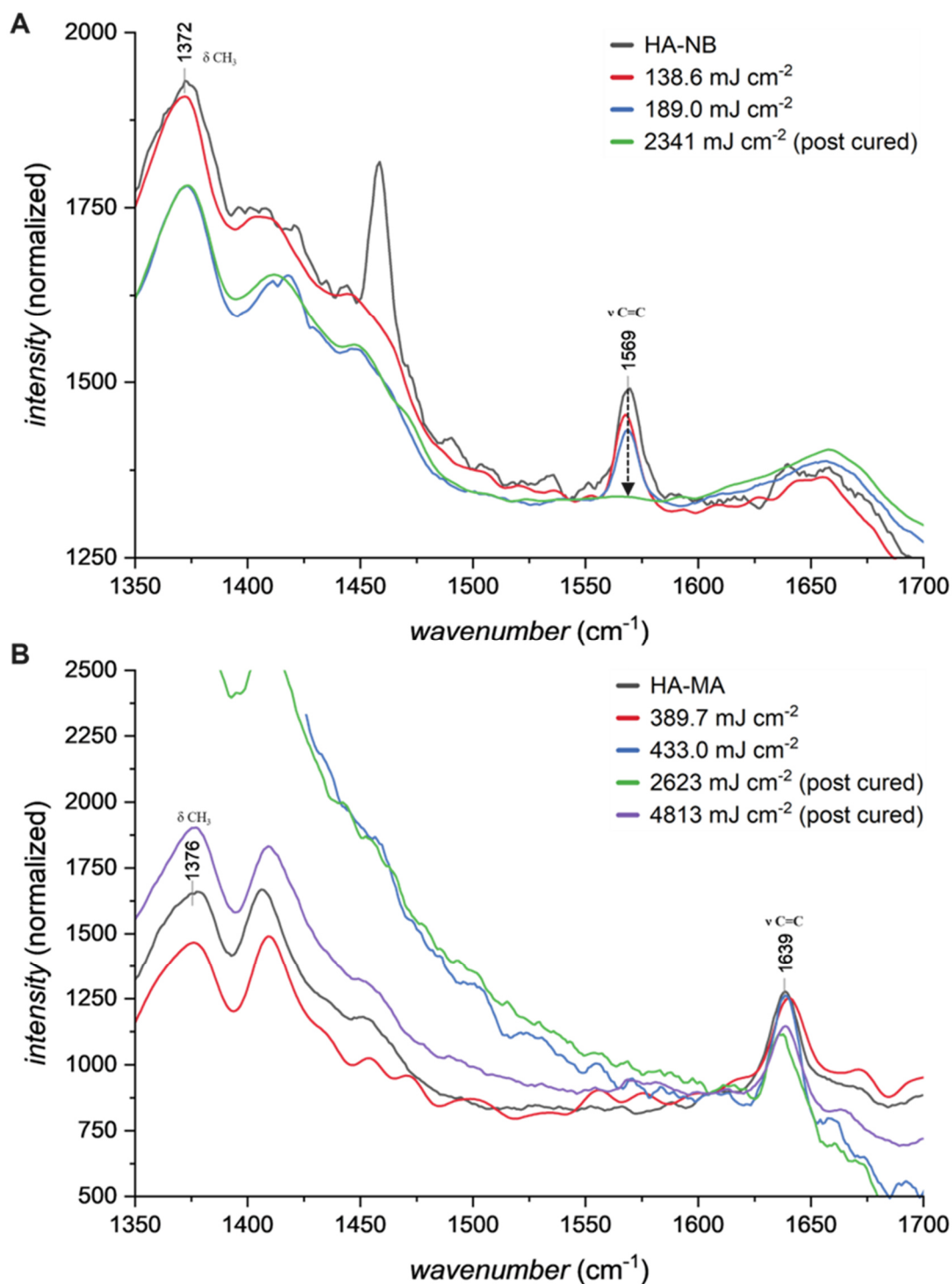


Figure 52. Raman spectra of HA-NB and -MA resins before and after VP. **A.** Raman spectra of HA-NB polymers and printed or post-cured scaffolds. The printed scaffolds were subjected to a light dose 138.6 and 189 mJ cm^{-2} and the total light dose for post-cured (5 min) scaffolds was 2341 mJ cm^{-2} . Decrease of the alkene stretching band at 1570 cm^{-1} indicates the decrease of norbornene groups upon crosslinking. Only in post-cured scaffolds, the stretching band has almost disappeared, indicating a nearly quantitative consumption of norbornene vinyl groups. The spectra were scaled to the methyl deformation band at 1373 cm^{-1} . **B.** Raman spectra of HA-MA polymers and printed or post-cured scaffolds. The printed scaffolds were subjected to a light dose of 389.7 and

433.0 mJ cm⁻². Post-cured scaffolds were exposed to a total light dose of 2623 mJ cm⁻² (5 min) or 4813 mJ cm⁻² (10 min). No decrease in alkene stretching band at 1641 cm⁻¹ is observed for printed scaffolds indicating that only a few methacrylate groups were crosslinked. Only in post-cured scaffolds has the stretching band decreased. The spectra were scaled to the carbonyl deformation band at 1375 cm⁻¹.

3.2.8. Physical characterization of printed scaffolds

The swelling of the printed scaffolds (DPBS, 37 °C) was quantified (**Figure 53A–C**, **Table 4–Table 6**). HA-NB scaffolds showed swelling degrees between 10.3 ± 3.1 and 21.8 ± 5.1% with decreasing light dose. HA-MA scaffolds showed swelling degrees between 40.9 ± 4.3 and 48.1 ± 1.9%. The higher swelling of HA-MA scaffolds reflects their lower degree of conversion after VP. The printed HA-NB (medium) scaffold showed a swelling between 19.7 ± 1.9% and 34.4 ± 5.0% with decreasing light dose. The storage moduli of the prints were measured by dynamic mechanical analysis (DMA) in a strain sweep test (**Figure 53D–F**). The highest stiffness was obtained for HA-NB structures, with storage moduli between 9.4 ± 2.7 and 14.6 ± 0.4 kPa with increasing light dose. Photopolymerization in cell culture medium resulted in softer gels (7.8 ± 1.3 to 9.7 ± 0.5 kPa). HA-MA prints showed a storage modulus between 1.0 ± 0.1 and 1.4 ± 0.1 kPa. The stiffness in printed HA-MA gels is in alignment with the low conversion and the high swelling.

In summary, hydrogels printed via radical thiol-ene reaction outperformed the HA-MA resin in terms of mechanical stability. Stable HA-NB prints with dimensional error <5% could be obtained at 2.8-times lower light dose. The prints had a 14-times higher stiffness than HA-MA ones and did not need post-curing steps for stabilization.

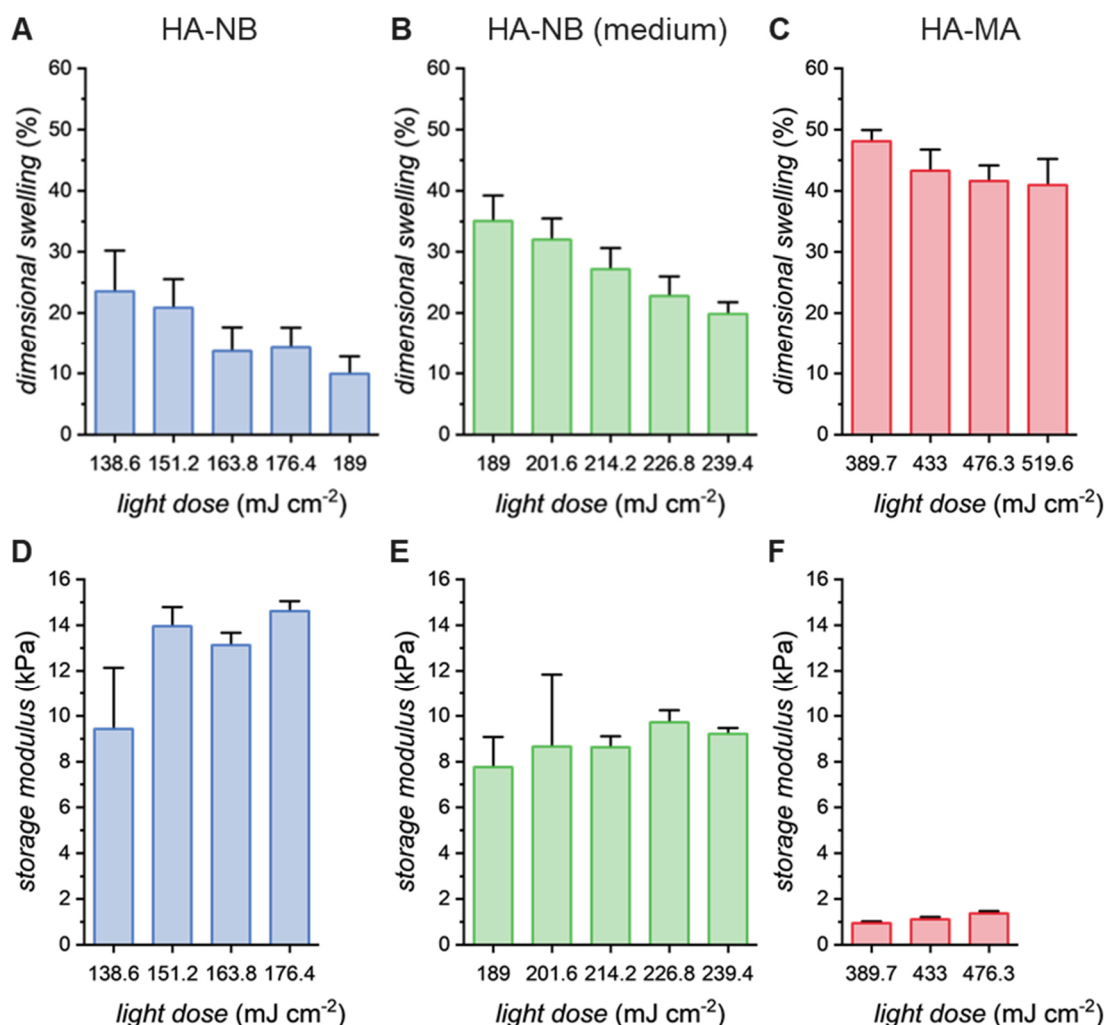


Figure 53. Dimensional swelling and storage modulus of scaffolds printed with different light doses using the HA-NB (A, D), HA-NB (medium) (B, E) and HA-MA (C,F) resins. **A–C.** Dimensional swelling of the prints was determined by quantified the changes in the scaffold dimensions after incubation in DPBS for 24 h at 37 °C. **D–F.** Storage modulus was determined of printed discs after incubation in DPBS for 24 h at 37 °C out of a strain sweep at 0.1% strain. Data are shown as mean $\pm$ SD. For each light dose, $N \geq 3$ prints were analyzed.

3.2.9. Volumetric printing of scaffolds with internal channels

The printability of internal channel structures in HA-NB resins in DPBS buffer and in medium was evaluated. A bifurcated internal channel structure with decreasing diameter from 1000 to 800, and 600, 400 and 200 μm within the $8 \times 5 \times 2 \text{ mm}^3$ rectangular scaffolds was printed (**Figure 54A, B**). The printed channels were imaged after swelling and injection of a fluorescein isothiocyanate dextran solution (FITC-Dex, 500 kDa). The full

width half maximum (FWHM) of the empty channels was quantified by fluorescence image analysis.

In all printed scaffolds, the 800 and 600 μm channels were visible. The channel width decreased with increasing light dose. The 800 μm channels printed in HA-NB in medium exhibited a higher and the 600 μm channels a lower width than scaffolds printed with HA-NB in DPBS (**Figure 54C-F, Table 7**). The 400 μm channel was only resolved at the lowest light dose in HA-NB resin in medium.

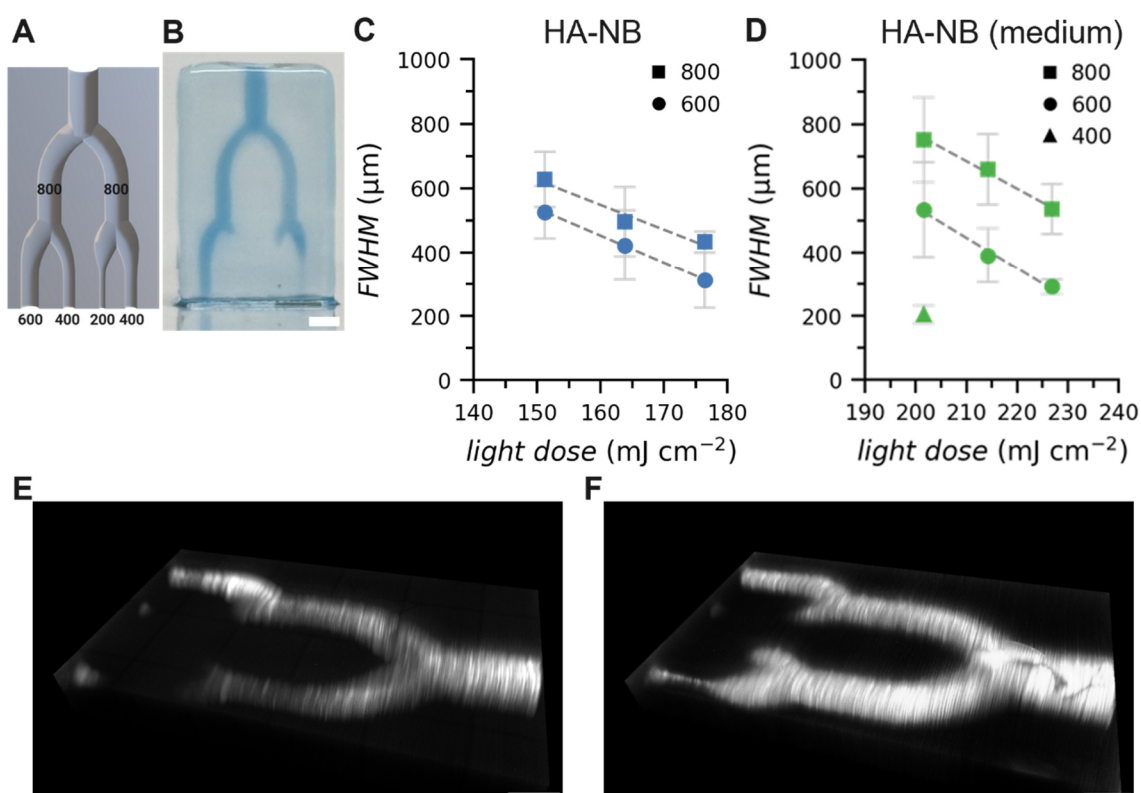


Figure 54. VP HA-NB scaffolds with internal channels in DPBS and medium. **A.** CAD model of the scaffold indicating the width of the channels in μm . **B.** Printed scaffold with channels perfused with blue dextran solution. **C, D.** FWHM of the internal channels as extracted from image analysis of printed HA-NB (**C**) and HA-NB (medium) (**D**) scaffolds after swelling in DPBS. Data are shown as mean $\pm$ SD. **E, F.** Reconstructed 3D fluorescence microscopy images of HA-NB (**E**) and HA-NB (medium) (**F**) scaffolds. Scale bar represents 1000 μm .

Table 7. FWHM of channels measured in swollen HA-NB and HA-NB (medium) scaffolds printed with different light doses. The theoretical model contained channels with 800, 600 and 400 μm diameter. Data are shown as mean $\pm$ SD.

Resin	Light dose (mJ cm^{-2})	$FWHM_{800\mu\text{m}}$ (μm)	$FWHM_{600\mu\text{m}}$ (μm)	$FWHM_{400\mu\text{m}}$ (μm)
HA-NB	151.2	627.3 ± 86.6	525.8 ± 81.3	-
	163.8	496.0 ± 108.0	422.8 ± 109.4	-
	176.4	433.1 ± 33.0	312.9 ± 87.2	-
HA-NB (Medium)	201.6	751.3 ± 131.4	533.6 ± 147.4	204.2 ± 28.0
	214.2	659.3 ± 109.9	390.9 ± 84.0	-
	226.8	535.0 ± 78.0	291.8 ± 23.3	-

3.2.10. Volumetric bioprinting with HA-NB bioresin

*In collaboration with Dr. Aleeza Farrukh at INM-Leibniz Institute for New Materials, the printability of HA-NB-based resin was tested in bioprinting experiments in combination with cells. She prepared the cell culture and performed the experiments for cell viability, cell morphology in bioprinted constructs and the corresponding analysis. Images and graphs shown in **Figure 55B-D** were prepared by her.*

The suitability of HA-NB for volumetric bioprinting was tested with L292 fibroblasts. For this purpose, the HA-NB resin was modified with the cell-adhesive peptide cyclo-(RGDfC) and mixed with a suspension of L292 fibroblasts (**Figure 55A**). A composition of 4% w/v HA-NB, 2.2 mM RGD peptide, 0.26% w/v DTT, and 0.05% w/v LAP was used. With this composition >93% of NB groups remain free for photocrosslinking. Upon mixing, the solution remained low viscous, enabling homogenization with the cell suspension by pipetting at low shear forces.

Rectangular scaffolds at high cell density (10^6 cells mL^{-1}) were printed at 400 nm using light doses between 226.8 and 252.0 mJ cm^{-2} and printing times between 29.3 and 32.6 s. With these light doses, stable and cured scaffolds were obtained. Lower light doses led

to undercured scaffolds. In comparison to printing with the HA-NB resin in medium, the addition of cells led to a slightly higher light dose needed for printing.

Uniform distribution of cells across the thickness of the VP-scaffold was observed in confocal z-stack images (Figure 55B). Live/dead assays performed on cells encapsulated for 1 day showed >90% cell viability, when the scaffolds were printed at a 226.8 mJ cm^{-2} (**Figure 55C**), proving the cytocompatibility at this printing condition. This high value of cell viability is in the range of other VP studies with gelatin-based bioresins (**Table 1**). Higher light doses (239.4 and 252.0 mJ cm^{-2}) led to a gradual drop of cell viability (**Figure 55C, Figure S3**). The observed photocytotoxicity at $>240 \text{ mJ cm}^{-2}$ disqualifies 4% w/v HA-MA as bioresin for VP, since this resin required doses $>240 \text{ mJ cm}^{-2}$ for printing. Embedded cells in the bioprinted HA-NB hydrogels remained homogeneously distributed and displayed increasing spreading up to areas of $\sim 350 \mu\text{m}^2$ at day 7 (**Figure 55D, E**). The printed scaffolds with encapsulated cells remained stable without visible degradation or change in shape for over 10 days. These results indicate that HA-NB bioresin with thiol-ene crosslinking is a convenient biomaterial for cytocompatible bioprinting and 3D encapsulation.

Using cell suspensions with lower cell density ($5 \times 10^5 \text{ cells mL}^{-1}$), stable cell-laden scaffolds with internal channels of approx. 300 to 600 μm swollen diameter were successfully printed in 26 s using light dose of 201.6 mJ cm^{-2} . **Figure 55F** and **G** show the image of the cell scaffold with channels perfused with FITC-Dex after staining of the cells with phalloidin.

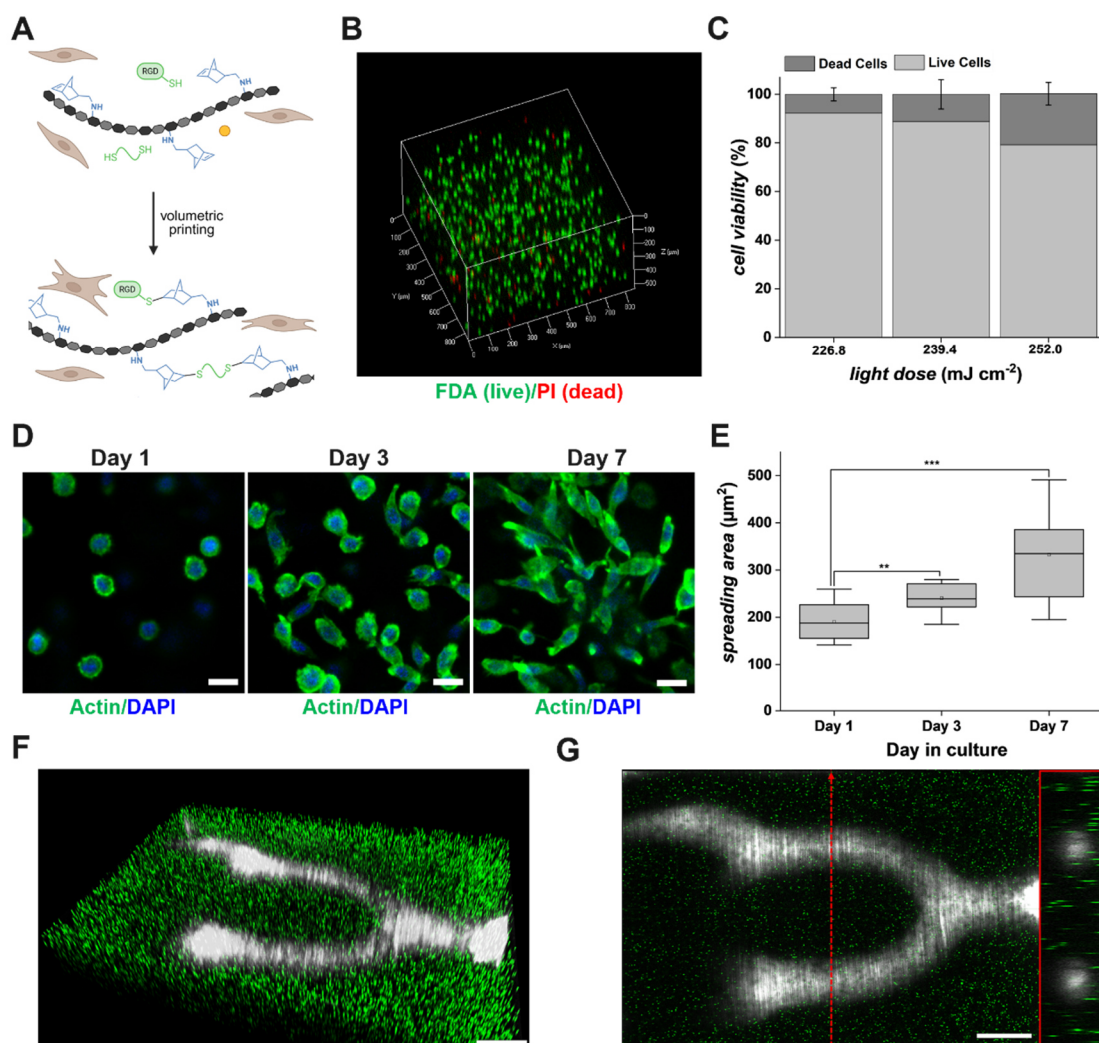


Figure 55. Bioprinting and cell encapsulation in the HA-NB scaffold. **A.** Schematic representation of cell-adhesive peptide (RGD) modified HA-NB resin for volumetric bioprinting. **B.** Z-stack fluorescence image of L929 fibroblasts encapsulated for 1 day in HA-NB scaffolds (composition: 4% w/v HA-NB, 2.2 mM RGD, 0.26% w/v DTT, and 0.05% w/v LAP) using a light dose of 226.8 mJ cm⁻². The live cells were stained with fluorescein diacetate (FDA) (green) and dead cells were stained with propidium iodide (PI, red). The image shows an area of 850 x 850 x 540 μm. **C.** Quantification of L929 cells viability after 1 day of encapsulation in HA-NB scaffold as a function of light dose. The viability was quantified from fluorescence z-stack images of cells after FDA/PI based assay. **D.** Morphology of L929 cells encapsulated in HA-NB scaffolds after 1, 3 and 7 days in culture. The fluorescence images were constructed from confocal z-stack images of scaffolds taken after staining of encapsulated cells with FITC-phalloidin (actin) and DAPI (nucleus). **E.** Spreading areas of encapsulated cells in HA-NB scaffolds were determined from fluorescently labeled F-actin (FITC-phalloidin) by measuring the cell area in the z-stack. Spreading area data are expressed as mean ± SD and statistical analyzed by using one-way ANOVA with Tukey-Kramer post hoc test ** $p < 0.001$, and *** $p < 0.0001$ comparing spreading areas on days 3 and 7, to day 1. **F.** The 3D fluorescence microscopy images of scaffold after bioprinting. Cells were stained for F-actin (TRITC-phalloidin), and the channels perfused with FITC-Dex. Scale bar represents 1000 μm. **G.** The corresponding 2D overview with orthogonal projection in the center of the z-stack image

for the bioprinted scaffold. The red arrow indicates the area for the orthogonal projection. Scale bar represents 1000 μm .

3.3. Discussion

Reported HA-MA-based resins have been applied to VP to print cell-free, cell- or bacteria-laden scaffolds.^[144, 158, 159] A high viscosity to render those resins printable was achieved either by using high molecular weight (1500 kDa)^[159] or by adding gelatin^[144] or Pluronic diacrylate^[158] as thermogelling agents. Scaffolds with internal perfusable channels up to 770 μm diameter have been printed at a light dose of 625 mJ cm^{-2} and 79.2 s print time in buffer and in the absence of cells.^[159] Printing in the presence of bacteria was performed using light doses of 120–140 mJ cm^{-2} with internal void structures in mm-range.^[158] Others report high viability of encapsulated cells without specifying printing conditions or achieved feature sizes.^[144] In this thesis, a low-viscosity (bio)resin based on HA-NB printable with high shape fidelity at light doses of 139–227 mJ cm^{-2} and 20–30 s print time containing internal channels down to approx. 200 μm and high viability of encapsulated cells is presented.

The step-growth mediated thiol-ene crosslinking offers higher spatiotemporal control over the network formation step than acrylate-mediated crosslinking, and is exclusively restricted to the illumination period and is not affected by dark curing.^[182] In this study, this led to an improvement in printability and required light doses from 390 to 139 mJ cm^{-2} . This is similar to reported results where a 50% lower dose (137 vs. 464 mJ cm^{-2}) and 4-times lower photoinitiator concentration was required for Gel-NB in comparison to the equivalent Gel-MA resin to achieve a 2-times higher conversion of functional groups after printing and a 3-times stiffer scaffold.^[76]

With the HA-NB resin, structures with internal channels with diameters between 310–630 μm were printed. The smallest channel which was printable had a theoretical diameter of 600 μm . Using Gel-NB resins, other authors have reported negative features between 120–780 μm ^[76, 156, 157]. Using Gel-MA, features between 100–900 μm have been reported.^[76, 134, 151, 152, 154, 155] For high-viscosity HA-MA resins, features down to 800 μm are reported.^[159] Although a comparison of absolute resolution between the different reports is not possible since the printed patterns and conditions are not equivalent, our low-

viscosity HA-NB resins compete in similar range with established high-viscosity systems, many of which require post-processing steps.^[109] In many reported examples the positive resolution is reported as the minimum distance that can be printed as end of spikes or cones. Values between 23–340 μm have been reported for thiol-ene Gel-based resins^[76, 77, 156, 157], and 42–240 μm for VP of Gel-MA^[76, 151, 154]. Such features, however, are of limited relevance in a scaffold bioprinting context.

Printing velocity in VP is coupled to light dose and the crosslinking kinetics of the resin, including the printing and potential post-processing exposure steps, which is also affected by initiator concentration. The print time for an $8 \times 5 \times 2 \text{ mm}^3$ scaffold with the HA-NB resin evaluated in this study was 18–23 s with light doses of 139–176 mJ cm^{-2} and no post-processing steps.

Bioprinting with HA-NB resin in the presence of RPMI 1640 medium with 10% Fetal Bovine Serum (FBS) required a 50 mJ cm^{-2} higher light dose. When fibroblasts were added, an additional increase of 38 mJ cm^{-2} was needed for a high cell density, and 12.6 mJ cm^{-2} for a lower cell density. This range is in agreement with other reports, where 20–200 mJ cm^{-2} higher light doses were needed in the presence of cells.^[76, 77, 157] In several reports 20–30% iodixanol is included in bioresins for tuning the RI which altered the gelation kinetics and stiffness of the hydrogels.^[87, 151, 157] The bioprinted scaffolds containing channels of 300–600 μm diameter in this work were obtained in the absence of iodixanol. With a cell density of $5 \times 10^5 \text{ cells mL}^{-1}$, perfusable channels with $\sim 300 \mu\text{m}$ diameter were printed in our study. Other reports show perfusable channels in bioprinted scaffolds with a theoretical diameter of 1500 μm using a resin composed of 6% w/v Gel-MA and MSCs.

Further optimization of the HA-NB bioresin to achieve higher resolution might be achievable with the aid of radical inhibitors, like TEMPO, which can reduce out-of-voxel curing.^[147, 201]

3.4. Conclusion

This chapter demonstrated VP of cell-laden scaffolds out of a low-viscosity HA-NB resin which undergoes radical thiol-ene reaction along with DTT as crosslinker. This is the first demonstration of VP of low-viscosity HA-based hydrogels without the addition of gelatin

or other thickening additives. The resin did not require post-exposure steps to achieve high stability. The light dose to print HA-NB with a low dimensional error was ~3-times lower than the dose needed to print HA-MA. HA-NB (with/without medium) scaffolds could be printed in less than 30 s, whereas printing of HA-MA required at least 50 s. Printed HA-NB (with/without medium) scaffolds were stable and swelling and storage modulus could be tuned between 10–35% and 8–14 kPa as function of the light dose during printing.

Perfusable prints with internal channels in swollen HA-NB scaffolds up to ~300 μm were obtained in DPBS buffer, up to ~200 μm in the presence of medium and up to 300 μm in the presence of cells. Fibroblasts were successfully encapsulated in HA-NB bioresin prints in less than 30 s. The bioprinted scaffold showed homogeneous cell distribution, high cell viability and cell spreading in RGD functionalized HA-NB. The results extend the available portfolio of bioresins for VP without the need of thickening agents. The low viscosity of HA-NB facilitates the combination of VP with other printing techniques to achieve higher flexibility, complexity and functionality in scaffold biofabrication.

3.5. Experimental section

3.5.1. Materials

If not stated otherwise, reagents were purchased from Sigma-Aldrich and used as received. HA sodium salt (40–50 kDa according to the manufacturer) was obtained from Biosynth s.r.o (Slovakia), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) from Carbolution Chemicals GmbH (Germany). 5-Norbornene-2-methylamine was purchased from TCI Chemicals (Belgium), DTT (purity 98%) from abcr GmbH (Germany) and D₂O for ¹H NMR spectroscopy was purchased from Deutero (Germany). Cyclo-RGDfC (RGD peptide, purity > 95%) was obtained from GeneCust (France). Dulbecco's Phosphate-Buffered Saline (DPBS) was obtained from Thermo Fisher Scientific (USA).

3.5.2. Methods

3.5.2.1. Synthesis of HA-NB and HA-MA

The synthesis of HA-NB and HA-MA was adapted from our previous report.^[182] The protocol was adapted to an automatic titration system (TitroLine7000, SI Analytics, Germany) which no longer required manual adjustment of the pH during the synthesis. HA-NB was synthesized as amide derivative using EDC and NHS coupling and 5-norbornene-2-methylamine and HA-MA using methacrylic anhydride under slightly basic conditions.

For HA-NB, HA (5.0 g, 12.5 mmol of HA repeating units) and NHS (2.9 g, 2 eq.) were dissolved in Milli-Q H₂O (500 mL) before EDC HCl (9.6 g, 4 eq.) was added as solid. After 30 min, 5-norbornene-2-methylamine (6.1 g, 4 eq.) was added dropwise to the reaction mixture. After the addition of the reagents, the pH was adjusted to 4.75 using 1.0 M HCl. Afterwards, the pH was automatically adjusted to 4.75 using 1.0 M NaOH during the whole reaction process. After 24 h, NaCl was added to obtain a final concentration of 2 M, and after 20 min the reaction mixture was precipitated dropwise in ice-cold EtOH (1000 mL). After filtration, the solid was redissolved in 100 mM NaCl, dialyzed against 100 mM NaCl (1 d) and Milli-Q H₂O (2 d), sterile filtered and lyophilized to obtain HA-NB as solid.

For HA-MA, HA (5.0 g, 12.5 mmol of HA repeating units) was dissolved in PBS (250 mL). The solution was cooled using an ice-bath and methacrylic anhydride (5.8 g, 3 eq.) added dropwise. The pH was adjusted automatically to 8.0 with 1.0 M NaOH (20 mL) and left to stir overnight. The reaction mixture was precipitated in ice-cold EtOH (500 mL), filtered, the precipitate redissolved in Milli-Q H₂O and dialyzed against Milli-Q H₂O for 3 d and sterile filtered. HA-MA was obtained after lyophilization as white solid.

3.5.2.2. ¹H NMR analysis

The degree of functionality of the polymers was determined by ¹H NMR spectroscopy on a Bruker Avance 300 spectrometer (USA) in D₂O at 298 K with a number of scans of 256 and a relaxation delay of 10 s. The spectra were analyzed using Bruker's TopSpin software with the ¹H NMR solvent peak (D₂O: δ = 4.79 ppm) as reference peak for the chemical shifts. The peak area of the acetyl proton signal of HA at 1.97 ppm was compared to the corresponding alkene protons of the attached NB or MA groups in the vinyl region (6.30–6.20 ppm) to calculate the degree of alkene functionalization per HA repeating unit.

3.5.2.3. Gel permeation chromatography

GPC was conducted using a PSS system (Polymer Standards Service, PSS, Germany) equipped with PSS SUPREMA LUX pre-column combined with a PSS SUPREMA LUX analytical column and RI and multi-angle static light scattering (MALLS) detectors. The mobile phase consisted of 0.07 M Na₂HPO₄ and 20% acetonitrile (pH = 9). Analysis was performed in the PSS WinGPC UniChrom Software (v8.31).

3.5.2.4. Raman spectroscopy

Raman spectroscopy measurements were carried out at ambient conditions on a LabRAM HR Evolution HORIBA Jobin Yvon A Raman microscope (France) using a 633 nm He-Ne laser (Melles Griot, IDEX Optics and Photonics, USA) equipped with 1800 lines per 1 mm grating. Polymers were measured as powder, printed scaffolds were dried at 40 °C before the measurement. Post-cured scaffolds were exposed to 405 nm, 7.35 mW cm⁻² for 5 or 10 min after printing.

3.5.2.5. Temperature-dependent measurement of complex viscosity

HA-NB was dissolved at 4.0% w/v either without or with 2.0% w/v gelatin in DPBS. The thermoresponsive behavior was measured using a DHR 3 rheometer (TA instruments, USA) equipped with a temperature control system and a 20 mm parallel plate geometry. The liquid polymer solutions were added to the lower plate, the upper plate was lowered, the solution trimmed and covered with silicon oil and a metal cover to avoid drying or disturbance during the measurement. The temperature was increased to 37 °C and after 5 min of soaking, an oscillatory shear rheology measurement was started using 1% strain and 2 Hz. Using a rate of 1 °C min⁻¹, the temperature was lowered to 4 °C which was held for 10 min and then subsequently increased back to 37 °C with a rate of 1 °C min⁻¹. The change in the complex viscosity was monitored over the temperature. The relation of complex viscosity η^* and complex modulus G^* , storage modulus G' , loss modulus G'' and frequency ω (in rad/s) can be defined as follows:

$$\eta^* = \frac{G^*}{\omega} = \frac{G' + iG''}{\omega} \quad (7)$$

3.5.2.6. Resin preparation

HA-NB or HA-MA was dissolved in DPBS on a shaker in the dark until complete dissolution. The solution was centrifuged (5 min, 3500 rpm) to remove any bubbles and any residue on the walls. To HA-NB, DTT was added from a 5% w/v stock solution to obtain final thiol:ene ratio of 1. LAP was added from a 1% w/v stock solution to HA-NB and HA-MA resins to obtain a final concentration of 0.05% w/v. After each addition, the solution was vortexed thoroughly to allow for homogeneous mixing. In resins containing medium or cells, 20% of DPBS was exchanged against RPMI 1640 medium with GlutaMAX™ supplemented with 10% FBS and Penicillin/Streptomycin with or without cells. The final concentration of HA-NB or HA-MA was 4% w/v.

3.5.2.7. Photorheology experiments to determine crosslinking kinetics of hydrogels

Rheological properties and curing kinetics of HA-NB and HA-MA resins were determined using a DHR 3 rheometer (TA instruments, USA) equipped with a 12 mm parallel plate

geometry and a 20 mm quartz lower plate connected to a UV/Vis illumination source (Omniscure S1500, Excelitas, USA) with a 400–500 nm filter installed to allow in situ curing of hydrogels at room temperature. The uncured hydrogel precursor solutions were added to the lower plate, the upper plate was lowered to a gap of 300 μm , covered with silicon oil and a metal cover to avoid drying or disturbance during the measurement. Strain sweeps (0.1 to 100% strain at frequency = 2 Hz) and frequency sweeps (0.01 to 100 Hz at strain = 1.0%) were performed on crosslinked hydrogels and to perform time sweeps within the linear viscoelastic region. Storage and loss moduli were recorded for 21 minutes with 1.0% strain and 2 Hz frequency. Irradiation of the sample (400–500 nm, 6.6 mW cm^{-2}) was started automatically by the TRIOS software after 1 minute. The gelation time t_{gel} was determined as time point where $G' = G''$. The stiffness of the hydrogels was determined as G' after 20 min of irradiation. In general, the light dose is the product of light intensity and exposure time. From the exposure profile of the light source with installed 400–500 nm filter used in the rheology experiments, the total intensity over the whole wavelength range can be retrieved. By multiplying this by the gelation time t_{gel} , the gelation dose in the rheology experiment can be calculated. It is important to acknowledge that, while the volumetric printer utilizes a narrow wavelength of 400 ± 1 nm, the profile of the 400–500 nm filter demonstrates peak intensities at 405 and 437 nm.

3.5.2.8. Absorbance measurement

HA-NB, HA-MA and HA-NB (medium) resins were prepared as described before. The resins were filled into a quartz cuvette with 10 mm path length. The absorbance was measured on a Varian Cary UV/Vis Spectrophotometer 4000 (USA) over the wavelength range of 500–200 nm. A 0.05% w/v LAP solution in DPBS was measured similarly. From the absorbance spectra, the absorption coefficient was calculated as follows. The absorption coefficient α can be expressed as

$$\alpha = 2.303 * \epsilon * c \quad (8)$$

With the molar extinction coefficient ϵ and concentration c of the absorbing species. The Lambert-Beer law expresses the light attenuation as

$$A = \log_{10} \left(\frac{I_0}{I_x} \right) = \varepsilon * c * d \quad (9)$$

with the absorbance A , the incident light intensity I_0 and light intensity at specific depth I_x , the molar extinction coefficient ε , concentration c and path length d . Taking together equation (8) and (9), the absorption coefficient α can be calculated as

$$\alpha = 2.303 * \frac{A}{d} \quad (10)$$

The absorption coefficient was calculated using the absorbance at 400 nm. One sample of each composition was analyzed.

3.5.2.9. Refractive index measurement

The RI of polymer solutions was measured using an Abbemat multiwavelength refractometer (Anton Paar, Austria). HA-NB and HA-MA were dissolved at 4.0% w/v in DPBS. The prism was covered with the solution and the RI at 20 °C was measured at different wavelengths (435.9–656.0 nm). The experimental data was fitted to the Cauchy equation which describes the wavelength-dependence of the RI in transparent materials:^[199]

$$RI = A + \frac{B}{\lambda^2} + \frac{C}{\lambda^4} \quad (\lambda \text{ in } \mu\text{m}) \quad (11)$$

With the Cauchy coefficients A , B and C . A describes the RI of the material at an infinite wavelength, and B and C the dispersions coefficients which describe how the RI changes in dependency of the wavelength. The Cauchy coefficients were used to determine the RI at 400 nm, which is the lasing wavelength of the printer. Three independent measurements were performed per solution.

3.5.2.10. Dose test and critical gelation threshold

450 μL of the HA-NB or HA-MA resin were loaded into a quartz cuvette and loaded into the volumetric printer (Tomolite Performance, Readily3D, Switzerland) with the help of a

specific holder which allows insertion of cuvettes instead of vials. With the built-in dose test function in the Apparite software, a logarithmic increase of time versus light intensity was calculated to generate a 7x7 matrix over a light dose range. For the first dose test, a wide range of light doses was used. For the HA-NB resin, the light dose range was set to 50–800 mJ cm⁻² and for the HA-MA resin to 250–1250 mJ cm⁻². Circular dots with a diameter of 0.5 mm and 0.5 mm spacing were projected onto the wall of the cuvette. 5 min after the light exposure, the cuvette was inspected and the dots where formation of hydrogel was visible marked as barely visible, small or big dot. The result of the first dose test was used to define a narrower light dose range for the second dose test which was then conducted and analyzed similarly. The dot with the lowest light dose determined the CGT where crosslinking of the resin was induced. 4 independent light dose tests for the HA-NB and HA-MA resin were performed and taken to calculate the CGT with 0.05% w/v LAP as photoinitiator.

3.5.2.11. Volumetric printing

Volumetric printing was performed using Tomolite Performance manufactured by Readily3D. 0.8–1 mL of resin were transferred into 10 mm vials and inserted into the volumetric printer and secured to fully cover the area where the scaffold will be projected. In the Apparite software, the RI of resins was set to 1.35, the absorption compensation to 0.3–0.4 cm⁻¹. The Power-to-Average-intensity-ratio was set to 6.0:1 so that the average light intensity during printing was 7.7–7.8 mW cm⁻².

3.5.2.12. Determination of printability and dimensional error

A scaffold with 8 mm height, 5 mm width and 2 mm thickness were used for the determination of printability. The scaffold contained channels with different diameters. First, the light dose determined during CGT was used for printing, then the light dose was varied between CGT -20% to CGT +90% to obtain a stable scaffold with the desired dimensions. After the exposure was completed, the vial was left in the printer for 5 min to allow for dark curing. The scaffold was removed from the vial and washed three times with 2 mL PBS to remove unreacted resin, transferred on a cover slip (25 mm) and imaged using a stereomicroscope (Nikon SMZ1270, 0.63X objective, USA). The dimensions were quantified using ImageJ software (v3.1). For the width, three points and for the length two

points were measured. The dimensional error for each light dose was calculated using the following equation:

$$\text{dimensional error (\%)} = \frac{d_{\text{Print}} - d_{\text{CAD}}}{d_{\text{CAD}}} * 100\% \quad (12)$$

Where d_{Print} is the length of the printed scaffold measured in ImageJ and d_{CAD} is the theoretical length of the CAD model. According to Jang et al., the dimensional error should be $<\pm 5\%$.^[200] When the dimensional error was $<-5\%$, the scaffold was defined as undercured, i.e. the printed dimension was smaller than the targeted dimension, whereas a $>+5\%$ error scaffold was overcured, i.e. the printed size was larger than the targeted size of the CAD model. The dimensional errors along the x and z axes were determined. Mean and standard deviation were calculated, and a linear fit performed using python. For each light dose ≥ 3 individual prints were analyzed.

3.5.2.13. Determination of dimensional swelling

The scaffold was imaged using a stereomicroscope after printing and after 24 h of incubation in 1 mL DPBS at 37 °C. The dimensions were measured as described in the section for the dimensional error. The dimensional swelling was calculated according to

$$\text{dimensional swelling (\%)} = \frac{d_{24\text{h}} - d_{0\text{h}}}{d_{0\text{h}}} * 100\% \quad (13)$$

Where $d_{0\text{h}}$ is the length measured immediately after printing and $d_{24\text{h}}$ the length measured after 24 h of incubation. The dimensions were measured at five points of the scaffold. For each light dose ≥ 3 prints were analyzed.

3.5.2.14. Determination of storage modulus of printed scaffolds

The storage modulus of printed scaffolds was analyzed using a DMA 850 (TA instruments, USA) equipped with 15 mm compression clamps. Discs with a diameter of 5 mm and a height of 3 mm were printed using the volumetric printer. The discs were incubated in 1 mL DPBS at 37 °C for 24 h. The diameter of the discs was measured with a caliper. The disc was placed between the plates, a preload of 0.01 N applied and after equilibration

for 5 min, a strain sweep from 0.01 to 20% was performed at a frequency of 1 Hz. The mean and standard deviation were calculated from the storage modulus at 0.1% strain of three discs.

3.5.2.15. Determination of channel width in printed scaffolds:

Volumetric printing was performed as described before using a scaffold of 8 x 5 x 2 mm³. The scaffold contained different channels: the first channel had a diameter of 1 mm which split in two channels with 0.8 mm diameter. The one channel split further in two channels with a diameter of 0.6 and 0.4 mm, the other one split in two channels with 0.4 and 0.2 mm diameter. In addition to the wash with DPBS, the channels were perfused with DPBS to remove uncured resin. The scaffolds were placed at 37 °C in 1 mL DPBS, which was exchanged one time after 30 min. After 23 h, DPBS was exchanged against 1 mL 0.5% FITC-Dex solution (500 kDa) and further incubated for 1 h. The scaffolds were imaged with a tile scan using a Celldiscoverer-7 microscope (Zeiss, Germany) equipped with a 5X objective. The plane was set manually to the middle of the scaffold where the 0.6 mm channel had the widest diameter. The channel diameter was analyzed in a semi-automatized matter. First, the coordinates of each channel were determined manually to define a region of interest (ROI) in ImageJ. The channel width was calculated as FWHM using python. The microscopy image file was loaded along with the coordinates to extract the ROI out of the image as intensity profile. A gaussian fit was performed to calculate the FWHM. For HA-NB and HA-NB (medium), ≥3 independent scaffolds were printed using three different light doses.

3.5.2.16. Cell culture

Fibroblast L929 cell line (ATCC) was cultured at 37 °C and 5% CO₂ in RPMI 1640 medium (Gibco, 61870-010) supplemented with 10% FBS (Gibco, 10270) and 1% P/S (Invitrogen) as established in the literature.^[26] For the bioprinting experiments, L929 cells (1 x 10⁶ or 5 x 10⁵ mL⁻¹ cells) were directly suspended in the HA-NB solution for photopolymerization. L929 cells were used between passages 7–15.

3.5.2.17. Bioprinting

The lyophilized HA-NB resin (61 mg mL⁻¹, 6.1 %w/v) and cyclo-RGDfC peptide (2 mg mL⁻¹, 3.3 mm) were weighed in the same autoclaved vial and dissolved in sterile DPBS buffer (pH 7.4). The solutions were prepared inside a sterile laminar flow, incubated for 1 h at 37 °C and used directly without further filtration. Stock solutions of DTT (5% w/v) and LAP (1% w/v) were also prepared in sterile DPBS buffer. These concentrations were kept constant during all cell experiments. The fibroblast cell suspension (1 x 10⁶ or 5 x 10⁵ cells mL⁻¹) in RPMI medium (200 µL) was added to the HA-NB/RGD solution (700 µL) prepared above. Immediately, the solution of LAP (50 µL, 0.05% w/v) and DTT (50 µL, 0.26% w/v) were added to the vial, carefully mixed with a pipette tip, and transferred to the autoclaved glass vials. The samples were loaded into the volumetric printer and a rectangle with 8 mm height, 5 mm width and 2 mm thickness were projected onto the wall of the cuvette. The light dose was varied between 201.6–252.0 mJ cm⁻² (400 nm, 7.75 mW cm⁻², 29–33 s) for photopolymerization. The bioprinted scaffolds were immediately transferred to a 6 well plate inside a sterile hood and washed three times with DPBS. The scaffolds were transferred to a 24 well plate containing complete RPMI medium (1 mL per well). The bioprinted scaffolds were incubated in medium at 37 °C and 5% CO₂, and the cultures were maintained for 1–7 days.

3.5.2.18. Live/Dead assay

L929 fibroblasts were cultured in HA-NB scaffolds for 1 day, and cell culture medium was removed. Samples were incubated for 5 min with fluorescein diacetate (40 µg mL⁻¹) and propidium iodide (30 µg mL⁻¹) in DPBS and washed twice with PBS. The bioprinted scaffolds were imaged with LSM 880 confocal microscope (Zeiss, Germany), using a EC Plan-Neofluar 10x/0.30 objective. For each sample, at least five independent z-stacks was analyzed (~1200–1400 cells per sample). Live and dead cells were counted manually in each slice of z-stack to calculate the percentage viability of each sample by using the Zen Blue (Version 3.5, Zeiss, Germany). Cell viability data are expressed as mean ± SD.

3.5.2.19. Fixation and staining

The HA-NB scaffolds were fixed with 4% PFA solution for 2 h at room temperature and washed with DPBS. Samples were blocked with 1% BSA solution for 1 h, followed by permeabilization with 0.5% Triton X-100 for 1 h. FITC or TRITC-phalloidin (1:200 in water, Thermo Fisher Scientific, USA) was used for staining fibers and DAPI (1:500 in water, Life Technology, USA) for staining the nucleus. Samples were incubated with staining probes for 5 h at room temperature and then washed twice with DPBS.

3.5.2.20. Morphology of fibroblast cells cultured in 3D hydrogel

Morphology of mouse fibroblast L929 encapsulated in HA-NB scaffolds for different culture durations (1, 3 and 7 days) were constructed from confocal z-stack of scaffolds taken after fixation and staining (FITC-phalloidin/DAPI). Spreading areas of suspended cells in 3D scaffolds was determined from fluorescently labelled actin fibers (FITC-phalloidin) by measuring the cell area in each slice (2 μm) of z-stack (~250–300 cells per sample) using Zen Blue (v3.5). Spreading area data are expressed as mean $\pm$ SD and statistical analyzed by using one-way ANOVA with a post hoc Tukey test.

3.5.2.21. Statistical analysis

Data were expressed as mean $\pm$ standard deviation, with a minimum sample size of $n \geq 3$ if not noted otherwise. Statistical analysis and the linear fit of the dimensional error and the FWHM were performed using python, all other statistical analyses for experiments were conducted using Origin2022 (OriginLab, USA).

4. Volumetric Printing of PVA-MA and PVA-NB Hydrogels

This chapter presents PVA-based resins and evaluates their suitability for volumetric printing. The prepolymers PVA-MA and PVA-NB with comparable substitution degrees are synthesized and characterized in terms of rheological and optical properties. The (volumetric) printability of 10% w/v PVA-based resins as function of light dose, absorption compensation, photoinitiator concentration, and dithiol crosslinker concentration (for PVA-NB systems) is analyzed. The targeted printed geometries are thin (350–500 μm) hydrogel layers with flat or semispherical shape, i.e. a geometry close to a contact lens. The z-resolution of the prints is quantified by imaging the cross-section using optical coherence tomography (OCT). Curved films of <350 μm central thickness and overcured edges were obtained at the tested illumination conditions and resin formulation. Strategies to improve these results in future experiments are discussed.

4.1. Introduction

DLP has been considered a suitable technology for the manufacture of customized, patient-specific contact lenses, as alternative to lathe cutting, and for the fabrication of multi-material devices with regions of different optical properties or containing intricate geometries like microfluidic channels.^[113, 165, 171, 174, 175, 202] This layer-by-layer fabrication approach has rendered contact lens geometries with commercial acrylate-based or HEMA/PEGDA-based materials and 200 μm thickness.^[113, 174] DLP printed contact lenses show surface defects in form of staircases of 35 μm resolution and requires post-processing steps for smoothing.^[113, 174]

Volumetric printing is considered a reasonable alternative towards printed optical lenses with improved surface quality and has been investigated for the fabrication of microlenses. For example, Two-Photon-Polymerization (2PP) has been studied for the fabrication of microlenses of acrylate-based resins with <300 μm dimensions (100–300 μm diameter, 70–200 μm height).^[176, 177] Lenses with a surface roughness <15 nm and an z-accuracy of <1 μm were reported.^[176, 177] Using tomographic volumetric printing, acrylate or silica glass nanocomposite lenses with 1.5–3 mm diameter and 1.5 mm height were printed in <1.5 min.^[179, 180] Silica glass lenses showed a surface deviation of

10–60 μm and printing resolution was characterized with a minimum feature sizes of 50 μm .^[179] TEMPO was added to the resin to reduce out-of-voxel curing. Webber and co-authors applied optical blurring of the light beam to reduce the striations and printed lenses with a surface deviation $\sim 1.4 \mu\text{m}$ in a resin based on diurethane dimethacrylate and PEGDA. Blurring of the light beam negatively impacted resolution by increasing the theoretical pixel size which led to a theoretical minimum feature size of 172.8 μm .

All the reported studies use hydrogel resin compositions with high monomer content which are swollen after printing. In this chapter, the applicability of tomographic VP for printing PVA-based contact lenses is explored, which are industrially manufactured as diluted water solutions in geometries very close to the final swollen form using photopolymerization processes.

PVA-MA and PVA-NB solutions have been printed into scaffolds of different geometries using different light-based printing processes. Kothari et al. fabricated drug-loaded patches for the eye surface using extrusion printing.^[175] The resin contained 15% w/v PVA-MA (180 kDa, $F_{\text{ene}} = 98.9\%$), 1–2% w/v HA-MA (47 kDa, $F_{\text{ene}} = 98.5\%$) and 2% w/v Irgacure 2959 as photoinitiator. The printed structure was stabilized by post-printing photocuring for 2 min (365 nm) and freeze thawing. Transparent eye patches with 15 mm diameter and 4 mm height were obtained. A resin containing 10% w/w PVA-MA (13–23 kDa, $F_{\text{ene}} \sim 15\%$) with 1% w/w Ponceau 4R as photoabsorber and Ru/SPS visible light photoinitiator was processed by DLP into $5 \times 5 \times 5 \text{ mm}^3$ scaffolds with positive and negative features in the 50–500 μm range.^[107] Authors used a layer thickness of 50 μm and 10 s exposure time per layer at 7.25 mW cm^{-2} . Using volumetric printing and a resin containing 1.5–3% w/v PVA-NB (47 kDa, $F_{\text{ene}} = 7.8\%$) and PEG2SH (2 kDa) in a thiol:ene ratio of 4:5 and 2 mM LAP, cell-laden scaffolds in mm-scale were printed.^[85] The resin was supplemented with 5% w/v gelatin to increase the viscosity. Cell-free scaffolds with perfusable channels with a diameter of ~ 0.9 –1 mm were printed using light doses between 100–140 mJ cm^{-2} . The scaffolds were post-cured for 5 min (405 nm, 10 mW cm^{-2}).

In a previous study in the group, 10% w/v vinyl sulfone-modified PVA (PVA-VS, 177 kDa, $F_{\text{ene}} = 1.5\%$) was used for molding-based fabrication of hydrogel CLs.^[106] The hydrogels

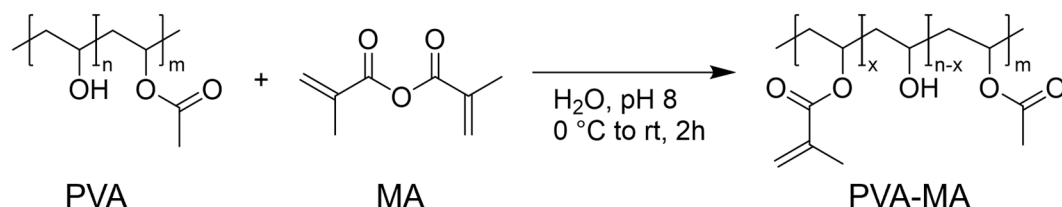
showed a storage modulus of 17.9 ± 0.1 kPa, an equilibrium water content (EWC) of $87.5 \pm 0.9\%$, oxygen permeability of 53.9 ± 2.4 barriers, transmittance of $99.1 \pm 0.5\%$ and refractive index of 1.336. CL laboratory prototypes with 11 mm diameter and 1.5 mm thickness were fabricated by molding. In this chapter, the suitability of PVA-MA and PVA-NB as photocrosslinkable materials at similar composition for the fabrication of contact lens-like geometries using volumetric printing will be investigated.

4.2. Results

4.2.1. Volumetric printing of PVA-MA scaffolds

4.2.1.1. Synthesis of PVA-MA

The synthesis of the photocrosslinkable PVA derivative PVA-MA was performed by reaction of the pendant hydroxyl groups with methacrylic anhydride (**Scheme 4**) following a reported protocol.^[107] The attachment of methacrylate (MA) groups was confirmed by ^1H NMR (**Figure 56A**). The signals of vinyl (6.1 ppm, 1H; 5.7 ppm, 1H) and methyl protons (2.1 ppm, 3H) confirmed the derivatization of the polymer. The ratio between the signal of the vinyl and the tertiary proton of the backbone (4.1 ppm, 1H) was used to calculate the degree of functionality. A F_{ene} value of $4.8 \pm 0.4\%$ was obtained. The functionalization was further confirmed by Raman spectroscopy (**Figure 56B**). The bands at 1712 and 1638 cm^{-1} were associated to the stretching vibrations of the carbonyl and carbon double bond, respectively, and confirmed the attachment of the MA group.^[203] The original degree of acetylation F_{acetyl} of the PVA ($\sim 14\%$) did not change during the reaction, according to the ^1H NMR spectrum. GPC analysis showed no changes in the molecular weight distribution of the PVA during methacrylation, indicating the absence of degradation or chain extension under the applied reaction conditions (**Figure 57**).



Scheme 4. Synthesis of photocrosslinkable PVA derivatives. PVA-MA was synthesized using methacrylic anhydride (MA) under basic conditions.

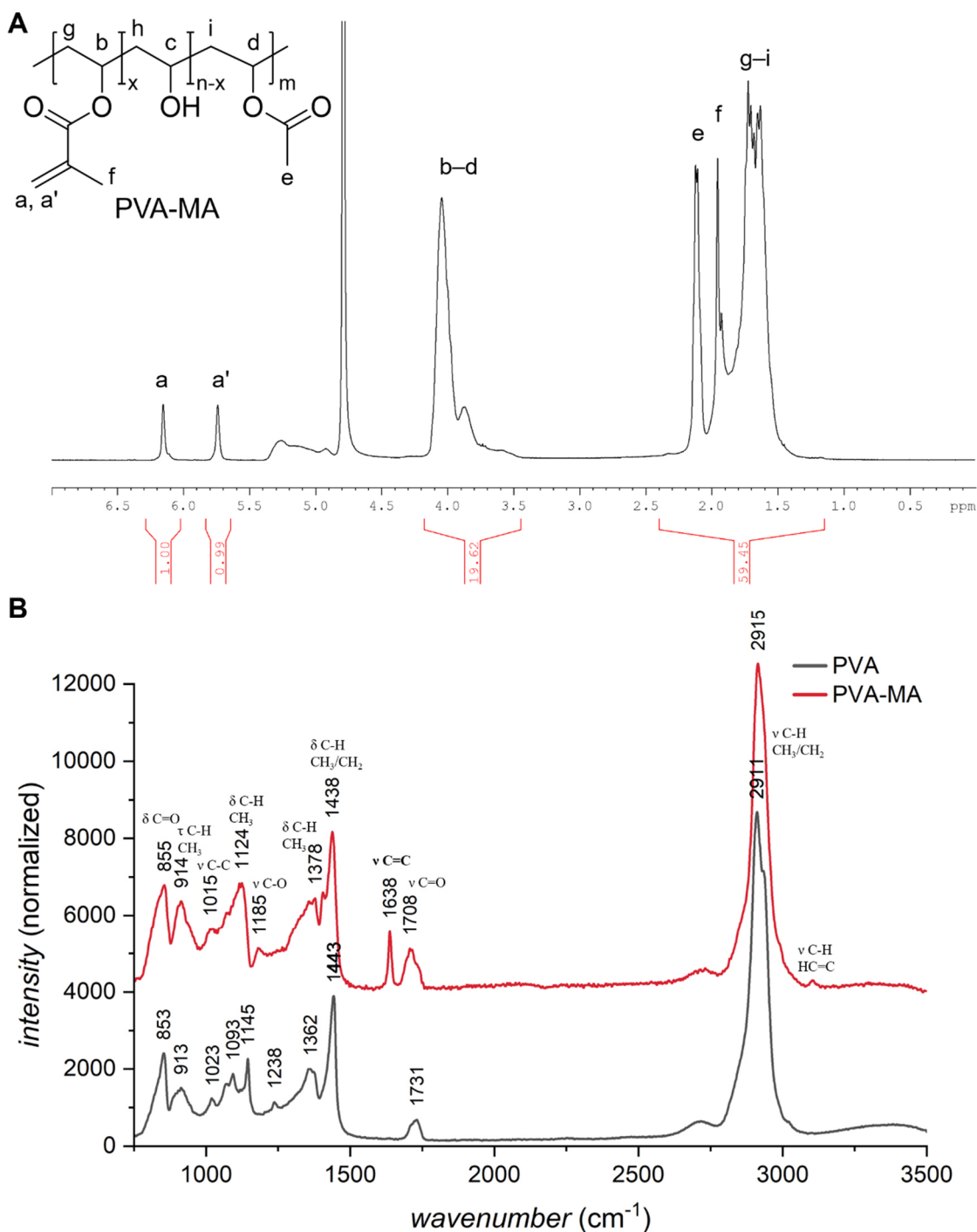


Figure 56. Characterization of PVA-MA. **A.** ¹H NMR spectrum of PVA-MA in D₂O ($\delta = 4.79$ ppm). The integral of one of the two vinyl protons 'a' from the MA group (6.1 ppm) was used as reference with $I_a = 1.00$ for calibration, which corresponds to one MA group. This integral was compared to the signal of the tertiary proton of the polymer backbone repeating unit 'b', 'c' and 'd' to calculate the degree of functionalization according to $F_{ene} = I_a / I_{b-d} = 1 / 19.62 = 0.05$. **B.** Raman spectra of PVA and PVA-MA polymers. Characteristic peaks corresponding to the methacrylate group are observed at 1708 and 1638 cm⁻¹ in the spectrum of PVA-MA, corresponding to the carbonyl and carbon double bond stretching vibrations, respectively.

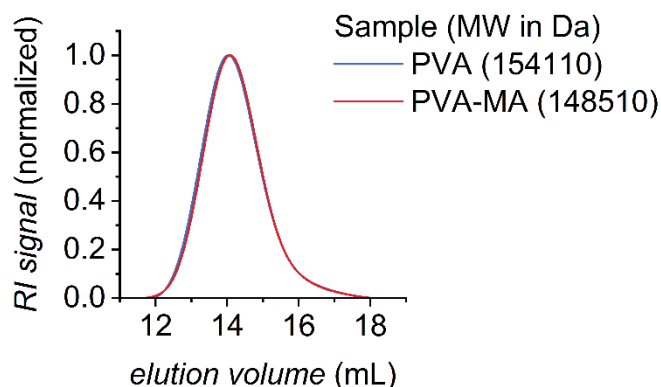


Figure 57. GPC elugrams of PVA and PVA-MA polymers. The molecular weight indicated in brackets was calculated using Pullulan standards.

4.2.1.2. Optical properties of PVA-MA resins and hydrogels

The UV/Vis spectrum of the 10% w/v PVA-MA resin containing 0.05% w/v LAP photoinitiator is shown in **Figure 58A**. The absorption maximum of LAP is visible at 370 nm. At 400 nm, the absorption coefficient of the resin was calculated to be 0.6 cm^{-1} . This value falls within an acceptable range for VP (an absorption coefficient $<1 \text{ cm}^{-1}$ is recommended to allow for sufficient light penetration^[195]).

The refractive index (RI) of the PVA-MA solution and the hydrogel after photopolymerization was measured within the wavelength range 435.9 to 656.0 nm (**Figure 58B**). After crosslinking, the hydrogel exhibits an overall lower RI in comparison to the polymer solution due to the higher water content after swelling. A RI of t 400 nm was calculated to be 1.359 was estimated for the printing wavelength of 400 nm using the Cauchy equation.^[199] The RI of crosslinked PVA-MA hydrogel at 880 nm will be used for OCT measurements described in section 4.2.1.7. Notably, the RI of polymer solutions was measured without the added photoinitiator or crosslinker was measured, since preliminary measurements in Chapter 3 confirmed that these do not measurably influence the RI.

After photocrosslinking, PVA-MA hydrogels appeared slightly opaque (**Figure 58C**). This effect is attributed to heterogeneities in the network structure of the PVA-MA hydrogel as consequence of the radical chain-growth crosslinking mechanism.

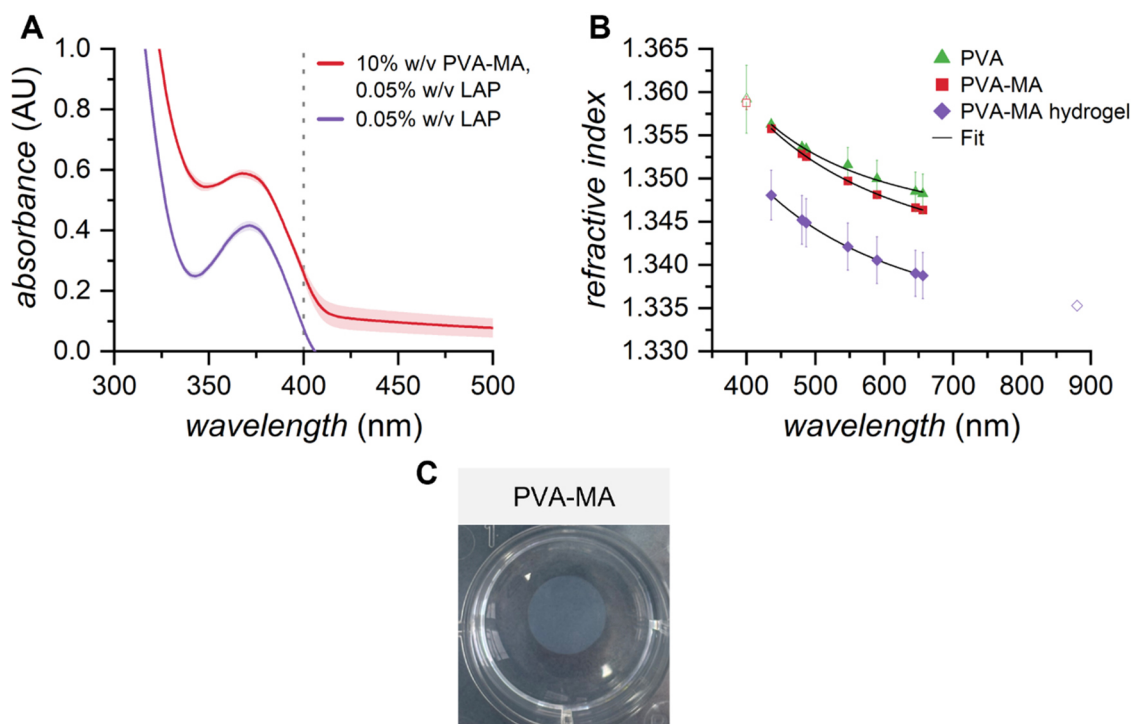


Figure 58. Optical properties of PVA-MA resin and hydrogel. **A.** UV/Vis absorbance spectrum of PVA-MA hydrogel precursor solutions. The hydrogel precursor solutions consisted of 10% w/v PVA-MA supplemented with 0.05% w/v LAP. The UV/Vis absorbance spectrum of 0.05% w/v LAP in Milli-Q H₂O is shown as reference. **B.** Refractive index of PVA and PVA-MA solutions at 10% w/v in Milli-Q H₂O and PVA-MA hydrogel measured at different wavelengths (closed symbols). The experimental results were fitted to the Cauchy model and used to calculate the refractive index at 400 nm for polymer solutions and at 880 nm for the PVA-MA hydrogel (open symbols). Data are shown as mean and standard deviation for $N = 3$ measurements. **C.** Image of crosslinked hydrogel placed in Milli-Q H₂O. The PVA-MA hydrogel appears slightly opaque.

4.2.1.3. Physicochemical properties of PVA-MA resins and hydrogels

The viscosity of 10% w/v PVA-MA solutions in water were characterized by rotational rheology. At a shear rate of 1 s^{-1} , a viscosity value of $0.49 \pm 0.03 \text{ Pa s}$ was obtained for PVA-MA solution (**Figure 59A**).

The photocrosslinking kinetics of the resin was studied by oscillatory shear rheology with in situ illumination. **Figure 59B** shows the evolution of G' and G'' during illumination at 400–500 nm. The crossover point where $G' = G''$ describes the transition from liquid- to solid-like behavior of the sample and defines the gelation point, t_{gel} .^[192] The resin showed a t_{gel} of $21.6 \pm 4.2 \text{ s}$ (**Table 8**) and a maximum storage modulus G' of $45 \pm 3 \text{ kPa}$ during

3 min exposure. A small decay in the storage modulus was observed at longer exposure times. This is attributed to syneresis, i.e. the expelling of water that became unbound from the polymeric network as consequence of the crosslinking-induced contraction of the hydrogel. The shrinking leads to a loss of contact between the hydrogel sample and the rheometer plate and this leads to a reduction of the measured G' value.^[143] This effect was observed independently from the rheometer settings.

Strain and frequency sweep experiments defined the experimental range for a linear viscoelastic response of the hydrogel (**Figure 59C, D**). PVA-MA hydrogels showed a frequency-independent storage modulus across the frequency range (0.1–100 Hz). At higher strain amplitudes, a decrease in G' along with increase in G'' was observed which is consistent with the onset of a nonlinear viscoelastic behavior outside of the linear viscoelastic region.

The equilibrium water (EWC) content for PVA-MA hydrogels was determined by gravimetry (**Table 8**). The EWC describes the percentage of water a hydrogel can hold when fully hydrated.^[166] PVA-MA hydrogels showed an EWC of $86.4 \pm 1.3\%$.

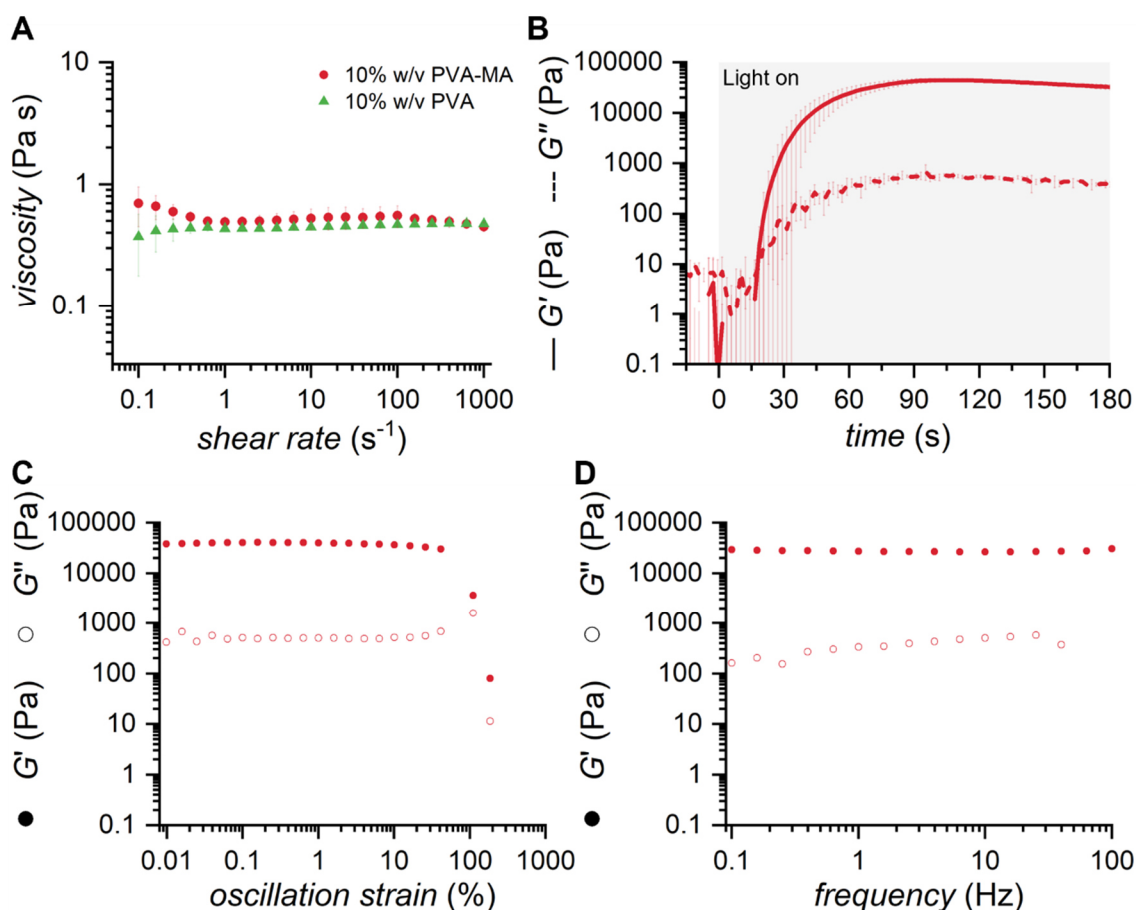


Figure 59. Rheological characterization of polymer solutions and resins. **A.** Viscosity of PVA and PVA-MA solutions at 10% w/v in Milli-Q H₂O over a shear rate from 0.1 to 1000 s⁻¹. Data are shown as mean $\pm$ SD for $N = 3$ measurements. **B.** Oscillatory shear rheology with in situ illumination (400–500 nm, 8 mW cm⁻²) of 10% w/v PVA-MA supplemented with 0.05% w/v LAP. The shaded region indicates when light was on. Measurements were performed at 0.1% strain and 1 Hz frequency. Data are shown as mean $\pm$ SD of $N = 3$ measurements. **C.** Representative strain sweep with increasing oscillation strain between 0.01 and 1000% at 1 Hz frequency. **D.** Representative frequency sweep with increasing frequency between 0.1 and 100 Hz at 0.1% strain.

Table 8. Rheological parameters, equilibrium water content EWC and critical gelation threshold (CGT) of PVA-MA hydrogels. Gelation point t_{gel} and maximum storage modulus G' determined from oscillatory shear rheology measurements, and CGT obtained in light dose tests.

Hydrogel	t_{gel} (s)	G' (kPa)	EWC (%)	CGT (mJ cm ⁻²)
PVA-MA	21.6 $\pm$ 4.2	45 $\pm$ 3 kPa	86.4 $\pm$ 1.3	175 $\pm$ 16

4.2.1.4. Identification of the critical gelation threshold for PVA-MA resins

The photoreactivity of the 10% w/v PVA-MA resin containing 0.05% w/v LAP at VP conditions was studied with the light dose test by projecting an array of dots with 0.5 mm diameter on the cuvette using increasing light doses. The diameter of the crosslinked dots was visible by eye due to the RI difference between the uncured and crosslinked resin. The onset of the crosslinking in the resin was monitored by quantifying the appearance of crosslinked hydrogel dots. The lowest light dose which led to the appearance of the dot was considered the critical gelation threshold (CGT) and used as a first indication of the reactivity of the resin for VP.^[147] The resin required a light dose of $175 \pm 16 \text{ mJ cm}^{-2}$ to induce gelation (**Table 8**). Increasing the LAP concentration from 0.05% to 0.1% decreased the CGT to 158 mJ cm^{-2} ($N = 1$).

A continuous increase in the area of the crosslinked dots was observed in the following 3 minutes after exposure of the PVA-MA resin (**Figure 60A**). This post-exposure curing is known as dark curing and is expected in radical crosslinking MA-based resins.^[182] This effect was also observed in crosslinking experiments performed in the rheometer when illumination was switched off shortly after t_{gel} . The storage modulus of the PVA-MA resin continued increasing by 180–240% during the following 2.3 min in the dark (**Figure 60C**) and led to hydrogels with a G' value around 80% of the G' value after full exposure.

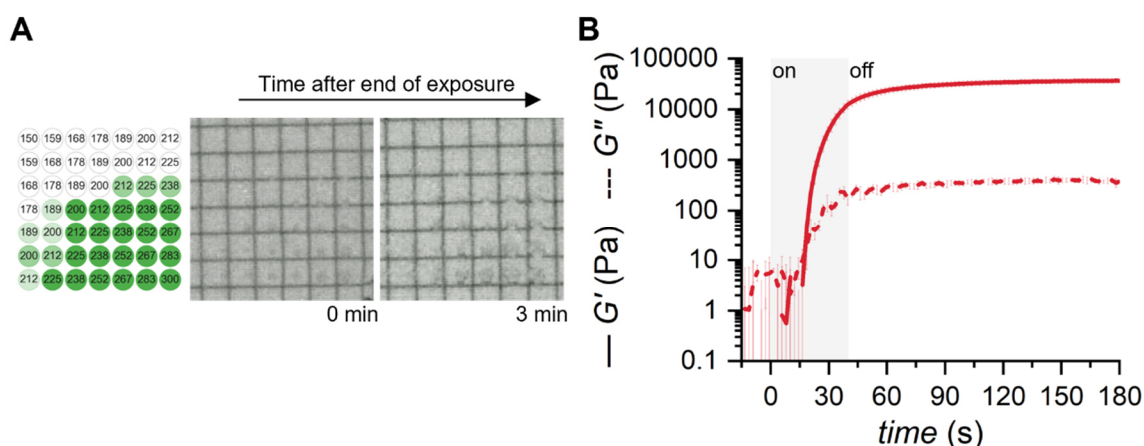


Figure 60. Post-exposure reactivity in PVA-MA resin. **A.** Illumination pattern used for the light dose test and images of the resin captured 0 and 3 min after the exposure using the build-in camera in the Apparite software. **B.** Oscillatory shear rheology with in situ illumination for 40 s (400–500 nm, 8 mW cm^{-2}) to monitor the after-exposure reactivity.

Measurements were performed at 0.1% stain and 1 Hz frequency. Data are shown as mean $\pm$ SD for $N = 3$. The resin contained 10% w/v PVA-MA and 0.05% w/v LAP.

4.2.1.5. Printability analysis of PVA-MA resin by VP

To evaluate the printing resolution of 10% w/v PVA-MA resins with 0.05% w/v LAP, discs with 12.5 mm diameter and 0.5, 0.35 or 0.2 mm thickness were printed using light doses between 189 and 340.2 mJ cm⁻², which is 8 to 94.4% above the CGT (175 ± 16 mJ cm⁻²). An absorption coefficient value of 0.6 cm⁻¹ was used for absorption compensation by the software of the printer. To evaluate printing fidelity as function of light dose, the diameter of the printed discs was quantified using a grid as reference scale, and their mechanical stability was assessed by testing if they could self-support on a spatula tip. Obtained prints with 12.5 ± 1 mm diameter, round borders and good mechanical stability were considered as cured samples with good shape fidelity. Samples were considered undercured when they showed a smaller diameter or had an incomplete form. Overcured samples had larger dimensions than targeted.

Discs with targeted 0.5 mm thickness printed at 208 mJ cm⁻² showed good shape fidelity and mechanical integrity (**Figure 61A**). Discs with 0.35 mm thickness required a light dose of 265 mJ cm⁻² to be printable with good shape fidelity. These light dose values were considered the optimum ones for the corresponding disc thickness. Discs with targeted 0.2 mm thickness were not obtained at good printing fidelity. While a light dose of 302 mJ cm⁻² rendered discs with diameter <12.5 mm and without mechanical stability, increasing the light dose to 340 mJ cm⁻² led to discs with overcuring at the center.

The higher light dose required for good printing fidelity in discs with smaller thickness and the failure in printing well-defined structures at 0.2 mm thickness has also been observed by other authors and explained based on the inhibitory effect of oxygen in the polymerization of MA groups.^[204] For small features, prolonged printing times led to blurring and compromised the printing quality.

In agreement with the observations in the light dose test, an evolution of the printed discs within the resin bath was observed post-exposure, indicating that the prints underwent

dark curing (**Figure 61B**). To investigate the consequences of dark curing in the quality of the prints, the radical inhibitor TEMPO at 0.05% w/v concentration was added to the printing vial right after exposure. At the exposure dose of 265 mJ cm^{-2} , discs with 0.35 mm thickness printed with TEMPO were softer, showed lower shape fidelity and less defined borders than discs printed in the absence of TEMPO (**Figure 61C**). These features are consistent with an inhibition of the dark curing, with a resulting lower crosslinking and lower stiffness of the overall print. It was concluded that dark curing was advantageous for printing PVA-MA under our working conditions.

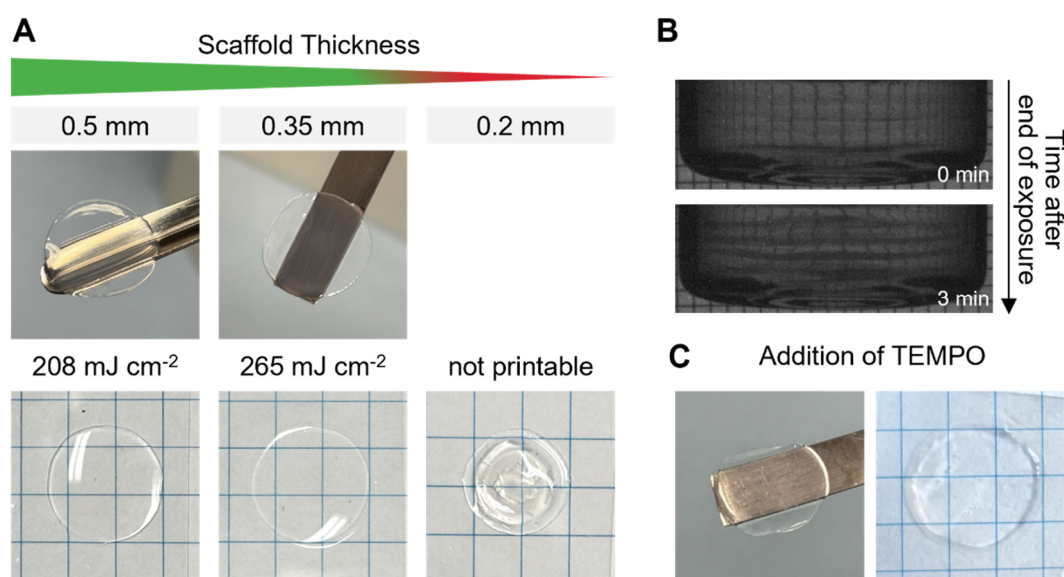


Figure 61. Printed PVA-MA discs. **A.** Images of printed discs with targeted thickness of 0.2, 0.35 and 0.5 mm. The grid corresponds to 5 mm. **B.** Image of printed discs of 0.35 mm thickness inside the cuvette at 0 and 3 min after illumination showing an increase in the contrast between the exposed and not exposed volumes with time. **C.** Printed discs with 0.35 mm thickness with addition of TEMPO after the exposure time, which led to suppression of dark curing and a softer hydrogel.

The PVA-MA discs of 0.5 mm thickness printed at the optical light dose of 208 mJ cm^{-2} were characterized by Raman spectroscopy to quantify the consumption of the polymerizable groups. The change in the intensity of the C=C stretching vibration mode at 1638 cm^{-1} was used for the quantification (**Figure 62**). In printed discs, the signal was visible after exposure, indicating that crosslinking did not proceed to full conversion, i.e. not all functional groups were fully consumed in the printing time. Full conversion was achieved in discs prepared by molding and illuminated at 400–500 nm, 8 mW cm^{-2} , 3 min.

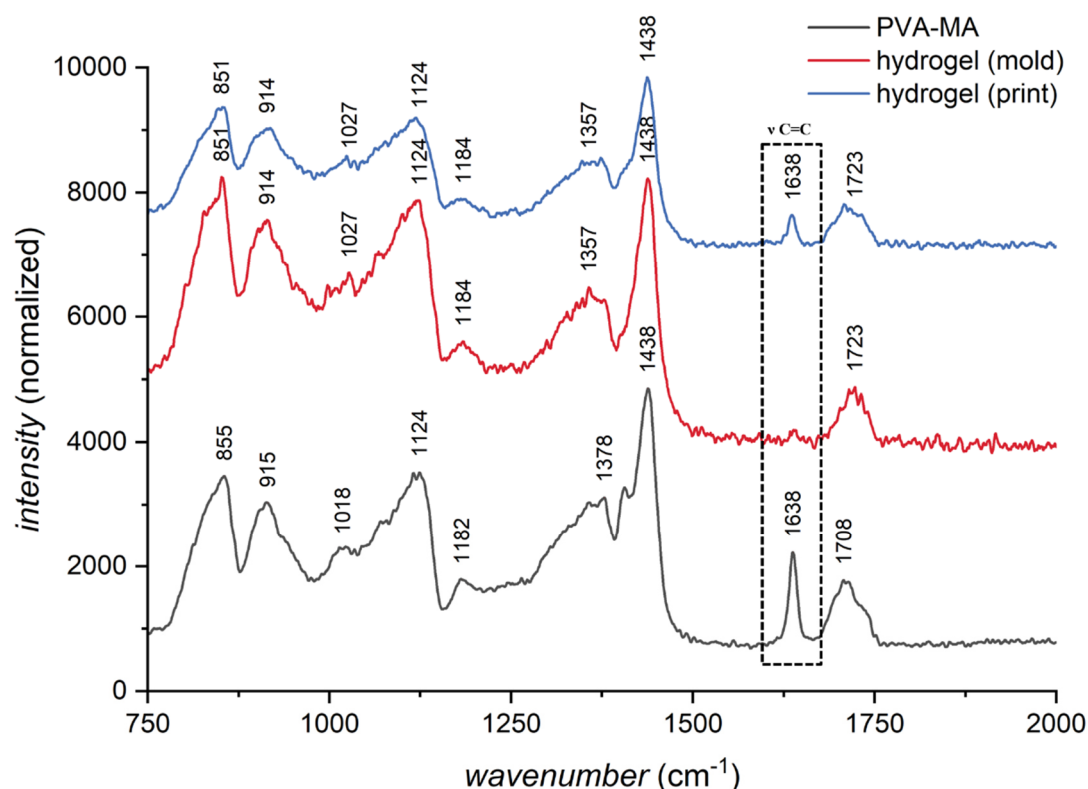


Figure 62. Raman spectra of uncrosslinked polymer, molded and printed PVA-MA resins. The intensity of the stretching vibration of the carbon double bond at 1638 cm^{-1} in the spectrum monitors the consumption of the functional groups during crosslinking. Spectra were scaled to $\nu_{\text{C-H}}$ (CH_3/CH_2) stretching mode at 2914 cm^{-1} and shifted vertically for clarity.

4.2.1.6. Vol μ metric printing of PVA-MA contact lenses

It was attempted to print a simplified contact lens geometry with 12.5 mm diameter, 3 mm height and uniform thickness of 0.35 mm (**Figure 63A**). Initial conditions were taken from the optimal dose needed to print the disc structure with 0.35 mm thickness. Starting with a light dose of 260 mJ cm^{-2} , prints were obtained at higher and lower exposure doses in steps of $\pm 10 \text{ mJ cm}^{-2}$. Conditions with good print fidelity were considered when the print had (i) a rounded and convex shape with empty interior, and (ii) was able to self-support when left on a planar surface.

Example images for the prints at different light doses are shown in **Figure 63B**. Light doses of 240 and 250 mJ cm^{-2} rendered a cured and mechanically stable print with rounded geometry. The top central part of the structure appeared thinner and softer than the

periphery. At higher doses (260 mJ cm^{-2}), an overcured print was obtained, with a square outer shape, with peripheral thick overhangs and a thinner central part of the lens. The distorted, square geometry of the overcured print was confirmed in the simulated light dose distribution using the software of the printing equipment (**Figure 63C**). According to the simulation, the resin receives 50% of the maximum light dose outside of the targeted printing volume. This strong deviation of the dose distribution vs. the targeted geometry was not observed in disc geometries.

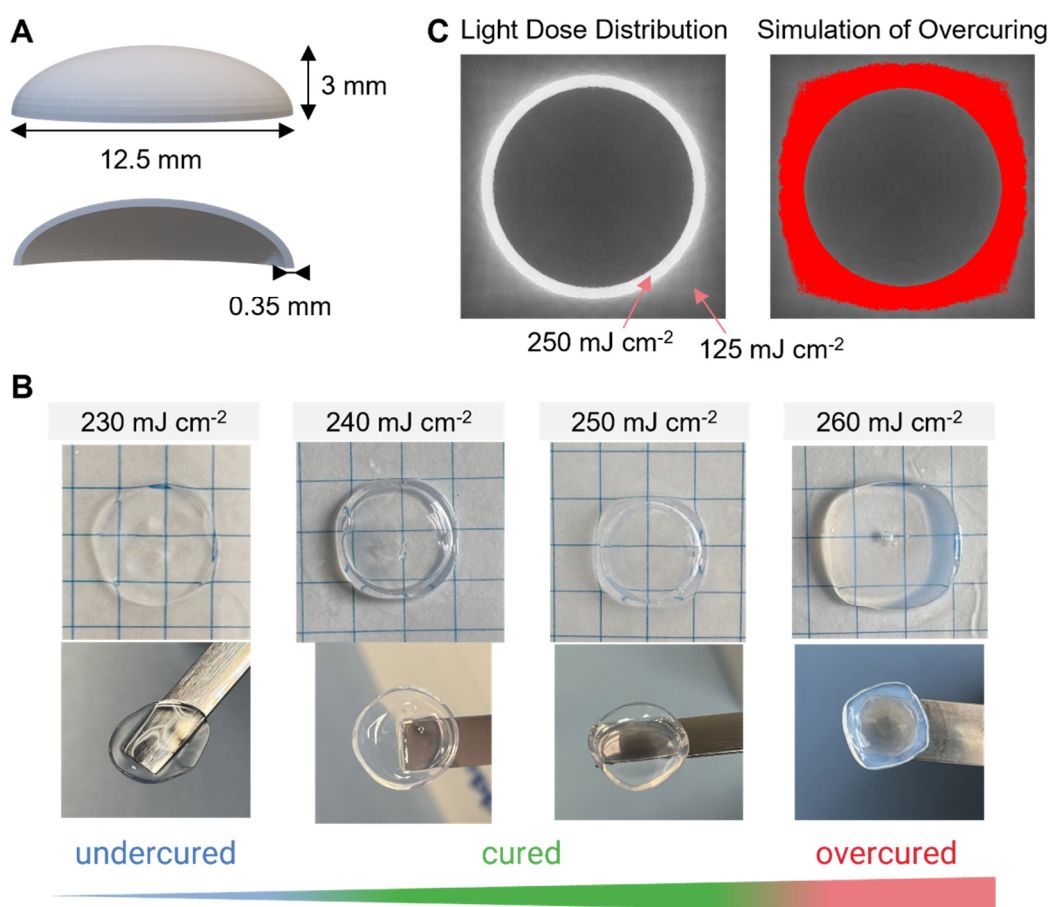


Figure 63. Printability of CL geometries. **A.** CAD model used for printing indicating the dimensions of the targeted lens. **B.** Printed PVA-MA structures using an absorption compensation of 0.6 cm^{-1} and varying light doses. The grid corresponds to 5 mm. **C.** Light dose distribution exported from the Apparite software and threshold setting in ImageJ to simulate overcuring conditions. The image represents the sliced light dose distribution at $h = 1.5 \text{ mm}$.

According to the thinning observed at the center part of the contact lens, the light dose received at the center of the vial was too low for sufficient crosslinking. To get higher doses at the center, a higher absorption compensation value can be set at the VP software. Absorption compensation values between 0.6 and 1.8 cm⁻¹ were tested (**Table 9, Figure S4A–B**). According to the software of the printed, the optimal light dose for printing with a resin with an absorption compensation of 0.6 cm⁻¹ was 240–250 mJ cm⁻², and a resin with 1.4 cm⁻¹ required a light dose of 130–140 mJ cm⁻² to be printed with good shape fidelity. A higher absorption compensation led to a more homogeneous, round light dose distribution according to the simulation of the software of the overcured structure (**Figure S4C**). This was also observed experimentally (**Table 9**). At absorption compensation 1.8 cm⁻¹ the scaffold did not show a square shape or thick overhangs on the outer periphery. However, overcuring occurred at the inner part of the print. Material was cured in out-of-voxel regions at the center. Further, no cured scaffold could be obtained, either no structure or an overcured scaffold was printed. It is hypothesized that the increased light exposure in the center of the vial leads to a locally increased heat accumulation due to exothermic character of the MA polymerization reaction. The higher temperature leads to a faster diffusion of radicals and to an auto-acceleration of the reaction.^[205] Additionally, the inner structure experiences reduced exchange with oxygen, thereby diminishing oxygen inhibition and further facilitating polymerization.

Table 9. Determination of printability for printing the model contact lens geometry using different absorption compensation and light doses. Macroscopic assessment of print quality and subsequent classification as undercured, cured or overcured printing conditions.

Absorption compensation (cm ⁻¹)	Light dose (mJ cm ⁻²)	Print description	Result
0.6	230	round, flat	undercured
	240–250	round, curved, hollow, self-supportive	cured
	260	square, curved, hollow, self-supportive	overcured
1.0	170	round, flat	undercured
	180–190	round, curved, hollow, self-supportive	cured
	220	square, hollow	overcured
1.4	120	round, flat, incomplete	undercured
	130–140	round, curved, hollow, self-supportive	cured
	150	square, hollow	overcured
1.6	100	round, flat, incomplete	undercured
	110–120	round, curved, hollow, self-supportive	cured
	130	square, hollow	overcured
1.8	70	round, flat, incomplete	undercured
	80	round, non-hollow	overcured

4.2.1.7. Characterization of the prints by optical coherence tomography

Optical coherence tomography (OCT) was used to image the cross-section of the printed lens-shape geometries. OCT is an imaging technique which relies on interferometric depth scanning using low-coherence light. It allows high-resolution cross-sectional imaging of objects by analyzing the interference between light reflected from the internal sample structures and a reference beam.

Semi-automated analysis of the OCT images provided a binary mask and thickness profile along the cross-section (**Figure 64A, B**). The thickness retrieved from the OCT image was compared to the theoretical thickness of the CAD model. **Figure 64A** shows the binary masks with the thickness deviation represented by a heat bar. These results correspond to samples printed using different absorption compensation. At first glance, all samples show similar features, with a lower thickness at the center of the scaffold compared to the targeted model, and thicker edges than expected. Prints at higher absorption compensation values showed a slightly higher thickness at the center of the print (**Figure 64C**): $222.4 \pm 26.6 \mu\text{m}$ with 0.6 cm^{-1} and $291.2 \pm 3.8 \mu\text{m}$ with 1.6 cm^{-1} .

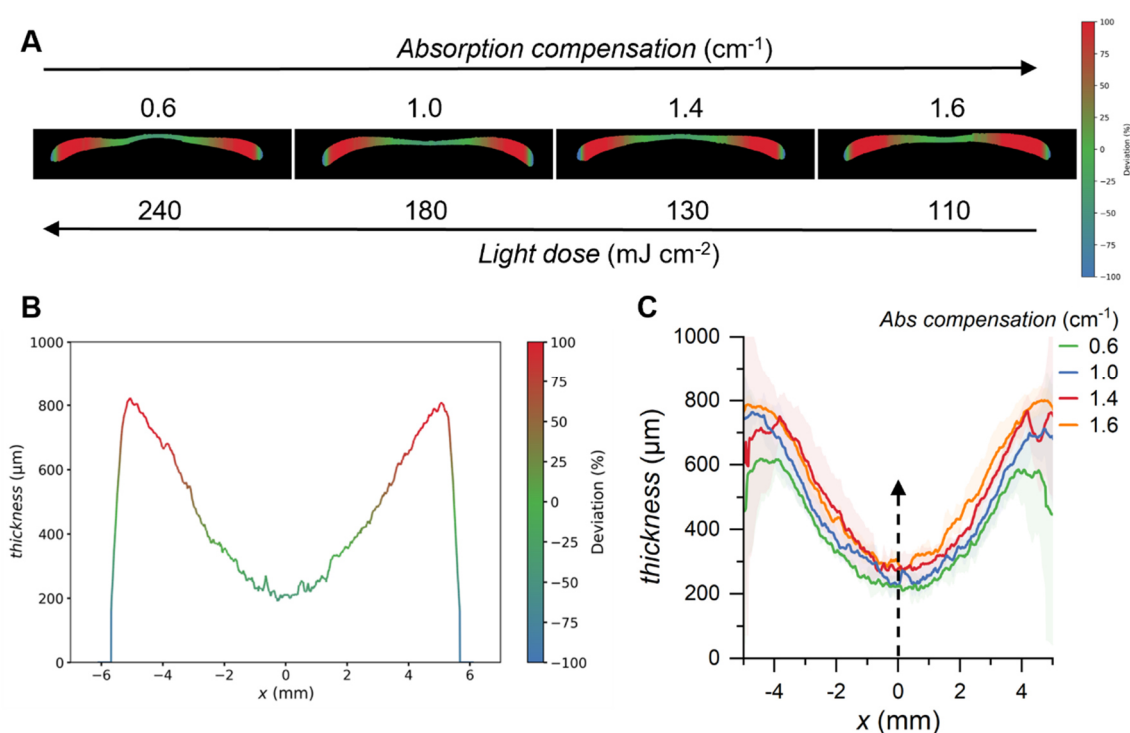


Figure 64. OCT imaging and cross-sectional analysis of printed scaffolds. **A.** Masks retrieved from OCT images of scaffolds printed with different absorption compensation and light dose settings. The binary masks were colored to illustrate the % deviation from the CAD model. **B.** Example plot for thickness along the x-axis retrieved from the processed OCT image. Values were shifted horizontally to set the center to $x = 0$. The colors indicate the % deviation from the CAD model at the specific x position. The scaffold was printed using a light dose of 240 mJ cm^{-2} and absorption compensation of 0.6 cm^{-1} . **C.** Thickness along the x-axis for different absorption compensation settings. Data are shown as mean $\pm$ SD.

Using OCT, the circular symmetry of the prints was quantified. For this purpose, OCT scans were acquired from prints at different radial positions. Identical dimensions were observed if the scan was shifted by 90°, but different thickness at the periphery of the lens was measured if the scan was shifted by 45° (**Figure 65A**). This agrees with the observed square pattern observed in the prints.

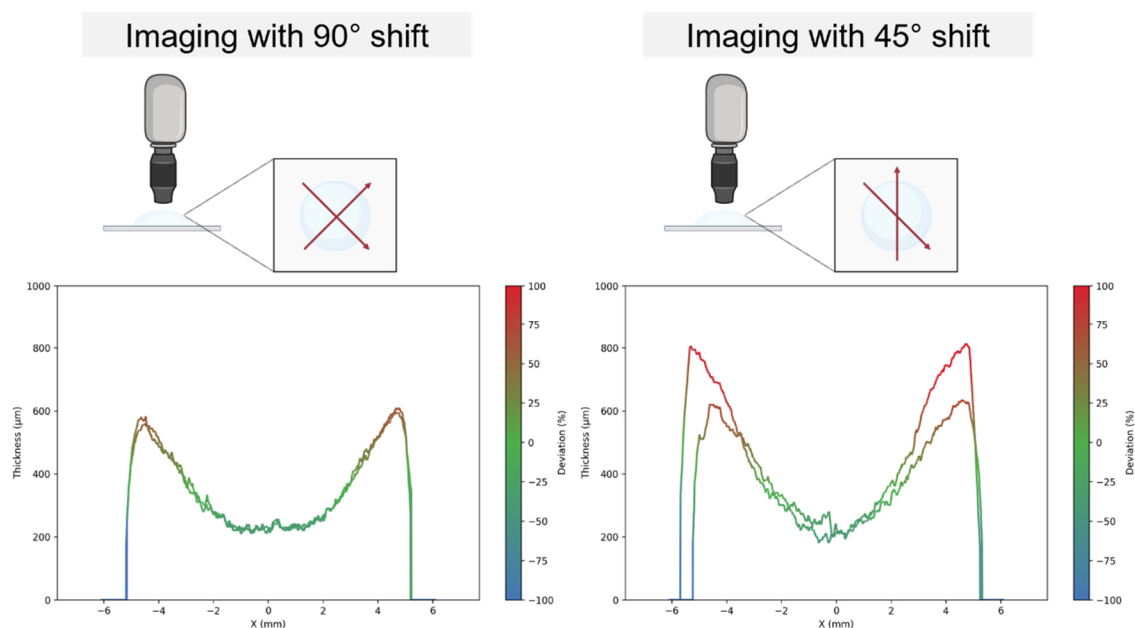


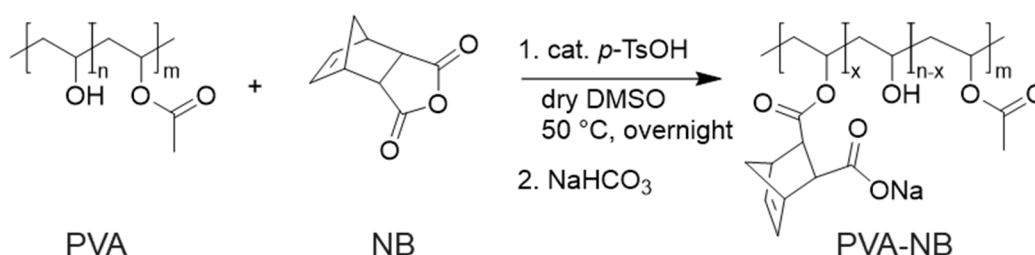
Figure 65. Analysis of radial homogeneity in printed structures. Overlay of different thickness profiles of OCT scans of the same scaffold acquired with 90° or 45° shift. Scaffolds were printed at a light dose of 130 mJ cm⁻² using an absorption compensation of 1.4 cm⁻¹.

4.2.2. Volumetric printing of thiol-ene PVA resins

In view of the dark curing and inhomogeneous crosslinking observed during volumetric printing with PVA-MA resins, it was investigated whether thiol-ene crosslinkable PVA-NB systems could provide better spatiotemporal control over the crosslinking kinetics and thereby achieve higher print fidelity. For this purpose, PVA was modified with NB groups, and a photocrosslinkable resin was formulated using LAP as photoinitiator and DTT as a model dithiol crosslinker.

4.2.2.1. Synthesis of PVA-NB

The synthesis of the photocrosslinkable PVA derivative PVA-NB by reaction with norbornene anhydride (**Scheme 5**) was performed according to a reported protocol.^[108] The NB functionalization was quantified by ¹H NMR (**Figure 66A**), where the signals corresponding to the alkene protons (6.3 ppm, 2H), the tertiary protons of the norbornene ring (3.3 ppm, 2H; 3.1 ppm, 2H) and the bridge protons (1.4 ppm, 2H) were visible. The F_{ene} was calculated by comparing the integral of the alkene protons to the tertiary proton of the PVA backbone (4.1 ppm, 1H) to be 4.3%. The degree of acetylation F_{acetyl} remained unchanged from the PVA starting material. The Raman spectrum of PVA-NB (**Figure 66B**) showed the stretching vibration of the double bond at 1574 cm⁻¹, which is characteristic for the alkene group of the norbornene ring.^[197] GPC analysis showed no changes in the molecular weight distribution during synthesis, indicating the absence of degradation or chain extension under the applied reaction conditions (**Figure 67**).



Scheme 5. Synthesis of photocrosslinkable PVA derivatives. PVA-NB was synthesized using norbornene anhydride (NB) and catalytic *p*-toluenesulfonic acid (*p*-TsOH) in dry DMSO.

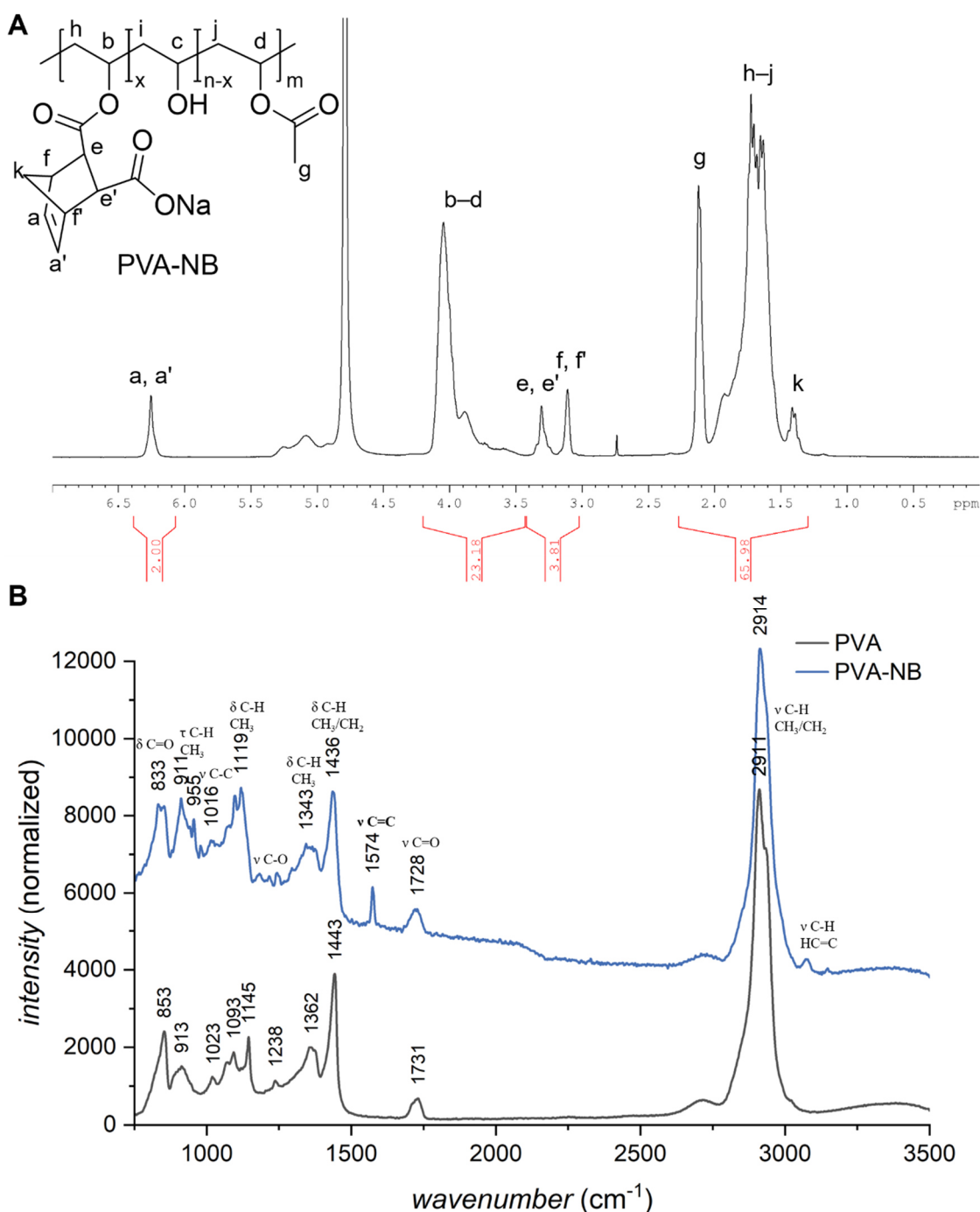


Figure 66. Characterization of PVA-NB. **A.** ^1H NMR spectrum of PVA-NB in D_2O ($\delta = 4.79$ ppm). The integral of two vinyl protons ‘a’ from the NB group (5.7 ppm) was used as reference with $I_a = 2.00$ for calibration, which corresponds to one NB group. This integral was compared to the signal of the tertiary proton of the polymer backbone repeating unit ‘b’ to calculate the degree of functionalization according to $F_{\text{ene}} = I_a / 2 / I_b = 1 / 23.18 = 0.04$. **B.** Raman spectra of PVA and PVA-NB polymers. The stretching vibration corresponding to the double bond of the NB group is observed at 1574 cm^{-1} confirming the derivatization.

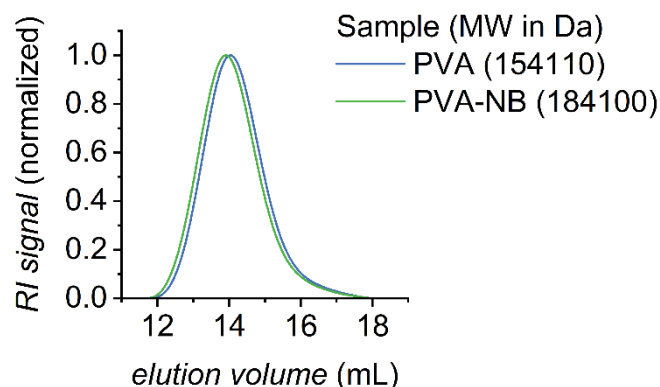


Figure 67. GPC elugrams of PVA and PVA-NB polymers. The molecular weight indicated in brackets was calculated using Pullulan standards.

To formulate a photocrosslinkable resin, a 10% w/v PVA-NB solution was supplemented with 0.05% w/v LAP and 0.57% w/v DTT as small dithiol model crosslinker. This corresponds to a thiol:ene ratio of 1 and a total thiol or ene concentration of 73.9 mM. The calculation of the required thiol concentration can be found in the experimental section.

4.2.2.2. Optical properties of PVA-NB resins and hydrogels

The UV/Vis spectrum of the PVA-NB resin is shown in **Figure 68A**. At 400 nm, the absorption coefficient of the resins was calculated to be 0.5 cm^{-1} . The RI of 10% w/v PVA-NB solutions at 400 nm was estimated to be 1.359 using the Cauchy equation (**Figure 68B**).^[199]

In contrast to the PVA-MA hydrogels, the PVA-NB hydrogels appeared transparent after crosslinking (**Figure 68C**). This effect can be attributed to a higher homogeneity of the radical step-growth crosslinked PVA-NB hydrogel.

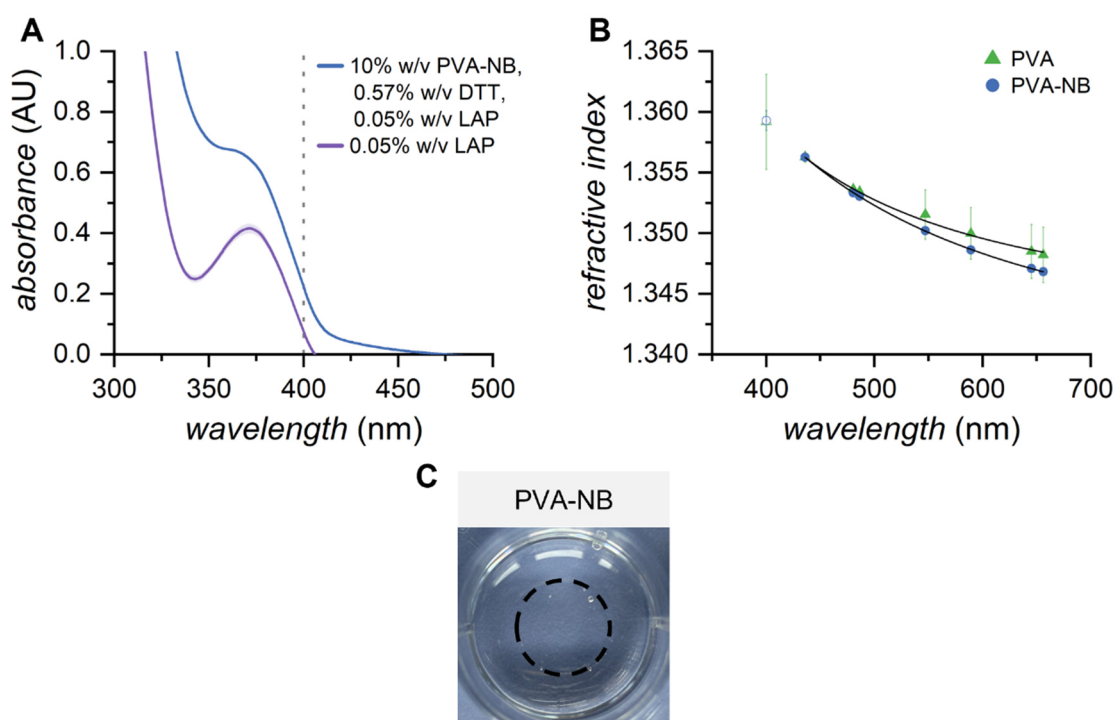


Figure 68. Optical properties of PVA-NB resin and hydrogel. **A.** UV/Vis absorbance spectrum of PVA-NB resin and the LAP solution. The resin consisted of 10% w/v PVA-NB supplemented with 0.57% w/v DTT and 0.05% w/v LAP. Data are shown as mean and SD for $N = 3$ independent samples. **B.** Refractive index of 10% w/v PVA-NB solutions in Milli-Q H_2O measured at different wavelengths (closed symbols). The RI of PVA solutions is also displayed for information. The experimental results were fitted to the Cauchy model and used to calculate the refractive index at 400 nm for the PVA-NB solution (open symbols). Data are shown as mean and SD for $N = 3$ independent samples. **C.** Photo of crosslinked PVA-NB hydrogel placed in H_2O . The hydrogel remains transparent.

4.2.2.3. Physicochemical properties of PVA-NB resins and hydrogels

The viscosity of 10% w/v PVA-NB solution in water at a shear rate of 1 s^{-1} was $0.31 \pm 0.04 \text{ Pa s}$ (**Figure 69A**). With this, the PVA-NB solutions showed a lower viscosity than PVA-MA solutions.

Figure 69B shows the evolution of the storage and loss shear moduli during photopolymerization of the resin at 400–500 nm monitored by oscillatory shear rheology. The resin showed a t_{gel} of $11.8 \pm 2.3 \text{ s}$, which is 1.8-times shorter than the PVA-MA resin (**Table 10**). The faster gelation is attributed to the faster radical thiol-ene step-growth reaction due to strain release from the NB unit upon addition of the thiyl radical to the vinyl group.^[52] The storage modulus G' of the PVA-NB hydrogel was 1.7-times lower than

for the PVA-MA hydrogels. Similar results were observed in thiol-ene vs. methacrylate crosslinked hyaluronic acid-based hydrogels in Chapter 2. PVA-MA crosslinking via a chain-growth mechanisms connects multiple vinyl groups leading to denser and stiffer crosslinking points. The thiol-ene step-growth mechanism only connects to NB groups through one dithiol. This leads to a lower clustering of crosslinks and a looser network structure. Intramolecular thiol-ene reactions could also form loops which do not contribute to the mechanical stiffness of the hydrogel network. Reaching higher stiffness values is potentially possible by increasing the molecular weight and the number of functional groups of the thiolated crosslinker.^[59, 66]

Strain and frequency sweeps of PVA-NB hydrogels (**Figure 69C, D**) showed a linear response within the range of 0.01 to 100% strain and 0.1 to 100 Hz. In comparison with PVA-MA hydrogels, the PVA-NB system shows a linear viscoelastic region over a wider (100 vs 1000%) strain range. This aligns with the expected higher homogeneity in thiol-ene hydrogels.

With an EWC of $97.4 \pm 0.2\%$, PVA-NB hydrogels show a 13% higher capacity to hold and uptake water than PVA-MA hydrogels ($86.4 \pm 1.3\%$). The looser network of PVA-NB hydrogels allows a higher water uptake and therefore higher EWC. Further, the charged carboxylic acid groups in the NB moiety can enhance the swelling and water uptake.

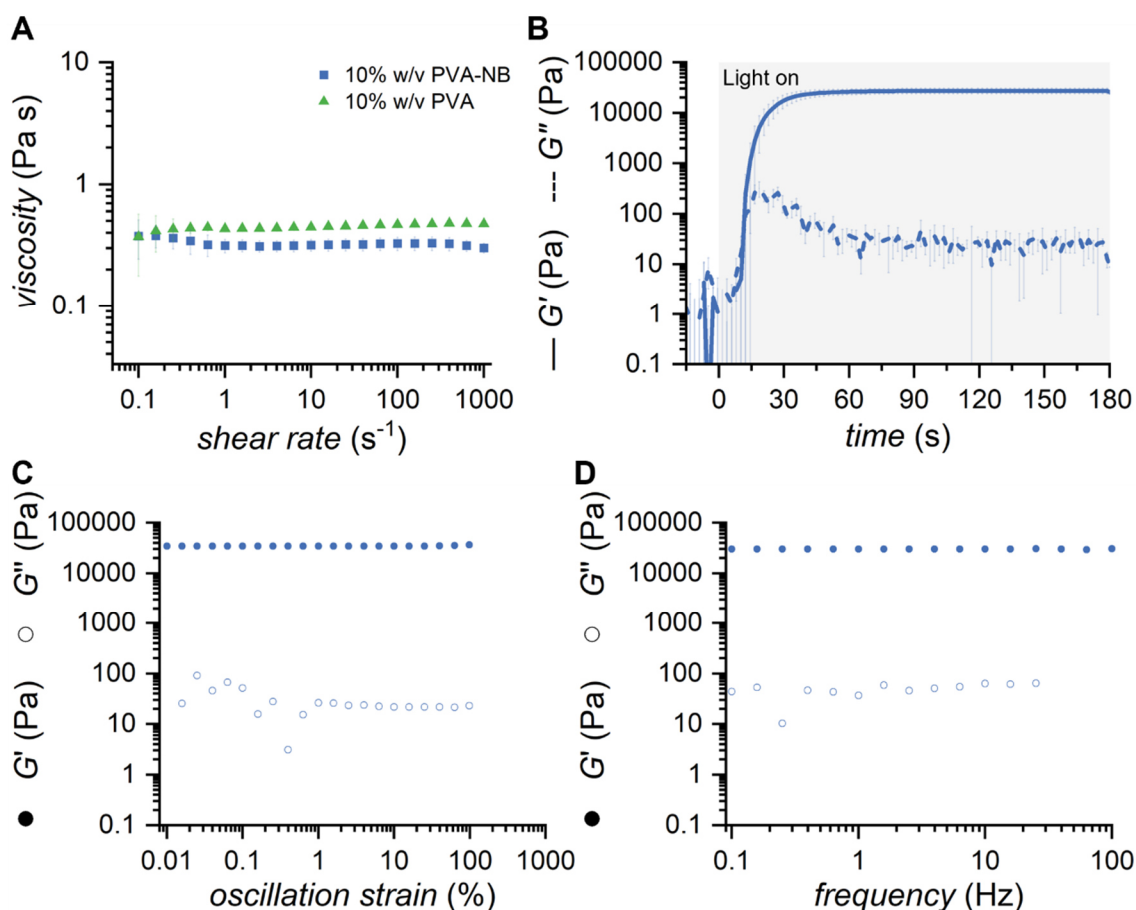


Figure 69. Rheological characterization of PVA-NB polymer solution and resin. **A.** Viscosity of PVA and PVA-NB solutions at 10% w/v in Milli-Q H₂O over a shear rate from 0.1 to 1000 s⁻¹. Data are shown as mean $\pm$ SD of $N = 3$ measurements **B.** Oscillatory shear rheology with in situ illumination (400–500 nm, 8 mW cm⁻²) of 10% w/v PVA-NB supplemented with 0.05% w/v LAP and 0.57% w/v DTT. The thiol:ene ratio in the hydrogel was 1. Shaded region indicates when light was on. Measurements were performed at 0.1% strain and 1 Hz frequency. Data are shown as mean $\pm$ SD of $N = 3$ measurements. **C.** Representative strain sweep with increasing oscillation strain between 0.01 and 1000% at 1 Hz frequency. **D.** Representative frequency sweep with increasing frequency between 0.1 and 100 Hz at 0.1% strain.

Table 10. Rheological characterization and equilibrium water content EWC of PVA-NB/DTT hydrogels. Gelation point t_{gel} and maximum storage modulus G' determined from oscillatory shear rheology measurements.

Hydrogel	t_{gel} (s)	G' (kPa)	EWC (%)	CGT (mJ cm ⁻²)
PVA-NB	11.8 $\pm$ 2.3	27.2 $\pm$ 3.2	97.4 $\pm$ 0.2	108 $\pm$ 0

4.2.2.4. Identification of the critical gelation threshold for PVA-NB resin

The PVA-NB/DTT resin required a CGT of $108 \pm 0 \text{ mJ cm}^{-2}$ (**Table 10**). This is 1.6-times lower than for the PVA-MA resin. No change in the dot area was observed in the PVA-NB/DTT resin after the end of the exposure (**Figure 70A**). This is supported by the photorheology experiment with shorter exposure time: the storage modulus of the PVA-NB/DTT resins plateaus immediately when light is switched off (**Figure 70B**), and the reached storage modulus G' is only 51% when compared to full crosslinking.

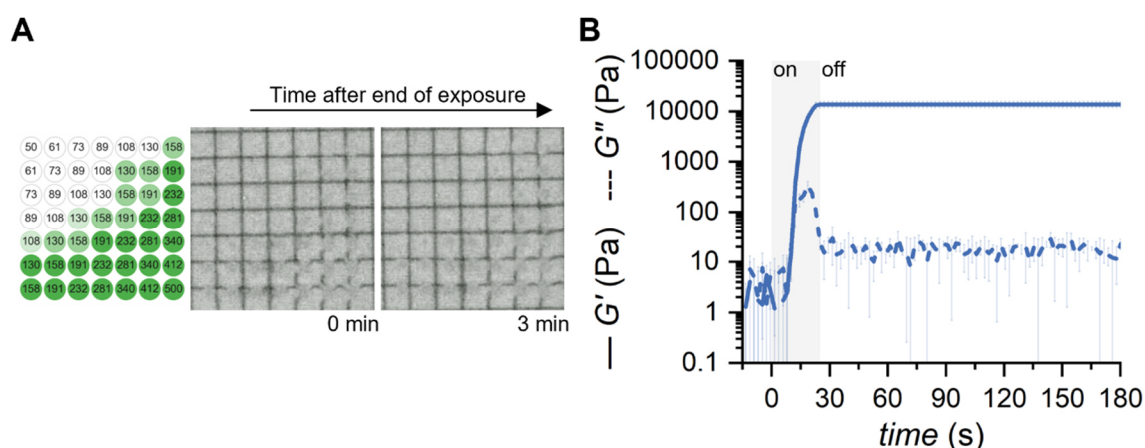


Figure 70. Post-exposure reactivity in PVA-NB resin. **A.** Crosslinked dots after illumination with different light doses during the light dose test for the PVA-NB resin. Images were captured 0 and 3 min after the exposure using the built-in camera in the Apparite software. **B.** Oscillatory shear rheology measurements with in situ illumination (400–500 nm, 8 mW cm⁻²) to observe the after-exposure reactivity. Samples were irradiated for 25 s. Measurements were performed at 0.1% strain and 1 Hz frequency. Data are shown as mean $\pm$ SD for $N = 3$. The samples contained 10% w/v PVA-NB, 0.05% w/v LAP and 0.57% w/v DTT corresponding to a thiol:ene ratio of 1.

4.2.2.5. Printability analysis of the PVA-NB resin

Discs with 12.5 mm diameter and 0.5 mm height printed at light dose of 140 mJ cm^{-2} (CGT +30%) were sticky and mechanically unstable but showed a larger diameter than targeted (**Figure 71A**). Raman spectroscopy showed a 50% consumption of the ene groups according to the decrease of the stretching vibration mode at 1574 cm^{-1} in the spectrum in comparison to the PVA-NB powder (**Figure 72**). It was assumed that the high reactivity of the thiol-ene system led to out-of-voxel polymerization and loss of shape fidelity at the used light dose. To reduce the reactivity of the resin, the concentration of photoinitiator

in the resin was decreased to 0.02% w/v. Discs with the targeted diameter but stickiness and poorly defined edges were obtained at 293 mJ cm⁻². Higher light doses (312–390 mJ cm⁻²) led to larger diameter and less sticky prints. Resins with 0.01% w/v LAP concentration printed at light dose of 1100 mJ cm⁻² (print time 142 s) led to sticky discs with the targeted diameter. Exchanging DTT by PEG2SH (1 kDa) in the resin did not improve the printability (**Figure 71B**). Printed discs remained soft and showed larger diameter.

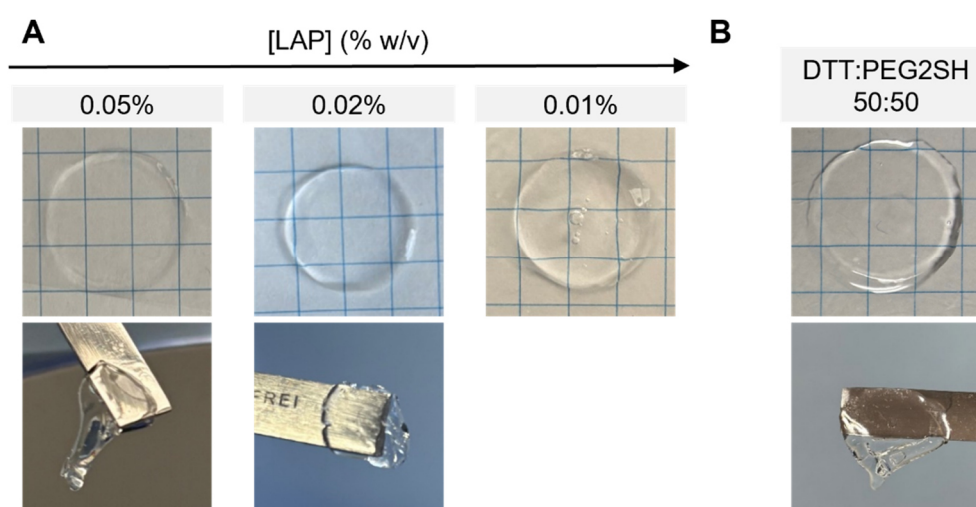


Figure 71. Printing PVA-NB discs with 0.5 mm height. **A.** Disc printed with different LAP concentrations do not exhibit good shape fidelity. **B.** Discs printed with a mixture of DTT and PEG2SH (1 kDa) show are less sticky but still show low shape fidelity. Grid represents 5 mm.

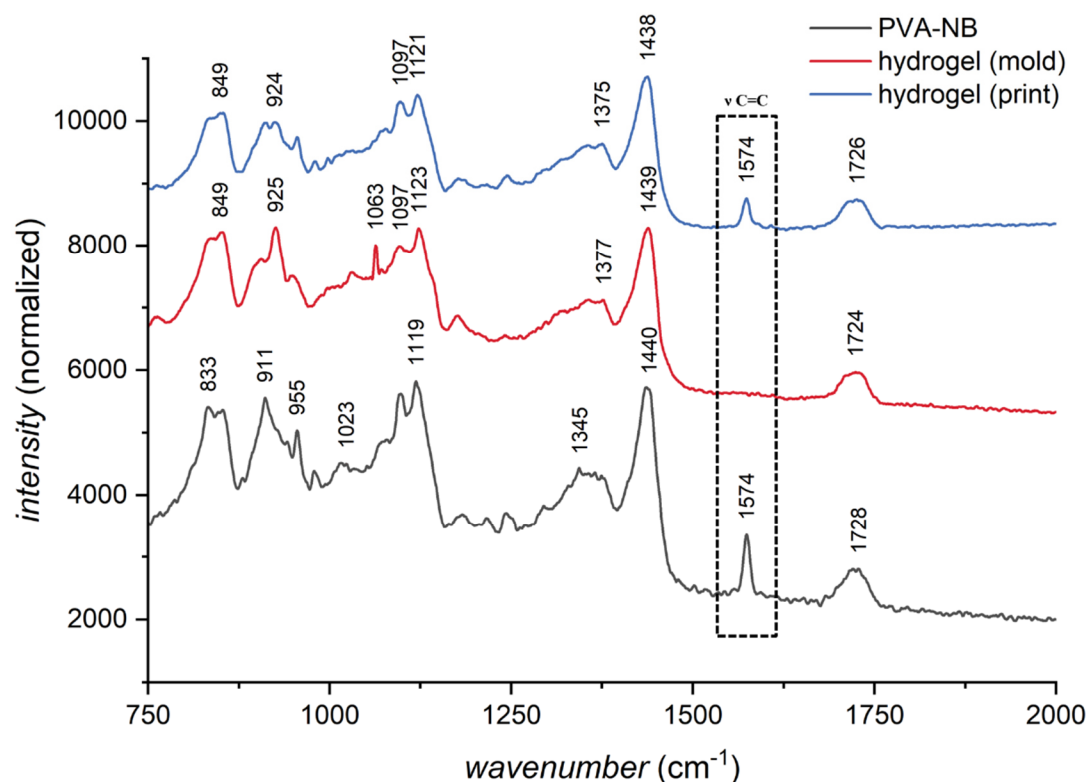


Figure 72. Raman spectra of uncrosslinked polymer, molded and printed PVA-NB hydrogels. The change in intensity in the stretching vibration of the carbon double bond at 1574 cm⁻¹ in the spectrum is used to monitor the consumption of the functional groups during crosslinking.

4.3. Discussion

In this work, 10% w/v PVA-MA hydrogel discs with 12.5 mm diameter and 0.35 thickness were printed using light doses 208–265 mJ cm⁻² in 26–35 s using VP. PVA-MA with molecular weight ~150 kDa and viscosity ~0.5 Pa s was used for these studies. Attempts to increase the printing resolution with thinner discs failed. The higher light dose required to overcome oxygen inhibition effects led to polymerization outside of the thinner disc. To reduce the impact of oxygen inhibition caused by continuous diffusion of oxygen at the air-resin interface and achieve crosslinking at lower light doses, the resin volume above the printed structure could be increased, or the printing vial could be purged with an inert gas in the future. To our knowledge, PVA-MA-based resins have not been applied to VP yet and, therefore, the printing fidelity achieved cannot be compared to other studies. DLP printed 10% w/w PVA-MA with lower molecular weight (13–23 kDa) and very low

viscosity (~ 0.01 Pa s) was used to print structures with positive and negative features down to ~ 50 μm in the presence of photoabsorber to improve resolution and avoid overcuring.^[107]

The printed CL geometries showed thinner features than targeted at the center of the CL geometry, and larger overhangs at the periphery. Attempts to improve the quality of the prints by absorption compensation were not successful. Further measures to improve the printing fidelity in the future could be:

- Lower LAP concentrations reduce the absorbance, reactivity of the resin and the dark curing effect. However, this can also lead to an overall lower crosslinking degree and, therefore, to prints with lower mechanical stability.
- Addition of radical quenchers such as TEMPO after exposure can stop the dark curing reaction in unintended areas.^[44] However, this also lowers the mechanical stability of the prints. As demonstrated by Thijssen et al., the TEMPO-to-photoinitiator concentration needs optimization to find a good compromise between fidelity and stability of the prints.^[147]
- Exploring different molecular weight, F_{ene} and polymer concentrations could improve the printability. Resins described for light-based 3D printing use lower polymer content and are based on PVA-MA with lower MW and higher F_{ene} but where only employed in extrusion printing.^[107] Overall, polymer content, MW and F_{ene} must be balanced to minimize post-exposure reactivity while maintaining sufficient reactivity and mechanical properties. In VP, a higher MW or higher polymer concentration is expected to increase viscosity thereby reduce diffusion of reactive species in non-targeted regions.
- Additives like *N*-methyl diethanolamine can regenerate propagating radicals from inactive peroxy radical species and reduce oxygen inhibition or printing light dose without compromising the resin absorbance.^[205]
- Additives like gelatin or Pluronic which form a thermoresponsive physical gel could potentially decrease the diffusion of (macro)radicals but their compatibility with the targeted application as CL needs to be considered.

It was not possible to print scaffolds with high shape fidelity with the PVA-NB resin within the range of tested resin compositions and printing parameters. Out-of-voxel curing was observed at all tested doses and compositions. It is assumed that the reactivity of the resin was too high, i.e. the gelation threshold too low, for the tested exposure conditions. VP of PVA-NB has been described by Qiu and co-authors.^[85] In their formulation, 1.5–3% w/v PVA-NB (47 kDa, $F_{ene} = 7.8\%$) was printed using PEG2SH (2 kDa) as crosslinker, 2 mM LAP ($\sim 0.07\%$ w/v) and 5% w/v sacrificial gelatin as viscosity enhancer using light doses in the range of 100–140 mJ cm⁻². This concentration of functional groups is approx. 1.8 to 3.7-times lower than ours ($\sim 20\text{--}40$ vs. 74 mM in this work), i.e. the resin has a lower reactivity. In addition, the use of gelatin as thermogelling agent could reduce the mobility of polymer chains and radicals and decelerate the crosslinking reaction. In a follow-up study, $\sim 1.5\text{--}4\%$ w/v PVA-NB (47 kDa, $F_{ene} = 7\%$) was printed by 2PP into structures ranging from $\sim 150 \times 150 \times 150 \mu\text{m}^3$ to $300 \times 300 \times 29 \mu\text{m}^3$.^[206] Using a thiolated PVA (PVA-SH, 35 kDa, $F_{SH} = 4.3\%$) crosslinker bearing $\sim 10\text{--}35$ free thiols per chain led to hydrogels with reduced swelling and enhanced mechanical properties compared with DTT and PEG2SH, and to improved printing fidelity. At 4% w/v total polymer content, PVA-SH decreased the mass swelling ratio by $\sim 24\text{--}29\%$ and increased the compressive modulus to 14.9 kPa which was $\sim 2.4\text{--}$ and $\sim 1.5\text{--}$ times higher than for DTT or PEG2SH (2 kDa), respectively. The PVA-NB/PVA-SH resin was processable at writing speeds between $10\text{--}400 \text{ mm s}^{-1}$ over a broader laser power range than PEG2SH, with apparent minimal line widths of $\sim 0.6\text{--}0.8 \mu\text{m}$ after swelling. Printing with DTT was unsuccessful under the printing conditions. Potentially, using a resin combination based on PVA-NB and PVA-SH could improve the printing fidelity of CL geometries using VP.

As discussed in Chapter 1, Section 1.5.3., VP relies on the chemical non-linearity of crosslinking reactions where a threshold light dose must be surpassed to induce gelation. This threshold can be adjusted using radical inhibitors. Thijssen et al. studied the impact of TEMPO as radical inhibitor in radical thiol-ene resins for VP. Increasing the TEMPO-to-photoinitiator ratio from 0 to 1 in triallyl isocyanurate/pentaerythritol tetrakis(3-mercaptopropionate) resins had an inhibitory effect in the photocrosslinking and increased the gelation light dose from 25.7 mJ cm⁻³ to 713.5 mJ cm⁻³.^[147] The authors highlight that the addition of TEMPO was crucial to achieve a clear dose contrast between

in-part and out-of-part voxels. The printability could be further improved by increasing the gelation point to higher doses in out-of-voxel regions. This could be achieved by reducing the effective concentration of reactive species, for example by lowering F_{ene} , polymer concentration and/or photoinitiator concentration. With this, the CGT is increased which could prevent out-of-voxel curing. These adjustments may also reduce final conversion and crosslink density and therefore must be considered against the targeted mechanical properties. In our experiments no improvement was observed by exchanging DTT by PEG-dithiols with different MW. PVA-SH derivatives bear multiple thiol groups and act as multifunctional macrocrosslinkers rather than simple dithiols. The higher functionality is expected to increase effective crosslinking efficiency at lower exposure times, enabling higher stiffness which could improve shape fidelity and scaffold stability at low light doses.^[59, 206] In addition, replacing a portion of PVA-NB with PVA-SH maintains the overall polymer content while maintaining a comparable viscosity in solution and crosslink density after curing.

In this chapter, it was attempted to print PVA-MA hydrogels into CL-like geometries in one single step without support structures or layers. However, the printed structures showed low shape fidelity, with a thinner center and thicker periphery of the lens. A similar shape deviation was observed by Toombs et al., who printed a silica glass based aspherical lenses by VP. While the outer parts were larger than expected, the inner part of the lens showed a negative shape deviation.^[179] This deviation is related to the light attenuation across the vial, and the enhanced scattering and therefore blurring of the projected light at larger printing volumes.^[141, 205] Notably, VP in this work was carried out in 20 mm-vials and printed structures had a diameter of 12.5 mm, which is the maximum build volume achievable with the printing setup. Zhang and coworkers developed a tomographic volumetric printer with 65- and 100-mm diameter vials and a 405 nm projector.^[205] They demonstrate the fabrication of complex geometries with up to 60 mm length and 20 mm height and feature sizes of 1.25 to 1.5 mm with surface deviations down to 0.175 mm. According to the authors, this large build volume was enabled by (i) improved 3D light dose computations which account for diverging light beams and refractions, (ii) using low photoinitiator concentrations (0.5 mM TPO-L) and (iii) adding a non-absorbent radical scavenger (125 mM *N*-methyldiethanolamine) which prevented oxygen inhibition.

Other authors have used iterative processes to optimize the quality of the printed structure and minimize form errors by adjusting the light dose distribution to compensate for under- or overcured regions.^[178] Although the algorithm for the light dose distribution cannot be adjusted using the commercial volumetric printer in this work, the obtained cross-sectional thickness profile could be used to re-design the 3D model to account for the observed structural deviations.

4.4. Conclusion

In this chapter, 10% w/v PVA-MA and PVA-NB resins were employed in VP and their suitability to print contact lens shaped geometries was evaluated. Both resins exhibited a viscosity <1 Pa s and were printed without adding viscosity enhancers to be compatible with the application scenario as CL. Photorheology and light dose tests demonstrated the post-exposure reactivity of PVA-MA resin during 3 min after exposure, which was not observed for PVA-NB. Discs with 12.5 mm diameter and 0.35–0.5 mm height were printed in less than 35 s out of the PVA-MA resin. Thinner discs could not be printed. CL-like geometries were attempted. OCT imaging of the cross-section revealed an inhomogeneous radial thickness profile. This could not be corrected neither by addition of radical scavengers nor by changes in the absorption compensation values in the software of the printer. A PVA-NB resin was also developed and tested. Using DTT or PEG2SH or different LAP photoinitiator concentrations did not lead to printed scaffolds with desired dimensions and form stability. Further optimization would be required to render the 10% w/v PVA-MA and PVA-NB resins printable in useful CL-like structures.

4.5. Experimental section

4.5.1. Materials

Polyvinyl alcohol (Mowiol 18-88, Mw 130,000 Da) was purchased from Sigma-Aldrich (Germany), dithiothreitol (purity 98%) from abcr GmbH (Germany) and *cis*-5-norbornene-*endo*-2,3-dicarboxylic anhydride (norbornene anhydride) from Fisher Scientific (Germany). D₂O for ¹H NMR spectroscopy was obtained from Deutero (Germany). Thiolated PEGs were obtained from Creative PEGWorks (USA). All other reagents were obtained from Carl Roth GmbH (Germany) and Sigma-Aldrich (Germany).

4.5.2. Methods

4.5.2.1. Synthesis of PVA-MA

The synthesis of PVA-MA was adapted from a previously published protocol.^[107] Briefly, PVA (5.00 g, 0.11 mol repeating units RU) was dissolved at 5% in Milli-Q H₂O at 80 °C. After cooling to 0 °C with an ice-bath, methacrylic anhydride (1.75 mL, 0.1 eq.) was added dropwise under vigorous stirring. The pH was adjusted to pH = 8.0 with 1 M NaOH for 2 h using an automatic titration system (Titroline7000, SI Analytics, Mainz, Germany). The reaction mixture was precipitated in ice-cold acetone (200 mL) and left on ice for 1 h. The supernatant was decanted, and the precipitate washed several times with acetone. The crude product was redissolved in Milli-Q H₂O, transferred in dialysis membranes (MWCO 3.5 kDa, SpectraPor) and dialyzed against Milli-Q H₂O for 2 days with two consecutive changes of the dialysis water per day. After lyophilization, PVA-MA (4.2 g) was obtained as white solid.

4.5.2.2. Synthesis of PVA-NB

PVA-NB was synthesized according to a protocol by Qin et al.^[108] Glassware was flame-dried glassware at 60 °C in Ar atmosphere. PVA (10.00 g, 0.23 mol RU) and *p*-toluene sulfonic acid monohydrate (0.03 g, 0.001 eq.) were dissolved in anhydrous DMSO (250 mL) under Ar. After 2 h, the solution was heated to 50 °C. Norbornene anhydride (2.00 g, 0.05 eq.) was dissolved in anhydrous DMSO (50 mL) under Ar and added dropwise to the PVA solution. The reaction was left under stirring at 50 °C overnight. The reaction mixture was transferred to a dialysis membrane (MWCO 3.5 kDa, SpectraPor) and

dialyzed against 0.01 M NaHCO₃ for 1 d, and against Milli-Q H₂O for 2 d (2 changes/d). Lyophilization yielded PVA-NB (8.5 g) as white solid.

4.5.2.3. ¹H NMR analysis

¹H NMR spectra of PVA-MA and PVA-NB polymers were acquired using a Bruker Avance 300 spectrometer (USA) at 298 K with a number of scans of 256 and a relaxation delay of 10 s. Chemical shifts are reported in parts per million (δ ppm) with the ¹H NMR solvent peak (D₂O: δ = 4.79 ppm) as reference. Analysis of spectra was conducted using TopSpin (v3.5, Bruker, USA). Polymers were dissolved at 20 mg mL⁻¹ in D₂O. Spectra were analyzed in TopSpin (Bruker, UA).

4.5.2.4. Determination of degree of functionality

To determine the degree of acetyl (F_{acetyl}) and alkene functionalization (F_{ene}) of PVA, PVA-MA and PVA-NB, the integral of the acetyl (2.1 ppm) or the alkene protons (6.30–5.7 ppm) was compared to the one of the tertiary proton of the PVA backbone (4.0 ppm). The signal of the acetyl protons appeared merged with the signal of the methylene protons of the PVA backbone. The F_{acetyl} was calculated by

$$F_{\text{acetyl}} = \frac{\frac{I_{\text{acetyl}}}{3H}}{I_{\text{backbone-CH}}} = \frac{\frac{I_{\text{acetyl+backbone-CH}_2} - 2H}{3H}}{I_{\text{backbone-CH}}} \quad (14)$$

The F_{ene} in the modified PVA polymers was calculated by

$$F_{\text{ene}} = \frac{\frac{I_{\text{vinyl}}}{2H}}{I_{\text{backbone-CH}}} \quad (15)$$

4.5.2.5. Raman spectroscopy

Raman spectroscopy measurements were performed on a LabRAM HR Evolution HORIBA Jobin Yvon A Raman microscope (France) at ambient conditions using a 633 nm He-Ne laser (Melles Griot, IDEX Optics and Photonics, USA) using 1800 lines per 1 mm grating.

Polymers were measured as powder. Molded and printed scaffolds were dried at 40 °C overnight in an oven before the measurement.

4.5.2.6. Gel permeation chromatography

GPC was performed with a PSS system (Polymer Standards Service, PSS, Germany) equipped with a combination of a PSS SUPREMA LUX pre-column and a PSS SUPREMA LUX analytical column and with refractive index detector. The mobile phase consisted of 0.1 M NaCl, 30% MeOH. Pullulan standards (0.18–14560 kDa, ReadyCal, Agilent, USA) were used for molar mass calibration. The PVA samples were dissolved at 2 mg mL⁻¹ in the mobile phase. Analysis was performed in the PSS WinGPC UniChrom Software (v8.31).

4.5.2.7. Viscosity measurements

The viscosity of PVA, PVA-MA and PVA-NB solutions at 10% w/v in Milli-Q H₂O were measured using a DHR 3 rheometer (TA instruments, USA) equipped with a 20 mm parallel plate geometry and a temperature control system. After pipetting the polymer solutions onto stainless steel lower plate, the upper plate was lowered to a gap of 200 μm and the sample and geometry covered with a metal cover to avoid drying or environmental disturbances during the measurement. After 5 min of temperature equilibration of the sample, a flow sweep measurement with shear rate increasing from 0.1 to 1000 s⁻¹ was performed. Measurements were performed at $T = 22$ °C. Three independent samples were measured for each composition ($N = 3$).

4.5.2.8. Calculation of molecular weight per alkene unit

The apparent average molecular weight (MW_{average}) of the repeating units (RU) in the functionalized PVA derivatives was calculated for each batch by weighting the molecular contributions of the unmodified PVA RU (MW_{PVA}), the acetylated PVA RU ($MW_{\text{PVA-acetyl}}$), and the ene-modified PVA RU ($MW_{\text{PVA-ene}}$) according to the respective degrees of functionality F_{acetyl} and F_{ene} , obtained from ¹H NMR analysis. The calculation is given in eq. 16

$$MW_{average} = MW_{PVA} * (1 - F_{ene} - F_{acetyl}) + MW_{PVA-acetyl} * F_{acetyl} + MW_{PVA-ene} * F_{ene} \quad (16)$$

From the $MW_{average}$ per RU, the molecular weight per alkene unit MW_{ene} can be calculated as follows:

$$MW_{ene} = \frac{MW_{average}}{F_{ene}} \quad (17)$$

The MW_{ene} will be used to calculate the required amount of thiolated crosslinker for the PVA-NB resin to obtain a final thiol:ene ratio of 1.

4.5.2.9. Calculation of thiol concentration for hydrogel precursor solution

PVA-NB resins were formulated with equal number of moles of alkene n_{ene} and thiol n_{SH} functional groups, corresponding to a thiol:ene ratio of 1. For a final polymer concentration C_{PVA-NB} = 10% w/v, the volume V of the solution and the in eq. 17 calculated molecular weight per alkene unit MW_{ene} , n_{ene} and n_{SH} can be re-expressed as

$$n_{ene} = \frac{C_{PVA-NB} * V}{MW_{ene}} = n_{SH} \quad (18)$$

Considering that the used crosslinker is bifunctional and therefore $n_{SH} = 2 * n_{dithiol}$, the necessary concentration of the dithiol molecule in the final solution in % w/v can be calculated by

$$C_{dithiol} = \frac{n_{SH} * MW_{dithiol}}{2 * V} \quad (19)$$

Substitution of eq. (18) for n_{SH} , $C_{dithiol}$ can be expressed as

$$C_{dithiol} = \frac{C_{PVA-NB} * MW_{dithiol}}{2 * MW_{ene}} \quad (20)$$

4.5.2.10. Formulation of the PVA resins

PVA-MA or PVA-NB were dissolved in Milli-Q H₂O on a shaker in the dark until a transparent solution was obtained. Stock solutions of LAP (1.0% w/v) in Milli-Q H₂O were prepared and added to the PVA solutions to obtain different photoinitiator concentrations. To the PVA-NB resins, DTT was added from a 5.0% w/v stock solution to a final thiol:ene ratio of 1. Solutions were centrifuged to remove bubbles trapped in the solution.

4.5.2.11. Oscillatory shear rheology with *in situ* photocrosslinking

The photocuring kinetics and mechanical properties of PVA-MA and PVA-NB hydrogels were analyzed by oscillatory shear rheology with *in situ* illumination using a DHR 3 rheometer (TA instruments, USA) in parallel plate geometry. A 12 mm stainless steel top plate and a 20 mm transparent quartz lower plate were used. A UV/Vis illumination source (Omnicure S1500, Excelitas, USA) with 400–500 nm filter installed was used for crosslinking.

38 μ L of the hydrogel precursor solution were pipetted on the lower plate, the upper plate was lowered to a gap of 300 μ m. The sample was surrounded with silicone oil and covered with a metal cover to avoid drying or disturbances from the environment during the measurement. In photocrosslinking kinetics experiments, the shear storage and loss moduli were recorded at 0.1% strain and 1 Hz frequency over 4 min. After 1 min, irradiation of the sample (400–500 nm, 8 mW cm⁻²) was started by the TRIOS software and maintained until the end of the measurement. The gelation time t_{gel} was determined as the timepoint of the crossover of shear storage and loss moduli, $G' = G''$. The maximum shear storage modulus of the hydrogels was determined as maximum value of G' during the exposure time and denominated G'_{max} . For time sweep experiments with shorter illumination period, the PVA-NB samples were exposed for 25 s, and PVA-MA samples for 40 s.

Strain (0.01 to 100% strain at 1 Hz frequency) and frequency sweeps (0.1 to 100 Hz at 0.1% strain) were performed on hydrogels to determine the linear viscoelastic region.

All measurements were performed in triplicates ($N = 3$) at room temperature.

4.5.2.12. Determination of equilibrium water content

Flat hydrogel discs of PVA-MA and PVA-NB (12 mm diameter, 350 μm height) were prepared by molding of 40 μL precursor solution in the rheometer and crosslinking using a UV/VIS source (Omnicure S1500, 400–500 nm, 8 mW cm^{-2} , 3 min). After crosslinking, the hydrogels were submerged in 1 mL Milli-Q H_2O and allowed to swell at 37 $^\circ\text{C}$ for 24 h. The hydrated samples were dry blotted using filter paper and the swollen weight recorded (W_s). After drying at 40 $^\circ\text{C}$ overnight in an oven, the samples were weighed again to obtain the dry weigh (W_d). The EWC in percentage was calculated as:

$$\text{EWC (\%)} = \frac{W_s - W_d}{W_s} * 100\% \quad (21)$$

Measurements were performed in triplicates ($N = 3$) and mean and standard deviation were given.

4.5.2.13. Absorbance measurements

The absorbance of the polymer solutions and resins was measured in a Varian Cary UV/Vis Spectrophotometers 4000 (USA). A quartz cuvette with 10 mm path length (d) was filled with the solutions, and the absorbance recorded within the wavelength range 500–200 nm. From the absorbance spectra, the absorption coefficient α at 400 nm was calculated by

$$\alpha = 2.303 * \frac{A_{400\text{nm}}}{d} \quad (22)$$

where $A_{400\text{nm}}$ is the absorbance value at 400 nm.

4.5.2.14. Determination of the refractive index

The RI of polymer solutions was measured using an Abbemat multiwavelength refractometer (Anton Paar, Austria). 1 mL were pipetted onto the prism of the

refractometer, and the refractive index in the wavelength range 435.9–656.0 nm was measured. The values for the RI were fitted according to the Cauchy equation:^[199]

$$RI = A + \frac{B}{\lambda^2} + \frac{C}{\lambda^4} (\lambda \text{ in } \mu\text{m}) \quad (23)$$

Where A, B and C describe the Cauchy coefficients. The coefficients were used to calculate the refractive index at 400 nm for polymer solutions and at 880 nm for hydrogels.

4.5.2.15. Dose test and critical gelation threshold

450–500 μL of the resin were transferred to a quartz cuvette with 1 mm path length. The cuvette was placed in the volumetric printer (Tomolite Performance, Readily3D, Switzerland) and exposed to a 7 x 7 matrix of dots in static mode. The projected dots had a diameter of 0.5 mm and a spacing of 0.5 mm and were illuminated with different light doses. After exposure, the cuvette was left 3 min inside of the printer to monitor dark curing. The cuvette was inspected by eye. The dot with the lowest light dose that led to the formation of a crosslinked hydrogel visible at the surface of the cuvette was defined as CGT.

4.5.2.16. Vol%metric printing

Volumetric printing was performed on a Tomolite Performance (Readily3D, Switzerland) using 20 mm glass vials (Readily3D, Switzerland) filled with 1–2 mL of resin. The vial was transferred in the printer and secured. The STL-file of the CAD model was loaded in the Apparite software and respective light dose and absorption compensation defined. After printing, the vial was left inside of the printer for 3 min to allow for dark curing if not stated otherwise. The printed scaffolds along with the uncured resin were transferred to a petri dish and quickly washed with Milli-Q H_2O to remove uncured resin.

4.5.2.17. Optical coherence tomography

Imaging of printed scaffolds was performed on a Thorlabs OCT System with a LSM03 scan lens. Printed scaffolds were placed below the scan lens on a glass cover slip, and an OCT image was acquired through the center of the scaffold with field of view with 14 mm

length and 2.69 mm height. The focus was set to image the top of the scaffold. The OCT image was exported as Tiff-Image with the refractive index set to 1.34 (calculated at 880 nm) and further analyzed in python.

4.5.2.18. Processing of OCT images in Python

The Tiff-Images were opened in python to convert into a binary mask. To facilitate the transformation, sides with scattering and noise were cropped, bright lines removed, and the brightness adjusted to optimize the mask towards the top center of the image. Due to differences in brightness of the imaged scaffold, not all parts could be transferred into the mask. The created binary mask was converted into a skeleton and the thickness along the x-axis exported as CSV-file. The python code for skeletonization was generated in collaboration with Dr. Christian Müller (INM). Representative images generated are shown in **Figure S5**. The x-axis was shifted horizontally to set the center of the mask to $x = 0$. The thickness was plotted over the x-axis. In addition, for each position, the measured thickness was compared to the theoretical thickness of the STL-file, the deviation calculated and displayed as color code in the thickness plot and in the mask. To calculate the center thickness, a range of ± 0.5 mm around $x = 0$ was taken into account.

5. Conclusion and Outlook

In this doctoral thesis, hydrogels (photo)crosslinked via radical thiol-ene reaction were developed and compared to methacrylated analogues. Hyaluronic acid (HA) and polyvinyl alcohol (PVA) were functionalized with pendant reactive groups including allyl ether (AE), norbornene (NB) and methacrylate (MA) moieties. The polymer and hydrogel precursor solutions as well as the crosslinked hydrogels were characterized by different methods to compare rheological and mechanical properties, (photo)crosslinking kinetics and reactivity, precursors stability and conversion. The hydrogel precursor solutions were employed as resins in digital light processing (DLP) and tomographic volumetric printing (VP), which represent an established and an emerging light-based 3D printing technique for biofabrication. The printability of the thiol-ene and methacrylate resins was compared and reactivity, i.e. exposure time or light dose, and resolution analyzed. To simulate a bioprinting scenario, the influence of cell medium and cells on the printability in VP was studied. The PVA-based resins were investigated from the perspective of the use of VP for contact lens fabrication.

The main conclusions of the work presented are the following:

- i. Hyaluronic acid can be modified with AE, NB or MA groups in straightforward 1-step reactions in aqueous medium to yield HA with controllable degrees of functionalities between 36 and 47%. The modified HA chains show differences in their stability: while HA polymers modified with AE or NB groups showed no detectable changes in the ^1H NMR spectrum for >15 months, the HA-MA was not stable during storage as solid at room temperature. The stability differences are relevant for shelf-life of the solid polymers.
- ii. Photorheology experiments showing a 4.6-times (Chapter 2) and 3.3-times (Chapter 3) faster t_{gel} of HA-NB in comparison to HA-MA align well with the lower exposure times needed for DLP-based printing and lower light dose in VP of these systems. A similar agreement was observed in PVA derivatives. This result validates rheological

studies for mimicking and predicting printability and exposure conditions, which has been proposed by others.^[85, 116, 147, 161, 207]

- iii. Photorheology experiments can quantify the extent and timescale of dark curing phenomena in photocrosslinkable systems with different radical polymerization mechanisms. In thiol-ene hydrogels, photocrosslinking is restricted to the illumination time period, while MA hydrogels exhibit a high post-exposure reactivity with extended crosslinking beyond illumination. This phenomenon is independent of the polymer backbone and solution viscosity within the tested range, as it was observed in HA and PVA hydrogels with viscosities between <0.1 and 0.5 Pa s. Post-exposure reactivity leads to polymerization in off-target regions and reduces resolution and shape fidelity in printed structures of MA-functionalized resins. Thiol-ene resins offer higher spatiotemporal control thereby improving precision and shape fidelity in 3D printing.
- iv. HA-NB resins of the compositions used in this work allowed DLP printing of 1-mm layers in 2–3 s with positive and negative features down to $100\text{ }\mu\text{m}$. With these parameters, the HA-NB resin achieves finer feature resolution than previously reported HA-based resins for DLP printing ($>360\text{ }\mu\text{m}$) which required comparable or longer exposure times (2.5–30 s) for substantially thinner layers ($100\text{--}300\text{ }\mu\text{m}$).^[7, 83, 84, 99] With this it proves potential as resin for bioprinting as others report HA- and gelatin-based bioresins at comparable or longer exposure times (2.5–60 s) where high cell viability was achieved.^[7, 83, 84, 116, 117, 133]
- v. The fast and efficient thiol-ene reaction allowed printing of HA-NB hydrogels with viscosity <0.1 Pa s for the first time. Scaffolds with perfusable channels down to $\sim 300\text{ }\mu\text{m}$ were printed at light doses between $139\text{--}176\text{ mJ cm}^{-2}$ and total print times of 18–23 s without the need of additives or post-exposure steps. Those results establish rapid crosslinking via the radical thiol-ene reaction as viable strategy to enable VP of

hydrogels using low-viscosity resins. The HA-NB is printable using similar light doses as reported methacrylate ($117\text{--}464\text{ mJ cm}^{-2}$)^[76, 143, 152, 155] or thiol-ene ($56\text{--}320\text{ mJ cm}^{-2}$)^[76, 77, 156, 157] gelatin-based resins and considerably faster than previously reported HA-based resins for VP which required a light dose of 625 mJ cm^{-2} . These results expand the portfolio of available resins for VP beyond gelatin-based systems.

- vi. Printing in the presence of cell medium and cells leads to a higher absorbance of the resin and the need of a 1.1- to 1.3-times higher light dose for VP. The fast and efficient photocrosslinking of the HA-NB bioresin allows printing at cytocompatible doses $<240\text{ mJ cm}^{-2}$ maintaining high cell viability in $<30\text{ s}$. This aligns with reported gelatin-based bioresins which required light doses of $185\text{--}500\text{ mJ cm}^{-2}$ when printing was carried out in the presence of cells.^[76, 77, 151] The thiol-ene modification further allows functionalization of the hydrogel with adhesive peptides or with alternative dithiol crosslinkers to achieve hydrogels with tunable mechanical properties, degradability and biofunctionality. These results are promising for cell scaffolds for tissue engineering applications.
- vii. A PVA-MA resin at 10% w/v concentration and 150 kDa could be volumetric printed into discs with 0.35 mm thickness but failed for printing curved and hollow structures like CL geometries. A 10% w/v PVA-NB resin was not printable under the tested conditions. Screening of resins at different polymer concentration, molecular weights and degree of functionalities, or in the presence of radical inhibitors or different thiol crosslinkers, as well as improvement in the algorithm for calculating the light dose distribution are necessary to reveal the real potential of VP of PVA hydrogels into CL forms.

This thesis provides quantitative information for assessing the impact of the most commonly used photo-initiated radical reactions in light-based 3D printing. The side-by-side comparison of thiol-ene based resins to the methacrylated counterparts, which are the gold

standard in the bioprinting community, with comparable molecular weight, concentration and degree of functionality, advances the understanding and quantitative proof of to which extent crosslinking mechanisms influence not only photocrosslinking kinetics but also printability. Of special interest is here the thiol-ene resin HA-NB which was employed in both, DLP and VP only with minor adjustments of the resin composition which facilitates transfer between different methodologies for specific biomedical applications.

References

- [1] "Bioprinting: Organe aus dem 3D-Drucker statt Organspende", in *VDI nachrichten*, 2024.
- [2] "Organe aus dem 3D-Drucker – Start-up meldet Erfolg in Tierversuchen", in *Handelsblatt*, 2025.
- [3] K. S. Lim, J. H. Galarraaga, X. Cui, G. C. J. Lindberg, J. A. Burdick, T. B. F. Woodfield, *Chemical Reviews* **2020**, 120, 10662.
- [4] S. Mantha, S. Pillai, P. Khayambashi, A. Upadhyay, Y. Zhang, O. Tao, H. M. Pham, S. D. Tran, *Materials (Basel)* **2019**, 12.
- [5] S. Sojdeh, A. Panjipour, A. Yaghmour, Z. Arabpour, A. R. Djalilian, *Gels* **2025**, 11, 724.
- [6] M. Dattilo, F. Patitucci, S. Prete, O. I. Parisi, F. Puoci, *Journal of Functional Biomaterials* **2023**, 14, 55.
- [7] T. Lam, T. Dehne, J. P. Kruger, S. Hondke, M. Endres, A. Thomas, R. Lauster, M. Sittlinger, L. Klope, *Journal of Biomedical Materials Research. Part B, Applied Biomaterials* **2019**, 107, 2649.
- [8] A. Herrmann, R. Haag, U. Schedler, *Advanced Healthcare Materials* **2021**, 10, e2100062.
- [9] Z. Kafrashian, S. Bruck, P. Rogin, M. Khamdan, H. Farrukh, S. Pearson, A. Del Campo, *Advanced Materials* **2025**, 37, e2309166.
- [10] J. Feng, Y. J. Zheng, S. Bhusari, M. Villiou, S. Pearson, A. del Campo, *Advanced Functional Materials* **2020**, 30.
- [11] S. Chatterjee, P. Upadhyay, M. Mishra, S. M, M. R. Akshara, K. N, Z. S. Zaidi, S. F. Iqbal, S. K. Misra, *RSC Advances* **2020**, 10, 36751.
- [12] C. S. A. Musgrave, F. Fang, *Materials (Basel)* **2019**, 12, 261.
- [13] Y. Lai, X. Xiao, Z. Huang, H. Duan, L. Yang, Y. Yang, C. Li, L. Feng, *International Journal of Molecular Sciences* **2024**, 25, 12567.
- [14] K. Zoller, D. To, A. Bernkop-Schnurch, *Biomaterials* **2025**, 312, 122718.
- [15] J. Malda, J. Visser, F. P. Melchels, T. Jungst, W. E. Hennink, W. J. Dhert, J. Groll, D. W. Huttmacher, *Advanced Materials* **2013**, 25, 5011.
- [16] P. Guo, L. Dong, B. Xue, Y. Cao, J. Yang, *Polymer Science & Technology* **2025**, 1, 812.
- [17] Y. L. Zhao, J. F. Stoddart, *Langmuir* **2009**, 25, 8442.
- [18] C. Li, A. Iscen, L. C. Palmer, G. C. Schatz, S. I. Stupp, *Journal of the American Chemical Society* **2020**, 142, 8447.
- [19] S. Bernhard, M. W. Tibbitt, *Advanced Drug Delivery Reviews* **2021**, 171, 240.
- [20] G. Xie, P. Li, Z. Zhao, X. Y. Kong, Z. Zhang, K. Xiao, H. Wang, L. Wen, L. Jiang, *Angewandte Chemie. International ed. in English* **2018**, 57, 16708.

- [21] D. D. Chang, W. H. Yan, Y. D. Yang, Q. C. Wang, L. Zou, *Dyes and Pigments* **2016**, 134, 186.
- [22] J. Hauptstein, L. Forster, A. Nadernezhad, H. Horder, P. Stahlhut, J. Groll, T. Blunk, J. Tessmar, *Macromolecular Bioscience* **2022**, 22, e2100331.
- [23] R. Pappalardo, M. Boffito, C. Cassino, V. Caccamo, V. Chiono, G. Ciardelli, *ACS Omega* **2024**, 9, 45774.
- [24] S. J. Wang, G. N. Nawale, O. P. Oommen, J. Hilborn, O. P. Varghese, *Polymer Chemistry* **2019**, 10, 4322.
- [25] A. de Miguel-Jimenez, B. Ebeling, J. I. Paez, C. Fink-Straube, S. Pearson, A. Del Campo, *Macromolecular Bioscience* **2023**, 23, e2200419.
- [26] J. I. Paez, A. Farrukh, R. Valbuena-Mendoza, M. K. Wlodarczyk-Biegun, A. Del Campo, *ACS Applied Materials & Interfaces* **2020**, 12, 8062.
- [27] A. P. Malafaia, R. Sobreiro-Almeida, J. M. M. Rodrigues, J. F. Mano, *Biomaterials Advances* **2025**, 167, 214105.
- [28] C. T. Huynh, F. Liu, Y. Cheng, K. A. Coughlin, E. Alsberg, *ACS Applied Materials & Interfaces* **2018**, 10, 25936.
- [29] S. Bian, M. He, J. Sui, H. Cai, Y. Sun, J. Liang, Y. Fan, X. Zhang, *Colloids and Surfaces. B, Biointerfaces* **2016**, 140, 392.
- [30] I. Kopyeva, R. P. Brady, C. A. DeForest, *Nature Reviews Bioengineering* **2025**, 3, 159.
- [31] Y. J. Zheng, M. K. L. Han, Q. Jiang, B. Li, J. Feng, A. del Campo, *Materials Horizons* **2020**, 7, 111.
- [32] C. A. DeForest, K. S. Anseth, *Nature Biotechnology* **2011**, 3, 925.
- [33] M. P. Sekar, S. Suresh, A. Zennifer, S. Sethuraman, D. Sundaramurthi, *ACS Biomaterials Science & Engineering* **2023**, 9, 3134.
- [34] C. Loebel, N. Broguiere, M. Alini, M. Zenobi-Wong, D. Eglin, *Biomacromolecules* **2015**, 16, 2624.
- [35] S. Lee, G. Choi, Y. J. Yang, K. I. Joo, H. J. Cha, *Carbohydrate Polymers* **2023**, 313, 120895.
- [36] B. R. Nelson, B. E. Kirkpatrick, C. E. Miksch, M. D. Davidson, N. P. Skillin, G. K. Hach, A. Khang, S. N. Hummel, B. D. Fairbanks, J. A. Burdick, C. N. Bowman, K. S. Anseth, *Advanced Materials* **2024**, 36, e2211209.
- [37] I. Condo, S. M. Giannitelli, D. Lo Presti, B. Cortese, O. Ursini, *Gels* **2024**, 10, 442.
- [38] S. Asim, C. Tuftee, A. T. Qureshi, R. Callaghan, M. L. Geary, M. Santra, V. Pal, I. Namli, G. H. F. Yam, I. T. Ozbolat, M. Rizwan, *Advanced Functional Materials* **2025**, 35.
- [39] S. C. P. Norris, S. M. Delgado, A. M. Kasko, *Polymer Chemistry* **2019**, 10, 3180.
- [40] A. Romano, I. Roppolo, E. Rossegger, S. Schlogl, M. Sangermano, *Materials (Basel)* **2020**, 13, 2777.

- [41] P. E. Farahani, S. M. Adelmund, J. A. Shadish, C. A. DeForest, *Journal of Materials Chemistry. B* **2017**, 5, 4435.
- [42] X. Zhang, W. Xi, G. Gao, X. Wang, J. W. Stansbury, C. N. Bowman, *Acs Macro Letters* **2018**, 7, 852.
- [43] B. D. Fairbanks, M. P. Schwartz, C. N. Bowman, K. S. Anseth, *Biomaterials* **2009**, 30, 6702.
- [44] P. Chansoria, R. Rizzo, D. Rutsche, H. Liu, P. Delrot, M. Zenobi-Wong, *Chemical Reviews* **2024**, 124, 8787.
- [45] C. A. Murphy, K. S. Lim, T. B. F. Woodfield, *Advanced Materials* **2022**, 34, e2107759.
- [46] M. D. Lechner, K. Gehrke, E. H. Nordmeier, "*Makromolekulare Chemie: Ein Lehrbuch für Chemiker, Physiker, Materialwissenschaftler und Verfahrenstechniker*", Springer Berlin Heidelberg, Berlin, Heidelberg, 2014.
- [47] C. C. Lin, C. S. Ki, H. Shih, *Journal of Applied Polymer Science* **2015**, 132.
- [48] S. M. Montgomery, C. M. Hamel, J. Skovran, H. J. Qi, *Extreme Mechanics Letters* **2022**, 53, 101714.
- [49] N. B. Cramer, C. N. Bowman, R. S. o. Chemistry, "Thiol-ene and Thiol-yne Chemistry in Ideal Network Synthesis", in *Thiol-X Chemistries in Polymer and Materials Science*, A. Lowe and C. Bowman, Eds., The Royal Society of Chemistry, 2013, p. 0.
- [50] R. Simic, J. Mandal, K. Zhang, N. D. Spencer, *Soft Matter* **2021**, 17, 6394.
- [51] B. Tieke, "*Makromolekulare Chemie: eine Einführung*", Wiley-VCH, Weinheim, 2014.
- [52] A. B. Lowe, *Polymer Chemistry* **2010**, 1, 17.
- [53] H. Shih, C. C. Lin, *Biomacromolecules* **2012**, 13, 2003.
- [54] B. H. Northrop, R. N. Coffey, *Journal of the American Chemical Society* **2012**, 134, 13804.
- [55] Z. Jian, S. Mecking, *Angewandte Chemie. International ed. in English* **2015**, 54, 15845.
- [56] M. V. Bermeshev, P. P. Chapala, *Progress in Polymer Science* **2018**, 84, 1.
- [57] K. F. Long, N. J. Bongiardina, P. Mayordomo, M. J. Olin, A. D. Ortega, C. N. Bowman, *Macromolecules* **2020**, 53, 5805.
- [58] C. E. Hoyle, C. N. Bowman, *Angewandte Chemie. International ed. in English* **2010**, 49, 1540.
- [59] J. Van Hoorick, A. Dobos, M. Markovic, T. Gheysens, L. Van Damme, P. Gruber, L. Tytgat, J. Van Erps, H. Thienpont, P. Dubruel, A. Ovsianikov, S. Van Vlierberghe, *Biofabrication* **2020**, 13, 015017.
- [60] D. P. Nair, M. Podgórski, S. Chatani, T. Gong, W. X. Xi, C. R. Fenoli, C. N. Bowman, *Chemistry of Materials* **2014**, 26, 724.

- [61] S. Chatani, D. P. Nair, C. N. Bowman, *Polymer Chemistry* **2013**, 4, 1048.
- [62] K. S. Anseth, J. A. Burdick, *MRS Bulletin* **2002**, 27, 130.
- [63] H. Shih, C. C. Lin, *Macromolecular Rapid Communications* **2013**, 34, 269.
- [64] J. Van Hoorick, P. Gruber, M. Markovic, M. Rollot, G. J. Graulus, M. Vagenende, M. Tromayer, J. Van Erps, H. Thienpont, J. C. Martins, S. Baudis, A. Ovsianikov, P. Dubruel, S. Van Vlierberghe, *Macromolecular Rapid Communications* **2018**, 39, e1800181.
- [65] C. Zhao, Z. Wu, H. Chu, T. Wang, S. Qiu, J. Zhou, Q. Zhu, X. Liu, D. Quan, Y. Bai, *Biomacromolecules* **2021**, 22, 2729.
- [66] T. Gockler, S. Haase, X. Kempter, R. Pfister, B. R. Maciel, A. Grimm, T. Molitor, N. Willenbacher, U. Schepers, *Advanced Healthcare Materials* **2021**, 10, e2100206.
- [67] Z. Munoz, H. Shih, C. C. Lin, *Biomaterials Science* **2014**, 2, 1063.
- [68] B. D. Fairbanks, M. P. Schwartz, A. E. Halevi, C. R. Nuttelman, C. N. Bowman, K. S. Anseth, *Advanced Materials* **2009**, 21, 5005.
- [69] A. A. Aimetti, A. J. Machen, K. S. Anseth, *Biomaterials* **2009**, 30, 6048.
- [70] J. J. Roberts, S. J. Bryant, *Biomaterials* **2013**, 34, 9969.
- [71] J. D. McCall, K. S. Anseth, *Biomacromolecules* **2012**, 13, 2410.
- [72] R. Rizzo, D. Ruetsche, H. Liu, M. Zenobi-Wong, *Advanced Materials* **2021**, 33, e2102900.
- [73] T. J. Tigner, S. Rajput, A. K. Gaharwar, D. L. Alge, *Biomacromolecules* **2020**, 21, 454.
- [74] S. Bertlein, G. Brown, K. S. Lim, T. Jungst, T. Boeck, T. Blunk, J. Tessmar, G. J. Hooper, T. B. F. Woodfield, J. Groll, *Advanced Materials* **2017**, 29, 1703404.
- [75] H. Genç, A. Cinciosi, R. Lohse, P. Stahlhut, J. Groll, C. Alexiou, I. Cicha, T. Jüngst, *Biomacromolecules* **2023**.
- [76] J. Duquesne, L. Parmentier, E. Vermeersch, F. Lemaire, J. W. Seo, R. I. Dmitriev, S. V. Vlierberghe, *Biofabrication* **2025**, 17, 025002.
- [77] A. Cinciosi, S. Stecher, M. Löffler, P. Bauer-Kreisel, K. S. Lim, T. B. F. Woodfield, J. Groll, T. Blunk, T. Jungst, *Advanced Healthcare Materials* **2023**, 12, e2300977.
- [78] B. G. Soliman, G. C. J. Lindberg, T. Jungst, G. J. Hooper, J. Groll, T. B. F. Woodfield, K. S. Lim, *Advanced Healthcare Materials* **2020**, 9, e1901544.
- [79] H. W. Ooi, C. Mota, A. T. Ten Cate, A. Calore, L. Moroni, M. B. Baker, *Biomacromolecules* **2018**, 19, 3390.
- [80] K. Guo, H. Wang, S. Li, H. Zhang, S. Li, H. Zhu, Z. Yang, L. Zhang, P. Chang, X. Zheng, *ACS Applied Materials & Interfaces* **2021**, 13, 7037.
- [81] G. F. Acosta-Velez, C. S. Linsley, M. C. Craig, B. M. Wu, *Bioengineering (Basel)* **2017**, 4, 11/1.
- [82] J. H. Galarraga, M. Y. Kwon, J. A. Burdick, *Scientific Reports* **2019**, 9, 19987.

- [83] A. P. Dhand, M. D. Davidson, J. H. Galarraga, T. H. Qazi, R. C. Locke, R. L. Mauck, J. A. Burdick, *Advanced Materials* **2022**, 34, e2202261.
- [84] J. H. Galarraga, A. P. Dhand, B. P. Enzmann, 3rd, J. A. Burdick, *Biomacromolecules* **2023**, 24, 413.
- [85] W. W. Qiu, J. Gehlen, M. Bernero, C. Gehre, G. N. Schaedli, R. Mueller, X. H. Qin, *Advanced Functional Materials* **2023**, 33, 2214393.
- [86] W. M. Gramlich, I. L. Kim, J. A. Burdick, *Biomaterials* **2013**, 34, 9803.
- [87] M. Winkelbauer, A. Hasenauer, D. Rutsche, H. Liu, J. Janiak, M. Nguyen, K. L. Christman, M. Zenobi-Wong, P. Chansoria, *Advanced Healthcare Materials* **2025**, 14, e2405105.
- [88] A. Fallacara, E. Baldini, S. Manfredini, S. Vertuani, *Polymers (Basel)* **2018**, 10, 701.
- [89] G. N. Iaconisi, P. Lunetti, N. Gallo, A. R. Cappello, G. Fiermonte, V. Dolce, L. Capobianco, *International Journal of Molecular Sciences* **2023**, 24.
- [90] P. Snetkov, K. Zakharova, S. Morozkina, R. Olekhovich, M. Uspenskaya, *Polymers (Basel)* **2020**, 12, 1800.
- [91] Z. Q. Luo, Y. Wang, J. B. Li, J. L. Wang, Y. R. Yu, Y. J. Zhao, *Advanced Functional Materials* **2023**, 33, 2306554.
- [92] D. Rebenda, M. Vrbka, P. Cipek, E. Toropitsyn, D. Necas, M. Pravda, M. Hartl, *Materials (Basel)* **2020**, 13, 2659.
- [93] E. M. Plaster, M. K. Eiken, C. Loebel, *Carbohydrate Polymer Technologies and Applications* **2023**, 6, 100360.
- [94] R. Hossain Rakin, H. Kumar, A. Rajeev, G. Natale, F. Menard, I. T. S. Li, K. Kim, *Biofabrication* **2021**, 13.
- [95] L. K. Shopperly, J. Spinnen, J. P. Kruger, M. Endres, M. Sittinger, T. Lam, L. Kloke, T. Dehne, *Journal of Biomedical Materials Research. Part B, Applied Biomaterials* **2022**, 110, 2310.
- [96] J. A. Burdick, C. Chung, X. Jia, M. A. Randolph, R. Langer, *Biomacromolecules* **2005**, 6, 386.
- [97] M. T. Poldervaart, B. Goversen, M. de Ruijter, A. Abbadessa, F. P. W. Melchels, F. C. Oner, W. J. A. Dhert, T. Vermonden, J. Alblas, *PLOS One* **2017**, 12, e0177628.
- [98] L. Ouyang, C. B. Highley, C. B. Rodell, W. Sun, J. A. Burdick, *ACS Biomaterials Science & Engineering* **2016**, 2, 1743.
- [99] A. Thomas, I. Orellano, T. Lam, B. Noichl, M. A. Geiger, A. K. Amler, A. E. Kreuder, C. Palmer, G. Duda, R. Lauster, L. Kloke, *Acta Biomaterialia* **2020**, 117, 121.
- [100] D. B. Unal, S. R. Caliri, K. J. Lampe, *Biomacromolecules* **2020**, 21, 4962.
- [101] C. Wang, H. Hao, J. Wang, Y. Xue, J. Huang, K. Ren, J. Ji, *Journal of Materials Chemistry. B* **2021**, 9, 4024.

- [102] M. D'Este, D. Eglin, M. Alini, *Carbohydrate Polymers* **2014**, *108*, 239.
- [103] A. Filimon, A. M. Dobos, M. D. Onofrei, D. Serbezeanu, *Polymers (Basel)* **2025**, *17*, 1016.
- [104] A. A. Sapalidis, *Symmetry* **2020**, *12*, 960.
- [105] M. M. Rahman Khan, M. M. H. Rumon, *Gels* **2025**, *11*, 88.
- [106] M. Puertas-Bartolome, I. Gutierrez-Urrutia, L. L. Teruel-Enrico, C. N. Duong, K. Desai, S. Trujillo, C. Wittmann, A. Del Campo, *Advanced Materials* **2024**, *36*, e2313848.
- [107] K. S. Lim, R. Levato, P. F. Costa, M. D. Castilho, C. R. Alcala-Orozco, K. M. A. van Dorenmalen, F. P. W. Melchels, D. Gawlitta, G. J. Hooper, J. Malda, T. B. F. Woodfield, *Biofabrication* **2018**, *10*, 034101.
- [108] X. H. Qin, K. Labuda, J. Chen, V. Hruschka, A. Khadem, R. Liska, H. Redl, P. Slezak, *Advanced Functional Materials* **2015**, *25*, 6606.
- [109] D. J. Whyte, E. H. Doeven, A. Sutti, A. Z. Kouzani, S. D. Adams, *Additive Manufacturing* **2024**, *84*, 104094.
- [110] L. Ouyang, C. B. Highley, W. Sun, J. A. Burdick, *Advanced Materials* **2017**, *29*, 1604983.
- [111] H. W. Kang, S. J. Lee, I. K. Ko, C. Kengla, J. J. Yoo, A. Atala, *Nature Biotechnology* **2016**, *34*, 312.
- [112] S. Kyle, Z. M. Jessop, A. Al-Sabah, I. S. Whitaker, *Advanced Healthcare Materials* **2017**, *6*, 1700264.
- [113] M. Hisham, A. E. Salih, H. Butt, *ACS Biomaterials Science & Engineering* **2023**, *9*, 4381.
- [114] N. Hedemann, A. Thomas, N. Tribian, A. K. Amler, S. Kruger, D. Holthaus, P. Huebbe, I. Florkemeier, J. Weimer, N. Maass, L. Kloke, D. Bauerschlag, M. T. van Mackelenbergh, *Biofabrication* **2025**, *17*, 025034.
- [115] M. H. Kim, C. C. Lin, *ACS Applied Materials & Interfaces* **2023**, *15*, 2737.
- [116] V. T. Duong, C. C. Lin, *Macromolecular Bioscience* **2023**, *23*, e2300213.
- [117] M. Wang, W. Li, J. Hao, A. Gonzales, 3rd, Z. Zhao, R. S. Flores, X. Kuang, X. Mu, T. Ching, G. Tang, Z. Luo, C. E. Garciamendez-Mijares, J. K. Sahoo, M. F. Wells, G. Niu, P. Agrawal, A. Quinones-Hinojosa, K. Egan, Y. S. Zhang, *Nature Communications* **2022**, *13*, 3317.
- [118] T. Grix, A. Ruppelt, A. Thomas, A. K. Amler, B. P. Noichl, R. Lauster, L. Kloke, *Genes (Basel)* **2018**, *9*.
- [119] A. P. Dhand, M. D. Davidson, J. A. Burdick, *Nature Reviews Bioengineering* **2025**, *3*, 108.
- [120] J. W. Choi, G. J. Kim, S. Hong, J. H. An, B. J. Kim, C. W. Ha, *Science Advances* **2022**, *12*, 13553.
- [121] M. Lee, R. Rizzo, F. Surman, M. Zenobi-Wong, *Chemical Reviews* **2020**, *120*, 10950.
- [122] R. Chaudhary, P. Fabbri, E. Leoni, F. Mazzanti, R. Akbari, C. Antonini, *Progress in Additive Manufacturing* **2023**, *8*, 331.

- [123] S. H. Kim, Y. K. Yeon, J. M. Lee, J. R. Chao, Y. J. Lee, Y. B. Seo, M. T. Sultan, O. J. Lee, J. S. Lee, S. I. Yoon, I. S. Hong, G. Khang, S. J. Lee, J. J. Yoo, C. H. Park, *Nature Communications* **2018**, 9, 1620.
- [124] R. Silva, R. C. Rebelo, C. T. B. Paula, P. Pereira, A. C. Fonseca, A. C. Serra, J. F. J. Coelho, *International Journal of Biological Macromolecules* **2025**, 306, 141389.
- [125] J. E. Ortiz-Cardenas, J. M. Zatorski, A. Arneja, A. N. Montalbino, J. M. Munson, C. J. Luckey, R. R. Pompano, *Organs-on-a-Chip* **2022**, 4.
- [126] L. Schittecatte, V. Geertsens, D. Bonamy, T. Nguyen, P. Guenoun, *MRS Communications* **2023**, 13, 357.
- [127] N. Bhattacharjee, C. Parra-Cabrera, Y. T. Kim, A. P. Kuo, A. Folch, *Advanced Materials* **2018**, 30, e1800001.
- [128] M. Caprioli, I. Roppolo, A. Chiappone, L. Larush, C. F. Pirri, S. Magdassi, *Nature Communications* **2021**, 12, 2462.
- [129] D. Xue, J. Zhang, Y. Wang, D. Mei, *ACS Biomaterials Science & Engineering* **2019**, 5, 4825.
- [130] S. Derakhshanfar, R. Mbeleck, K. Xu, X. Zhang, W. Zhong, M. Xing, *Bioactive Materials* **2018**, 3, 144.
- [131] C. Mandrycky, Z. Wang, K. Kim, D. H. Kim, *Biotechnology Advances* **2016**, 34, 422.
- [132] S. A. Skoog, P. L. Goering, R. J. Narayan, *Journal of Materials Science. Materials in Medicine* **2014**, 25, 845.
- [133] C. Yu, K. L. Miller, J. Schimelman, P. Wang, W. Zhu, X. Ma, M. Tang, S. You, D. Lakshminpathy, F. He, S. Chen, *Biomaterials* **2020**, 258, 120294.
- [134] P. N. Bernal, P. Delrot, D. Loterie, Y. Li, J. Malda, C. Moser, R. Levato, *Advanced Materials* **2019**, 31, e1904209.
- [135] Q. Thijssen, J. Toombs, C. C. Li, H. Taylor, S. Van Vlieberghe, *Progress in Polymer Science* **2023**, 147, 101755.
- [136] L. Stuwe, M. Geiger, F. Rollgen, T. Heinze, M. Reuter, M. Wessling, S. Hecht, J. Linkhorst, *Advanced Materials* **2024**, 36, e2306716.
- [137] V. Hahn, P. Rietz, F. Hermann, P. Müller, C. Barner-Kowollik, T. Schlöder, W. Wenzel, E. Blasco, M. Wegener, *Nature Photonics* **2022**, 16, 784.
- [138] A. Wolfel, C. Johnbosco, A. Anspach, M. Meteling, J. Olijve, N. F. Konig, J. Leijten, *Advanced Materials* **2025**, 37, e2501052.
- [139] L. Stoecker, G. Cedillo-Servin, N. F. Konig, F. V. de Graaf, M. Garcia-Jimenez, S. Hofmann, K. Ito, A. S. Wentzel, M. Castilho, *Advanced Materials* **2025**, 37, e2410292.
- [140] B. E. Kelly, I. Bhattacharya, H. Heidari, M. Shusteff, C. M. Spadaccini, H. K. Taylor, *Science* **2019**, 363, 1075.

- [141] J. Madrid-Wolff, J. Toombs, R. Rizzo, P. N. Bernal, D. Porcincula, R. Walton, B. Wang, F. Kotz-Helmer, Y. Yang, D. Kaplan, Y. S. Zhang, M. Zenobi-Wong, R. R. McLeod, B. Rapp, J. Schwartz, M. Shusteff, H. Talyor, R. Levato, C. Moser, *MRS Communications* **2023**, 13, 764.
- [142] P. N. Bernal, S. Florczak, S. Inacker, X. Kuang, J. Madrid-Wolff, M. Regehly, S. Hecht, Y. S. Zhang, C. Moser, R. Levato, *Nature Reviews Materials* **2025**, 10, 826.
- [143] D. Ribezzi, J. P. Zegwaart, T. Van Gansbeke, A. Tejo-Otero, S. Florczak, J. Aerts, P. Delrot, A. Hierholzer, M. Fussenegger, J. Malda, J. Olijve, R. Levato, *Advanced Materials* **2025**, 37, e2409355.
- [144] M. B. Riffe, M. D. Davidson, G. Seymour, A. P. Dhand, M. E. Cooke, H. M. Zlotnick, R. R. McLeod, J. A. Burdick, *Advanced Materials* **2024**, 36, e2309026.
- [145] P. Chansoria, D. Rutsche, A. Wang, H. Liu, D. D'Angella, R. Rizzo, A. Hasenauer, P. Weber, W. Qiu, N. B. M. Ibrahim, N. Korshunova, X. H. Qin, M. Zenobi-Wong, *Advanced Science (Weinheim)* **2023**, 10, e2300912.
- [146] S. Tisato, G. Vera, Q. Song, N. Nekoonam, D. Helmer, *Nature Communications* **2025**, 16, 6730.
- [147] Q. Thijssen, A. J. Ortega, R. P. Vieira, S. Van Vlierberghe, *Reactive & Functional Polymers* **2024**, 205, 106096.
- [148] D. Loterie, P. Delrot, "Volumetric 3D printing of elastomers by tomographic back-projection", (Preprint), 2018.
- [149] D. Loterie, P. Delrot, C. Moser, *Nature Communications* **2020**, 11, 852.
- [150] N. U. Dinc, A. Saba, J. Madrid-Wolff, C. Gigli, A. Boniface, C. Moser, D. Psaltis, *Nanophotonics* **2023**, 12, 777.
- [151] P. N. Bernal, M. Bouwmeester, J. Madrid-Wolff, M. Falandt, S. Florczak, N. G. Rodriguez, Y. Li, G. Grossbacher, R. A. Samsom, M. van Wolferen, L. J. W. van der Laan, P. Delrot, D. Loterie, J. Malda, C. Moser, B. Spee, R. Levato, *Advanced Materials* **2022**, 34, e2110054.
- [152] J. Gehlen, W. Qiu, G. N. Schadli, R. Muller, X. H. Qin, *Acta Biomaterialia* **2023**, 156, 49.
- [153] V. Sgarminato, J. Madrid-Wolff, A. Boniface, G. Ciardelli, C. Tonda-Turo, C. Moser, *Biofabrication* **2024**, 16.
- [154] M. Viola, M. G. Valverde, P. N. Bernal, J. P. van Trijp, J. Hak, G. Di Marco, M. Neumann, C. C. L. Schuurmans, C. F. van Nostrum, R. Masereeuw, S. M. Mihaila, J. Malda, R. Levato, T. Vermonden, *Advanced Functional Materials* **2024**, 34, 2406098.
- [155] M. B. Buchholz, P. N. Bernal, N. Bessler, C. Bonhomme, R. Levato, A. Rios, *Biofabrication* **2025**, 17.
- [156] M. Falandt, P. N. Bernal, O. Dudaryeva, S. Florczak, G. Grofibacher, M. Schweiger, A. Longoni, C. Greant, M. Assuncao, O. Nijssen, S. van Vlierberghe, J. Malda, T. Vermonden, R. Levato, *Advanced Materials Technologies* **2023**, 8, 2300026.

- [157] N. L. Pien, B. Bogaert, M. Meeremans, C. S. Popovici, P. Dubruel, C. De Schauwer, S. Van Vlierberghe, *Additive Manufacturing* **2025**, *109*, 104850.
- [158] M. R. Binelli, A. Kan, L. E. A. Rozas, G. Pisaturo, N. Prakash, A. R. Studart, *Advanced Materials* **2023**, *35*, e2207483.
- [159] D. Iudin, L. Gerridzen, N. B. P, C. C. L. Schuurmans, M. Neumann, L. Nguyen, M. J. van Steenberghe, J. Hak, W. Li, C. Casadidio, A. M. van Genderen, R. Masereeuw, R. Levato, Y. S. Zhang, B. G. P. van Ravensteijn, T. Vermonden, *Biomacromolecules* **2025**, *26*, 4108.
- [160] M. B. Buchholz, P. N. Bernal, N. Bessler, C. Bonhomme, R. Levato, A. Rios, *Biofabrication* **2025**, *17*, 031001.
- [161] P. C. Hall, D. Chasko, T. Wheeler, D. Ostojich, J. Aptecker, I. Liashenko, R. Luxenhofer, G. C. J. Lindberg, P. D. Dalton, *Advanced Materials Technologies* **2024**, *10*, 2401668.
- [162] Y. C. Oh, J. J. Ong, H. Alfassam, E. Diaz-Torres, A. Goyanes, G. R. Williams, A. W. Basit, *Drug Delivery and Translational Research* **2025**, *15*, 1595.
- [163] G. Grossbacher, M. Bartolf-Kopp, C. Gergely, P. N. Bernal, S. Florczak, M. de Ruijter, N. G. Rodriguez, J. Groll, J. Malda, T. Jungst, R. Levato, *Advanced Materials* **2023**, *35*, e2300756.
- [164] M. Xie, L. Lian, X. Mu, Z. Luo, C. E. Garciamendez-Mijares, Z. Zhang, A. Lopez, J. Manriquez, X. Kuang, J. Wu, J. K. Sahoo, F. Z. Gonzalez, G. Li, G. Tang, S. Maharjan, J. Guo, D. L. Kaplan, Y. S. Zhang, *Nature Communications* **2023**, *14*, 210.
- [165] F. Alam, M. Elsherif, B. AlQattan, M. Ali, I. M. G. Ahmed, A. Salih, D. S. Antonysamy, A. K. Yetisen, S. Park, H. Butt, *Advanced Engineering Materials* **2020**, *23*.
- [166] H. Sahabudeen, R. Machatschek, A. Lendlein, *Multifunctional Materials* **2021**, *4*, 042001.
- [167] K. Y. Wu, A. Dave, M. Carbonneau, S. D. Tran, *Micromachines (Basel)* **2024**, *15*, 856.
- [168] X. Ma, S. Ahadian, S. Liu, J. W. Zhang, S. N. Liu, T. Cao, W. B. Lin, D. Wu, N. R. de Barros, M. R. Zare, S. E. Diltemiz, V. Jucaud, Y. Z. Zhu, S. M. Zhang, E. Banton, Y. Gu, K. W. Nan, S. Xu, M. R. Dokmeci, A. Khademhosseini, *Advanced Intelligent Systems* **2021**, *3*, 2000263.
- [169] Y. Zhu, S. Li, J. Li, N. Falcone, Q. Cui, S. Shah, M. C. Hartel, N. Yu, P. Young, N. R. de Barros, Z. Wu, R. Haghniaz, M. Ermis, C. Wang, H. Kang, J. Lee, S. Karamikamkar, S. Ahadian, V. Jucaud, M. R. Dokmeci, H. J. Kim, A. Khademhosseini, *Advanced Materials* **2022**, *34*, e2108389.
- [170] "Sensimed - Triggerfish".
- [171] A. M, A. Saxena, D. Mishra, K. Singh, S. D. George, *Microsystems & Nanoengineering* **2025**, *11*, 59.
- [172] I. Rykowska, I. Nowak, R. Nowak, *Molecules* **2021**, *26*, 5577.
- [173] Y. Zhu, R. Nasiri, E. Davoodi, S. Zhang, S. Saha, M. Linn, L. Jiang, R. Haghniaz, M. C. Hartel, V. Jucaud, M. R. Dokmeci, A. Herland, E. Toyserkani, A. Khademhosseini, *Small* **2023**, *19*, e2207017.

- [174] F. Alam, M. Elsherif, B. AlQattan, A. Salih, S. M. Lee, A. K. Yetisen, S. Park, H. Butt, *ACS Biomaterials Science & Engineering* **2021**, 7, 794.
- [175] P. P. Kothari, S. Ch, S. G. Padaga, S. Biswas, *International Journal of Biological Macromolecules* **2025**, 319, 145307.
- [176] T. Gissibl, S. Thiele, A. Herkommer, H. Giessen, *Nature Photonics* **2016**, 10, 554.
- [177] L. Siegle, S. Ristok, H. Giessen, *Optics Express* **2023**, 31, 4179.
- [178] S. O'Halloran, A. Pandit, A. Heise, A. Kellett, *Advanced Science (Weinheim)* **2023**, 10, e2204072.
- [179] J. T. Toombs, M. Luitz, C. C. Cook, S. Jenne, C. C. Li, B. E. Rapp, F. Kotz-Helmer, H. K. Taylor, *Science* **2022**, 376, 308.
- [180] D. Webber, Y. J. Zhang, K. L. Sampson, M. Picard, T. Lacelle, C. Paquet, J. Boisvert, A. Orth, *Optica* **2024**, 11, 665.
- [181] J. A. Burdick, G. D. Prestwich, *Advanced Materials* **2011**, 23, H41.
- [182] T. Steudter, T. Lam, H. Pirmahboub, C. Stoppel, L. Kloke, S. Pearson, A. Del Campo, *Macromolecular Bioscience* **2025**, 25, e2400535.
- [183] J. Hauptstein, T. Bock, M. Bartolf-Kopp, L. Forster, P. Stahlhut, A. Nadernezhad, G. Blahetek, A. Zernecke-Madsen, R. Detsch, T. Jungst, J. Groll, J. Tessmar, T. Blunk, *Advanced Healthcare Materials* **2020**, 9, e2000737.
- [184] J. M. Heffernan, D. J. Overstreet, L. D. Le, B. L. Vernon, R. W. Sirianni, *Annals of Biomedical Engineering* **2015**, 43, 1965.
- [185] T. Wan, P. Fan, M. Zhang, K. Shi, X. Chen, H. Yang, X. Liu, W. Xu, Y. Zhou, *ACS Applied Bio Materials* **2022**, 5, 334.
- [186] M. Q. Li, Z. H. Tang, C. Wang, Y. Zhang, H. T. Cui, X. S. Chen, *Chinese Journal of Polymer Science* **2014**, 32, 969.
- [187] A. D. D. Cavalcanti, B. A. G. Melo, R. C. Oliveira, M. H. A. Santana, *Applied Biochemistry and Biotechnology* **2019**, 188, 527.
- [188] L. Shahzadi, F. Maya, M. C. Breadmore, S. C. Thickett, *ACS Applied Polymer Materials* **2022**, 4, 3896.
- [189] T. Borke, M. Najberg, P. Ilina, M. Bhattacharya, A. Urtti, H. Tenhu, S. Hietala, *Macromolecular Bioscience* **2018**, 18, 1700200.
- [190] L. Guo, Y. Chai, F. Zhou, P. Wang, *IOP Conference Series: Earth and Environmental Science* **2021**, 651, 042022.
- [191] H. Genc, A. Cianciosi, R. Lohse, P. Stahlhut, J. Groll, C. Alexiou, I. Cicha, T. Jungst, *Biomacromolecules* **2023**, 24, 1497.
- [192] C. Y. M. Tung, P. J. Dynes, *Journal of Applied Polymer Science* **1982**, 27, 569.
- [193] L. A. Sawicki, A. M. Kloxin, *Journal of Visualized Experiments* **2016**, e54462.

- [194] S. Bhusari, S. Sankaran, A. Del Campo, *Advanced Science (Weinheim)* **2022**, 9, e2106026.
- [195] Readily3D, **2023**.
- [196] C. A. Castor, A. Pontier, J. Durand, J. C. Pinto, L. Prat, *Chemical engineering science* **2015**, 131, 340.
- [197] Y. C. Pan, T. Li, Y. Zhou, L. Li, *Polymer* **2022**, 242, 124613.
- [198] A. Synytsya, A. Synytsya, P. Alexa, R. Wagner, M. Davidková, K. Volka, *Journal of Raman spectroscopy* **2011**, 42, 544.
- [199] M. R. McGrory, M. D. King, A. D. Ward, *Journal of Visualized Experiments* **2020**, 124, 9617.
- [200] K. J. L, T. A. J, C. Ye, J. Trujillo, M. Triplett, L. M. M, C. Robertson, W. Hynes, M. W. E, *Biomedical Materials* **2024**, 20, 015009.
- [201] X. Guo, R. A. Mittelstaedt, L. Guo, J. G. Shaddock, R. H. Heflich, A. H. Bigger, M. M. Moore, N. Mei, *Toxicology in Vitro* **2013**, 27, 1496.
- [202] B. Ozdalgic, A. K. Yetisen, S. Tasoglu, *Journal of 3D Printing in Medicine* **2022**, 6, 105.
- [203] N. L. B. Thai, H. T. Beaman, M. Perlman, E. E. Obeng, C. Du, M. B. B. Monroe, *ACS Applied Bio Materials* **2024**, 7, 7818.
- [204] Y. J. Zhang, H. de Haan, K. Houlahan, K. L. Sampson, D. Webber, A. Orth, T. Lacelle, L. Gaburici, R. Lam, B. Deore, C. Paquet, *Additive Manufacturing* **2025**, 109, 104844.
- [205] Y. Zhang, K. Houlahan, D. Webber, N. Milliken, K. L. Sampson, H. W. de Haan, H. Li, R. Vlaming, L. Gaburici, A. Orth, C. Paquet, *Advanced Materials* **2025**, 37, e08729.
- [206] W. Qiu, M. Bernero, M. E. Ye, X. Yang, P. Fisch, R. Muller, X. H. Qin, *Advanced Materials* **2026**, e10834.
- [207] A. P. Dhand, B. E. Kirkpatrick, M. Garay-Sarmiento, B. R. Nelson, C. E. Miksch, B. Meurer-Zeman, H. M. Zlotnick, A. Mandal, J. S. Lee, J. Cione, C. N. Bowman, K. S. Anseth, J. A. Burdick, *Science Advances* **2025**, 11, eadw9262.

Appendix

Table S1. Physicochemical properties of HA resins used for printing in selected reports.

Reactive group	F_{ene} (%)	MW _{HA} (kDa)	c(HA-X)	Crosslinker	Printing technique	Viscosity (mPa s)	Storage Modulus G' (kPa)	Young's modulus E (kPa)	xy Resolution (μ m)	Ref.
NB	50	60	3% w/w	PEG2SH	inkjet	8	0.8	-	-	[81]
NB	40	74	2% w/w	DTT	extrusion	-	~ 6	-	-	[82]
MA	100	88	0.6% w/w	dithiol-functionalized adamantane- β -cyclodextrin	DLP	~60 a)	~14 (IPN) ~11 (HA-NB only)	-	~300 (IPN) ~550 (HA-NB only)	[83]
NB	40		4.5% w/w							
NB	40	88	5% w/w	DTT	DLP	~200 a)	~0.01	-	~500	[84]
MA	14	1000	1-2% w/v	-	DLP	4-13 a)	-	1.3-4.5	~360	[99]
MA	22	90	5%	-	extrusion	<1000	~11	-	-	[98]
MA	5-7	1700	3% w/v	-	extrusion	-	2.6	-	-	[97]
SH	45	51	2.5% w/v	PEGDA PEG-diallyl	extrusion	~10	-	~5	-	[22]
NB	36-42	40-50	4% w/v	DTT	DLP	~20-60 a)	8-27	-	~100	This work
AE	40-47			-						
MA	39-42			-						

a) at 100 s⁻¹ shear rate

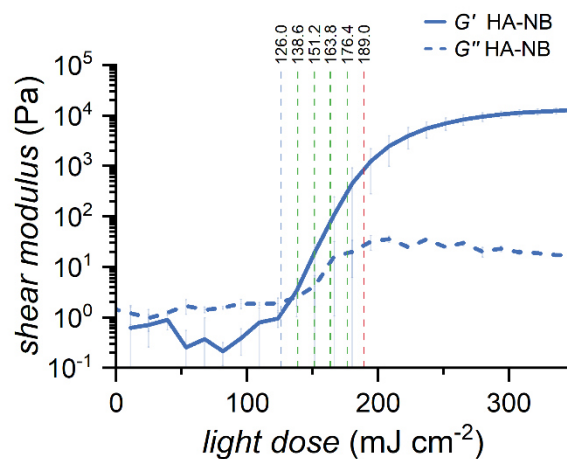


Figure S1. Photorheology curve of HA-NB resin. Evolution of shear storage modulus G' and loss modulus G'' of HA-NB resins during illumination (400–500 nm, 6.6 mW cm⁻²).

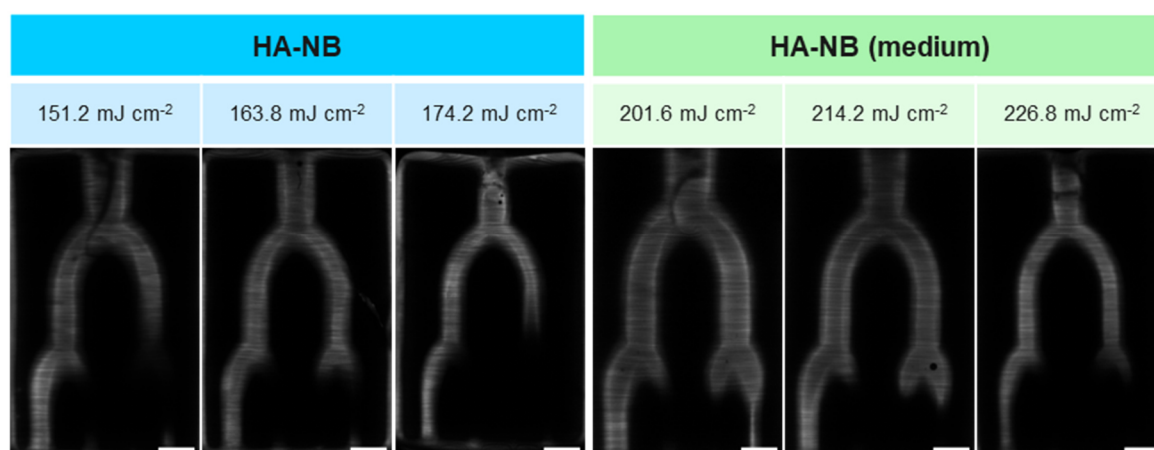


Figure S2. Microscopy images of channels in HA-NB and HA-NB (medium) scaffolds at different light doses. Channels are perfused with FITC-Dex for imaging and quantification of channel dimensions. Scale bar represents 1000 μ m.

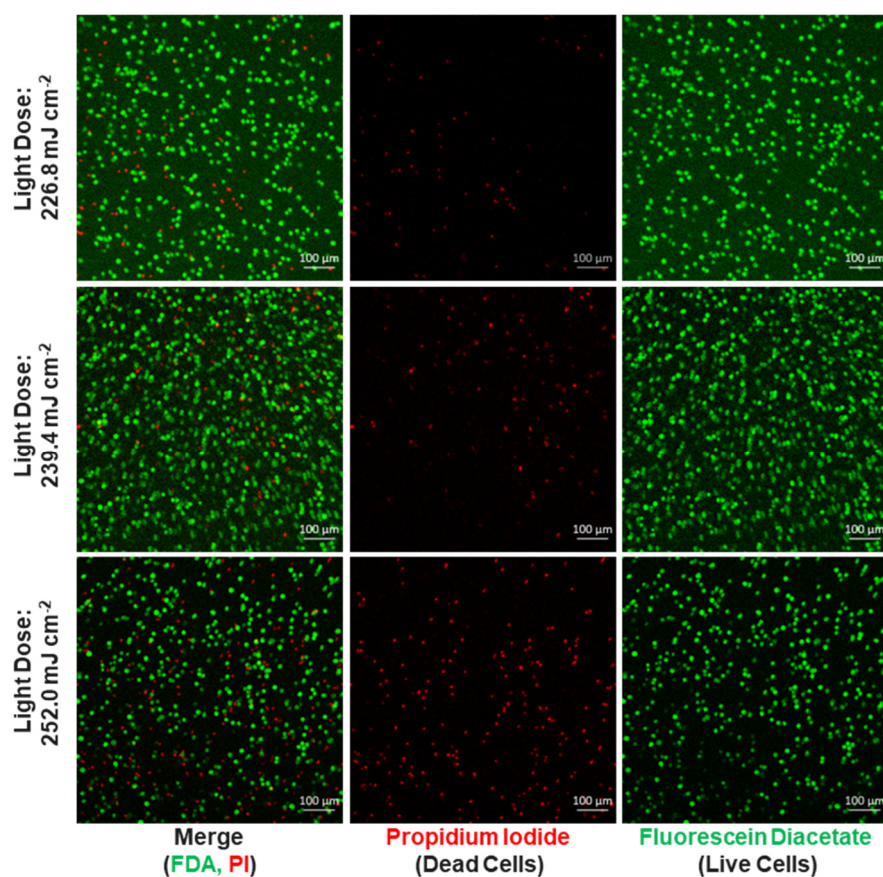


Figure S3. Representative images of viability of cells encapsulated in printed HA-NB scaffolds. Fluorescence image of L929 fibroblasts encapsulated for 1 day in HA-NB scaffolds, photopolymerized at different light doses (400 nm, 7.75 mW cm⁻², 29 – 31 s). The live cells were stained with fluorescein diacetate (FDA, green) and dead cells were stained with propidium iodide (PI, red).

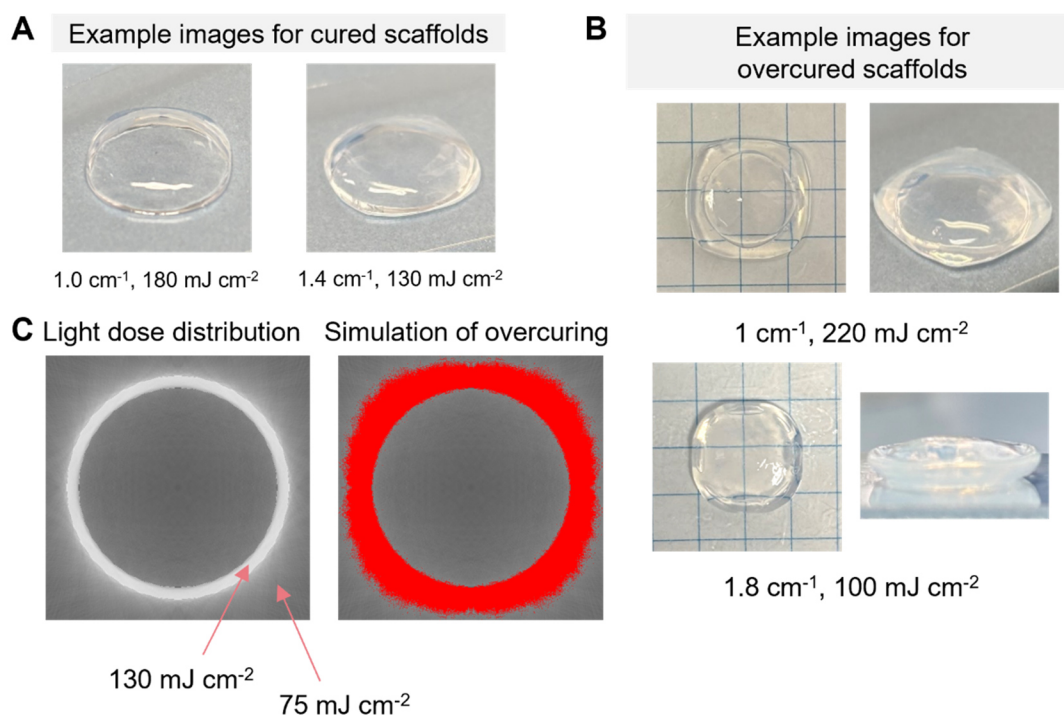


Figure S4. Printing of CL geometries with different absorption compensation. **A.** Example images for cured scaffolds using the optimal light dose for different absorption compensation settings. **B.** Example images for overcured scaffolds. With an absorption compensation of 1.0 cm^{-1} , the overcured scaffold shows a square outer geometry and hollow core. Overcured scaffolds printed with an absorption compensation of 1.8 cm^{-1} show a rounder outer geometry but contain additional crosslinked material in the core. **C.** Light dose distribution using an absorption compensation of 1.4 cm^{-1} exported from the Apparite software and threshold setting in ImageJ to simulate overcuring.



Figure S5. Processing of OCT images in python. The original Tiff-Image is first cropped and bright lines erased. The image is then transferred into a binary mask from which a skeleton is derived.