Optimized Photoclick (Bio)Resins for Fast Volumetric Bioprinting

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Supplementary Methods

Dose Test and VP optimization: Photoresin formulations prepared as indicated above were poured (~ 500-700 µL) into quartz cuvettes (CV10Q1400FS, Thorlabs) and left to physically gel at 4°C for 10-15 minutes. Cuvettes were then placed in a cuvette holder and then transferred onto a commercially available volumetric printer (Tomolite, Readily3D SA).\[^{1}\]

The Dose Test built-in function was used to project a matrix of dots (0.5 mm diameter, 0.5 mm spacing) within a variable broad dose range (see Figure S3, Supporting Information).

After a first broad-range screening, a second, more refined Dose Test was performed within a smaller dose range to better estimate the critical gelation threshold (CGT). For VP optimization, photoresins were filtered through a 0.45 µm filter to remove potentially scattering particles and 3-4 mL were transferred into the glass vial container. The photoresin was left to physically gel at 4 °C for 10-15 minutes. Printing was performed with light dose around the CGT estimated by Dose Test. The crosslinking of the desired object was monitored with a built-in camera and printing process was stopped when the generated structure became visible due to change in refractive index (Video S1, Supporting Information). Printed objects were washed in PBS prewarmed to 37 °C to remove uncrosslinked photoresin. 4-5 additional prints were performed to fine tune the optimal light dose to be delivered to the resin container in order to get the desired structure. Calculation of vCGT was done as previously reported,\[^{2}\] using the following equation:

\[
vCGT = I_{405}\alpha(\lambda_{405})e^{-\alpha(\lambda_{405})z}t_e
\]
where $I_{405}$ corresponds to the light intensity delivered by the printer at 405 nm, $\alpha(\lambda_{405})$ is the absorption coefficient of the photoinitiator LAP at the same wavelength, $z$ is the depth at the center of the photoresin volume and $t_e$ is the exposure time. LAP absorption coefficient is calculated using the equation:

$$\alpha(\lambda_{405}) = 2.3\epsilon(\lambda_{405})c$$

where $\epsilon(\lambda_{405})$ is the molar extinction coefficient of LAP at the VP excitation wavelength (30 M$^{-1}$ cm$^{-1}$ at 405 nm)$^{[3]}$ and $c$ is its molar concentration in the photoresin solution.

*Compression test.* Unconfined uniaxial compression tests were performed on a TA.XTplus Texture Analyzer (Stable Micro Systems) equipped with a 500 g load cell. Cylinder models of 2 mm height and 4 mm in diameter were generated by volumetric printing following the printing procedure described above. Fully-crosslinked control samples were prepared by filling PDMS ring molds (2 mm height and 4 mm inner diameter) with photoresin. The crosslinking was left to proceed in the photorheometer chamber for 20 minutes under the same light and temperature conditions used for photorheology. The samples were then placed between the compression plates and 0.1 g pre-load was applied to ensure full contact with the plates. Samples were left to relax for 2 minutes and then compress to 15% strain at a speed of 0.01 mm s$^{-1}$. Elastic compressive modulus was extrapolated by linear fitting of the initial linear region (0.5-5%) of the stress-strain curve.

*Degree of conversion.* Gel-NB (DS~50%) was analysed by $^1$H-NMR (Bruker Ultrashield 400MHz, 1024 scans) in D$_2$O. Following the procedure indicated above, cylindrical objects (25 mm height, 4 mm diameter) were printed by volumetric printing at their optimized critical gelation threshold (CGT) with 5% Gel-NB/PEG4SH and 2.5% Gel-NB/PEG4SH photoresins containing 0.05% w/v LAP. After extensive washing with distilled H2O prewarmed to 37°C, the cylindrical gels were freeze and lyophilized. Dried samples were then cut into small
pieces, left to swell in D₂O, centrifuged to remove air bubbles and placed into 4 mm magic angle spinning (MAS) rotor. ¹H-MAS-NMR spectra were then taken on a Bruker Ascend DNP (400 MHz) with a spinning rate of 3.2 kHz. Norbornene alkene peaks (~6.21-6.00 ppm) were normalized using phenylalanine protons (7.5-7.15 ppm) as internal standard.

**Table S1.** Table summarizing the results obtained for Gel-NB synthesis with different methods, Gel:CA ratio and scale.

| Method | Gel:CA ratio | Synthesis scale [g] | DS [mmol/g] | DS [%] | Grafting yield [%] |
|--------|--------------|---------------------|-------------|--------|--------------------|
| M1     | 100:1        | 2                   | 0.028 ± 0.002 | 8.7 ± 0.2 | 46.6 ± 3.5 |
|        | 50:1         | 2                   | 0.068 ± 0.004 | 20.9 ± 0.4 | 55.9 ± 3.7 |
|        | 10:1         | 2                   | 0.122 ± 0.006 | 37.6 ± 0.6 | 20.1 ± 1.0 |
| M2     | 100:1        | 2                   | 0.030 ± 0.003 | 9.2 ± 0.3 | 49.4 ± 5.3 |
|        | 50:1         | 2                   | 0.064 ± 0.003 | 19.6 ± 0.3 | 52.2 ± 2.6 |
|        | 10:1         | 2                   | 0.128 ± 0.010 | 39.5 ± 1.0 | 21.1 ± 1.6 |
| M3     | 50:1         | 10                  | 0.009 ± 0.001 | 2.9 ± 0.1 | 77.6 ± 10.0 |
|        | 100:1        | 10                  | 0.035 ± 0.004 | 10.8 ± 0.4 | 57.8 ± 7.2 |
|        | 50           | 10                  | 0.037 ± 0.005 | 11.4 ± 0.5 | 60.7 ± 8.7 |
|        | 100:1        | 2                   | 0.033 ± 0.002 | 10.3 ± 0.2 | 55.0 ± 3.5 |
|        | 50           | 2                   | 0.062 ± 0.009 | 19.0 ± 0.9 | 50.8 ± 7.5 |
|        | 100:1        | 10                  | 0.069 ± 0.002 | 21.1 ± 0.2 | 56.4 ± 2.0 |
|        | 50           | 50                  | 0.057 ± 0.002 | 17.4 ± 0.2 | 46.6 ± 1.7 |
|        | 10:1         | 2                   | 0.113 ± 0.006 | 34.6 ± 0.6 | 18.5 ± 1.0 |
|        | 50           | 10                  | 0.154 ± 0.008 | 47.3 ± 0.8 | 25.3 ± 1.3 |
|        | 10:1         | 50                  | 0.163 ± 0.003 | 50.1 ± 0.3 | 26.7 ± 0.5 |

**Table S2.** Volumetric printing parameters for 5% Gel-NB/PEG4SH (0.05% w/v LAP) and 2.5% Gel-NB/PEG4SH (0.05% w/v LAP).

| Printing Parameters | 5% Gel-NB/PEG4SH (0.05% w/v LAP) | 2.5% Gel-NB/PEG4SH (0.05% w/v LAP) |
|---------------------|----------------------------------|-----------------------------------|
| Light Dose          | 80 mJ cm⁻²⁻¹                     | 90 mJ cm⁻²⁻¹                       |
| Volumetric Absorbed Energy | 8.59 mJ cm⁻³⁻¹  | 9.69 mJ cm⁻³⁻¹  |
| Average Intensity   | 7.89 mW cm⁻²⁻¹                   | 7.89 mW cm⁻²⁻¹                     |
| Vial Turns | 1 | 1 |
|-----------|---|---|
| Rotation Speed | 35.5° s⁻¹ | 31.6° s⁻¹ |
| Projection Rate | 123 Hz | 110 Hz |
| Angle Step | 0.288 ° | 0.288 ° |
| Print Time | 10.1 s | 11.4 s |

Figure S1. Gel-NB vs Gel-MA. A) Photorheology comparison (average trace, n=3) of Gel-NB/PEG4SH and Gel-MA photoresins. Photoresin composed of 5% Gel-NB (DS~50%) and PEG4SH at 1:1 SH:NB molar ratio (total polymer content ~7.5%, 0.05% w/v LAP) shows a much faster photocrosslinking kinetic compared to Gel-MA (DS~55%) at 5% and 7.5% (0.05% w/v LAP), therefore generating fewer potentially harmful radicals to reach storage modulus plateau value. B) Dose test comparison showed a significantly lower critical gelation threshold (CGT) for Gel-NB/PEG4SH resin (aCGT: 80 mJ cm⁻², vCGT: 9.16 mJ cm⁻³) compared to Gel-MA (aCGT: 288-320 mJ cm⁻², vCGT: 33-36.7 mJ cm⁻³), confirming better performance of the photo-click thiol-norbornene-based resin.

Figure S2. NMR spectrum of Gel-NB. Norbornene alkene protons peak can be observed at ~6.21-6.00 ppm (red highlight), while it is absent in unmodified gelatin (Gel). Its integral is used to determine the DS in comparison with methyl protons of 3-(Trimethylsilyl)-1-propanesulfonic acid (DSS) internal standard (~0.5 to -0.5 ppm).
Figure S3. Dose Test. A) Illustration of Dose Test principle of operation (left). A 405 nm laser beam (light purple) is directed towards a digital-micromirror-device (DMD) which generates a grid of dots with varying light dose reported as purple shades (light purple = low light dose, dark purple = high light dose). The matrix of dots is projected towards a quartz cuvette containing the photoresin in static, non-rotating mode. When the light dose exceeds the critical gelation threshold (CGT), the crosslinked material becomes visible (right). After a first test performed in a broad light dose range (1 - 4096 mJ cm\(^{-2}\)), a second Dose Test is performed in a narrower range around the estimated CGT which is identified by the visible dot crosslinked with the minimal light dose. This refinement screening helps to better estimate the CGT for the following use of the photoresin in volumetric printing. B) Dose Test parameters used in this work reported as light dose per unit area (top row) and volumetric absorbed energy (bottom row) for Dose Test 1 and Dose Test 2.
Figure S4. Dose Test results for different photoresin formulations. Images of the cuvettes (top rows) are displayed as colored dots (bottom rows) with shades of blue from dark to pale blue referring to clearly visible to less visible crosslinked dot formation, respectively. Red circles highlight the dots obtained with the minimum light dose which identifies the critical gelation threshold (CGT). CGT values for each Dose Test are reported in areal unit (aCGT) referring to the light dose delivered from the printer and in volumetric unit (vCGT) referring to the volumetric energy absorbed by the photoresin. A) Results for photoresin composed of 5% Gel-NB with varying degree or substitution (DS), PEG4SH in 1:1 SH:NB molar ratio and 0.05% w/v LAP. B) Results for photoresin composed of Gel-NB (DS~50%) at varying concentrations, PEG4SH in 1:1 SH:NB molar ratio and 0.05% w/v LAP. For 10% Gel-NB formulation we observed phase separation when cooled down to 4°C. C) Results for photoresin composed of 5% Gel-NB (DS~50%) and PEG4SH at varying SH:NB ratio, and 0.05% w/v LAP. For 5:1 thiol excess we observed phase separation when cooled down to 4°C. D) Results for photoresin composed of 5% Gel-NB (DS~50%) and varying thiolated crosslinker in 1:1 SH:NB ratio, and 0.05% w/v LAP.
**Figure S5.** Light penetration. Dose Test performed on 10 mm cuvettes showed light penetration and subsequent gelation throughout the entire path length (~89% of VP build volume diameter) for both 2.5% and 5% Gel-NB/PEG4SH photoresin formulations with 0.05% w/v LAP.

**Figure S6.** Writing resolution, defined as the minimum distance at which two proximal features can be bioprinted without overlap, is estimated with the printing of an hollow cone structure. Image of the printed object perfused with high MW blue-dextran (Scale bar: 2 mm) and fluorescence imaging close ups upon TRITC-dextran perfusion (Scale bar: 200 µm).

**Figure S7.** Compression test on fully crosslinked (FC) and volumetric printed (VP) cylinders with 5% and 2.5% Gel-NB/PEG4SH photoresins with 0.05% w/v LAP. The resulting elastic modulus showed that the hydrogels obtained with volumetric printing at the critical gelation threshold have a lower stiffness (~35-40%) compared to their fully crosslinked counterparts.
Figure S8. Norbornene conversion. Comparison of norbornene alkene protons peak at ~6.21-6.00 ppm (red highlight) between Gel-NB (DS~50%) and hydrogels generated by volumetric printing with 5% Gel-NB(DS~50%)/PEG4SH and 2.5% Gel-NB(DS~50%)/PEG4SH photoresins. Phenylalanine protons (7.5-7.15 ppm, green highlight) were used as internal standard. The degree of conversion for the two photoresins was estimated to be around 8-14.5%.

Figure S9. 3D .stl models (top row) and respective volumetric printed object imaged in the glass vial photoresin container. From top left to bottom right: Pawn, Rook, Knight, Bishop, King, Queen, hollow ok-hand, glass, ETH logo, Klein bottle, Branch model. (Scale bar: 2 mm)
Figure S10. A) Schematic of cell experiments procedure and summary of resulting viability. B) C2C12 Live/Dead viability assay with representative images C) NHDF Live/Dead viability assay with representative images (Scale bar: 200 µm).

Supplementary References

[1] Readily3D. https://readily3d.com/.
[2] C. C. Cook, E. J. Fong, J. J. Schwartz, D. H. Porcincula, A. C. Kaczmarek, J. S. Oakdale, B. D. Moran, K. M. Champley, C. M. Rackson, A. Muralidharan, R. R. McLeod, M. Shusteff, Adv. Mater. 2020, 32, 2003376.
[3] M. Lee, R. Rizzo, F. Surman, M. Zenobi-Wong, Chem. Rev. 2020, 120, 10950-11027.