Photocrosslinking of hyaluronic acid-based hydrogels through biotissue barriers

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SECTION 1

Synthesis of chlorin p_6 trimethyl ester ***(2)***

An aliquot of 600 mg (0.061 mmol) of purpurin 18 methyl ester (**1)** (see **Fig. S1**) was dissolved in 30 ml of tetrahydrofuran, then aliquot of 20 ml of 0.9% aqueous solution of sodium hydroxide was added. The resulting reaction mixture was vigorously stirred for 30 min. Then, 400 µl of 36% aqueous solution of hydrochloric acid was added into the solution, and the resulting mixture was stirred for 5 min. After that, an excess amount of diazomethane in the form of solution in diethyl ether was added into the solution in portions. The formed aqueous layer was separated and the remaining solution was dried by evaporation. The residue was dissolved in 100 ml of chloroform and purified twice with water, then it was dried over anhydrous sodium sulfate and evaporated to dryness. The product was purified by flash chromatography using chloroform: methanol = 80: 1 system, then it was recrystallized in tetrahydrofuran: heptane = 1: 4 system. The reaction yield was 637 mg (96 %).

UV-Vis (CH_2Cl_2) λ_{max} (ε, $l \cdot mol^{-1} \cdot cm^{-1}$), nm: 668 (40900), 614 (4850), 532 (5490), 498 (9920), 402 (137000). ^{1}H-NMR spectra were recorded using BrukerDPX-300 spectrophotometer at 300 MHz frequency. ^{1}H-NMR spectrum (300 MHz, $CDCl_3$), δ: 9.67 (s, 1H, 10-H), 9.37 (s, 1H, 5-H), 8.60 (s, 1H, 20-H), 7.89 (m, 1H, 31-H), 6.16 and 6.02 (dd, 2H, 32-H), 5.14 (m, 1H, 18-H), 4.48 (m, 1H, 17-H), 4.16 and 4.10 (d, 6H, 131-CO_2CH_3 and 151-CO_2CH_3), 3.62 (q, 2H, 81-CH_2CH_3), 3.56 (s, 3H, 173-OCH_3), 3.49 (s, 3H, 121-H), 3.30 (s, 3H, 21-H), 3.12 (s, 3H, 71-H), 2.19 (m, 4H, 171-172-H), 1.80 (d, 3H, 181-H), 1.64 (t, 3H, 82-H), -1.38 (s, 2H, NH).

Synthesis of 13^1,15^1-dimethyl ether of chlorin p_6 ***(3)***

An aliquot of 600 mg (0.960 mmol) of chlorin p_6 trimethyl ester (**2**) was dissolved in 10 ml of acetone then 15 ml of 20% aqueous solution of hydrochloric acid were added under stirring and the mixture was vigorously stirred for 1 hour in the atmosphere of argon. The resulting mixture was diluted with 100 ml of chloroform and purified twice with water, then

was purified with 100 ml of a 0.5% aqueous solution of hydrochloric acid and water, dried over sodium sulfate and by evaporation. Product **3** was purified by column chromatography using the chloroform: methanol = 40: 1 system. Reaction yield was 557 mg (95 %).

^{1}H-NMR spectrum (300 MHz, $CDCl_3$), δ: 9.62 (s, 1H, 10-H), 9.39 (s,1H, 5-H), 8.60 (s, 1H, 20-H), 7.90 (m, 1H, 3-CH=CH_2), 6.15 and 6.05 (dd, 2H, 3-CH=CH_2), 5.13 (m, 1H, 18-H), 4.42 (m, 1H, 17-H), 4.17 and 4.11 (d, 6H, 13-CO_2CH_3 and 15-CO_2CH_3), 3.58 (q, 2H, 8-CH_2-CH_3), 3.49 (s, 3H, 12-CH_3), 3.33 (s, 3H, 2-CH_3),3.15 (s, 3H, 7-CH_3), 2.18 (m, 4H, 171-172-CH_2), 1.81 (d, 3H, 18-CH_3), 1.63 (t, 3H, 8-CH_2-CH_3), -1.40 (bs, 2H, NH).

Synthesis of 17^3-succinimide ester- 13^1,15^1-dimethyl ester of chlorin p$_6$ ***(4)***

An aliquot of 200 mg (0.327 mmol) 13^1,15^1- dimethyl ester of chlorin p$_6$ **3** was dissolved in 8 ml of N,N-dimethylformamide, then 42 mg (0.365 mmol) N-hydroxysuccinimide and 126 mg (0.656 mmol) of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride were added under stirring. The resulting reaction mixture was vigorously stirred for 30 minutes in argon atmosphere. The solution was diluted with 100 ml of chloroform, purified with 100 ml of a 0.5% aqueous solution of hydrochloric acid, then purified twice with water, dried over sodium sulfate and by evaporation. The product was used without additional purification.

Synthesis of 17^3-N-(methoxy-PEG$_{2000}$)-13^1,15^1- chlorin p$_6$ dimethyl ester ***(5)***

Finally, 17^3-N-(methoxy-PEG$_{2000}$)-13^1,15^1-chlorin p$_6$ dimethyl ester (Chl p6-PEG) **(5)** was synthesizedas followsaliquot of 220 mg (0,311 mmol) of 17^3-N-hydroxysuccinimide ester of 13^1,15^1-dimethyl ester of chlorin p$_6$ **4**was dissolved in 10 ml of chloroform, 289 mg (0.141 mmol) of monoamino PEG$_{2000}$ monomethyl ester and 50 mg (0.373 mmol) of diisopropylethylamine were added under stirring. The resulting reaction mixture was vigorously stirred in argon atmosphere for 20 minutes, then it was diluted with 100 ml of chloroform and purified twice with water, dried over sodium sulfate and by evaporation. The

product was purified by column chromatography using chloroform: methanol = 10: 1 system. Reaction yield was 245 mg (79 %).

^{1}H-NMR spectrum (500 MHz, $CDCl_3$), δ: 9.69 (s, 1H, 10-H), 9.47 (s,1H, 5-H), 8.67 (s, 1H, 20-H), 7.98 (m, 1H, 3-CH=CH_2), 6.33 and 6.15 (dd, 2H, 3-CH=CH_2), 5.93 (m, 1H, PEG-NH-CO), 5.15 (m, 1H, 18-H), 4.45 (m, 1H, 17-H), 4.22 and 4.16 (2×s, 6H, 13-CO_2CH_3 and 15-CO_2CH_3), 3.69-3.20 (m, 217H, 8-CH_2-CH_3 and PEG-CH_2, 12-CH_3, 2-CH_3, 7-CH_3), 2.24 (m, 4H, 17^1-17^2-CH_2), 1.81 (d, 3H, 18-CH_3), 1.69 (t, 3H, 8-CH_2-CH_3), -0.9 (m, 2H, NH) (**Fig. S2**).

Chromatography

HPTLC-Kieselgel 60 F254 plates (Merk, Germany) were used for thin-layer chromatography. Column chromatography and flash chromatography were performed on Silicagel 60 (40-63) (Merk, Germany).

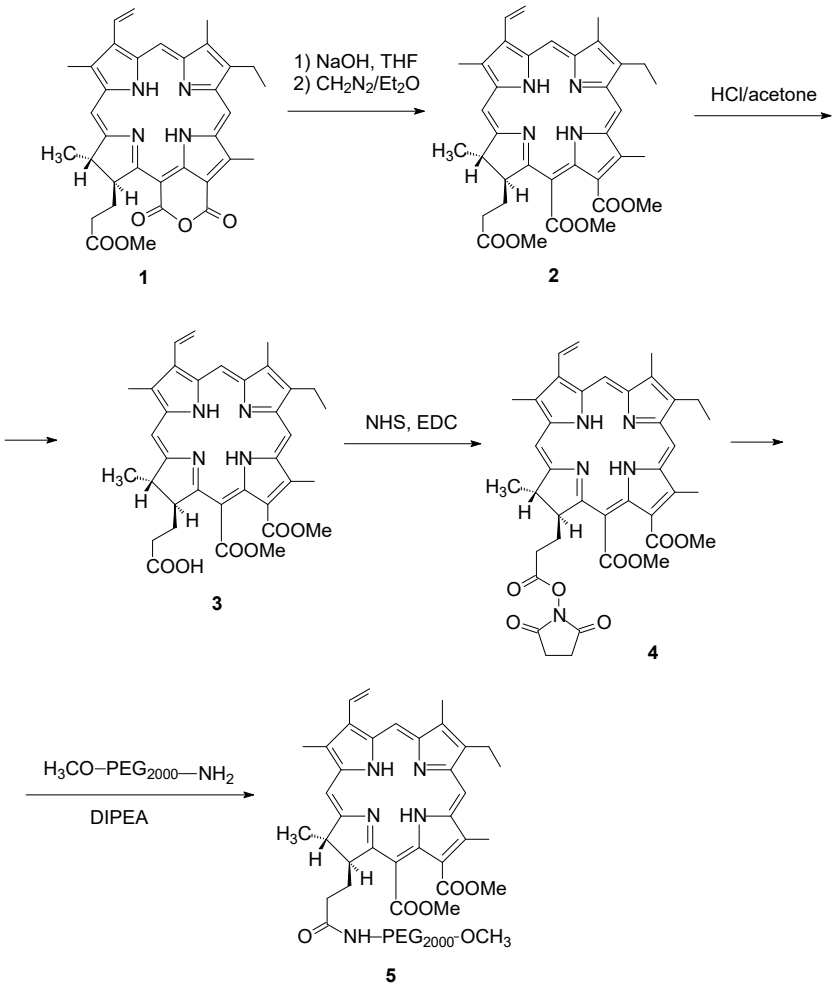

Figure S1. Synthesis of 17^3-N-(methoxy-PEG_{2000})-13^1,15^1- chlorin p_6 dimethyl ester

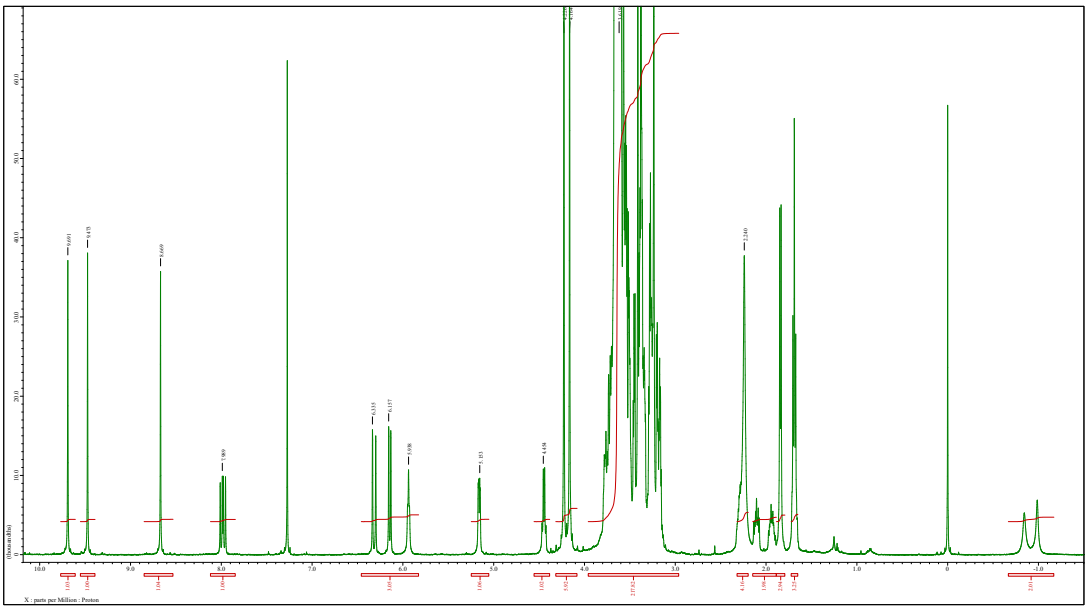

Figure S2. ^{1}H-NMR spectrum of 17^3-N-(methoxy-PEG_{2000})-13^1,15^1- chlorin p_6 dimethyl ester.

SECTION 2

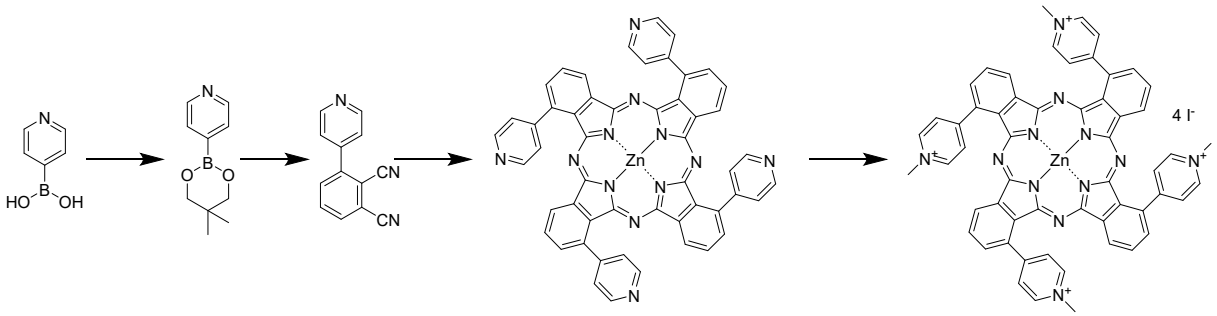

Figure S3. Synthesis of {4,4',4",4'"-(29H,31H-phthalocyanine-1,8,15,22-tetrayl-134 κ4N29,N30,N31,N32)tetrakis[1-methylpyridiniumato(2-)]}zinc(4+) tetraiodide (pyridinium phthalocyanine).

SECTION 3

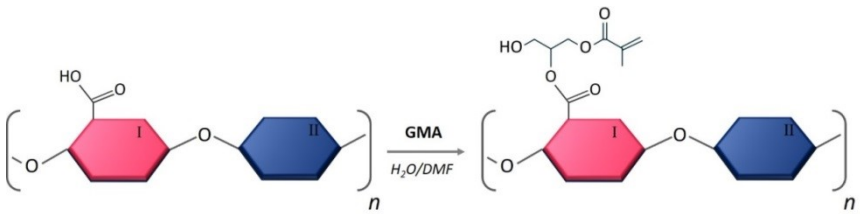

Figure S4. Modification of hyaluronic acid with glycidyl methacrylate: I – D-glucuronic acid, II – N-acetyl-D-glucosamine.

SECTION 4

Free radical detection was performed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH is a stable radical with strong absorption peak at 525 nm, which can decrease (the violet DPPH solution turns colorless) after reaction of DPPH with radicals or antioxidants. For tests solution of DPPH (380 μM) in ethanol was prepared. Then the solution was mixed with water solutions of examined samples in equal volumes thus decreasing DPPH concentration to 190 μM. Spectra were measured on a Cary 50 spectrophotometer.

The initiation efficiency of macrocycles is usually insufficient to cleave vinyl bonds, despite a high molar absorption coefficient. Typically, systems in which macrocycles are used

to initiate radical polymerization under red light irradiation are highly complex systems, such as a reversible addition-fragmentation chain transfer radical polymerization [1], using a special solvent [2] or a type II photoinitiator incorporated into the PS macrocycle [3], as well as a system requiring pre-irradiation [4] or prolonged irradiation [5]. In the current research, we proposed a type II photoinitiator complex consisting of a PS and a coinitiator for HA-based hydrogels photocrosslinking. The interaction between the excited PS and the coinitiator can occur through different pathways, including hydrogen abstraction or electron transfer, as well as with the involvement of both processes [6]. Amines and thiols are known to participate in photoinduced electron transfer followed by hydrogen atom abstraction. This leads to the formation of free N or S-centered radicals, responsible for double bond activation, allowing radical polymerization

In this study, we used triethanolamine (TEOHA), mercaptoethanol (ME), and dithiothreitol (DTT) as coinitiators. In order to evaluate the role of the studied compounds in photocrosslinking under red light, DPPH was used. We found that TEOHA had a negligible effect on the peak intensity of DPPH (**Fig. S5**), in contrast to ME, which caused complete color degradation (**Fig. S6**). The hydrogen abstraction in thiols, leading to reaction with DPPH, was suggested to be more efficient than that in amines. These data also correlate with literature data indicating that thiols are efficient coinitiators. [7,8] The study of radical generation in the system with PIs was performed with concentration of coinitiator ME equimolar to DPPH (190 μM), in order to prevent complete discoloration of DPPH **(Fig. S7).** The system containing Pht with ME had almost no impact on the intensity and position of the DPPH peak, even after prolonged 660 nm irradiation (**Fig. S7A-D**). Other photoinitiating systems, Chl-p6PEG + ME, and especially Chl e6-MGlu + ME, had more significant effect on the DPPH peak, but weaker than ME alone (**Fig. S7B, C**).

It is worth noting that this effect was enhanced as the exposure time increased. It is known that along with the electron transfer from the excited state of PS* to ME, leading to the

formation of ion-radical pairs, a hydrogen atom abstraction usually occurs [9]. This process competes with hydrogen atom abstraction from ME, which is then accepted by DPPH. We hypothesize that the smaller the change in the DPPH peak, the more efficient the system of PS with ME is in terms of initiating the reaction with HAGM (**Fig. S7E**). Therefore, Pht appears to be more efficient than both of the chlorins. It correlates with previously described data showing that phthalocyanines, especially those with metal ions (e.g. zinc or aluminum), possess a high redox potential, enabling effective electron/hydrogen abstraction from ME [10].

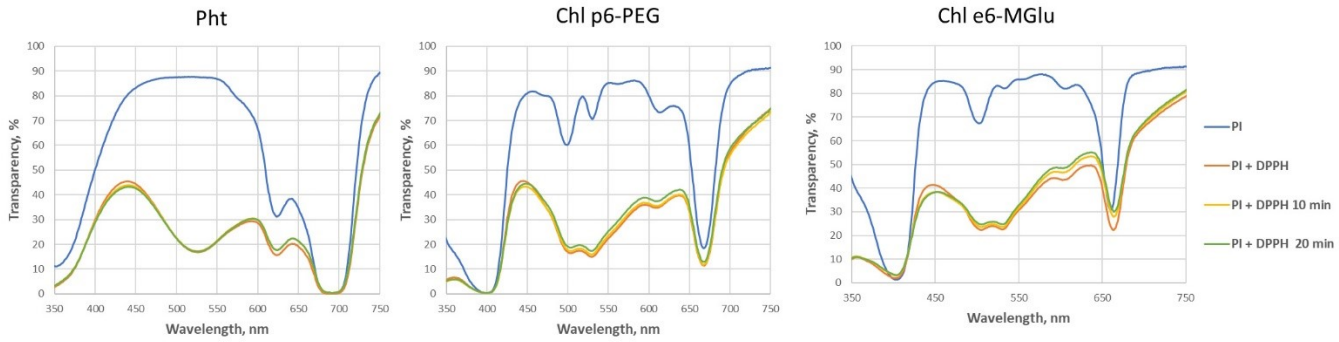

Figure S5. DPPH test with PIs.

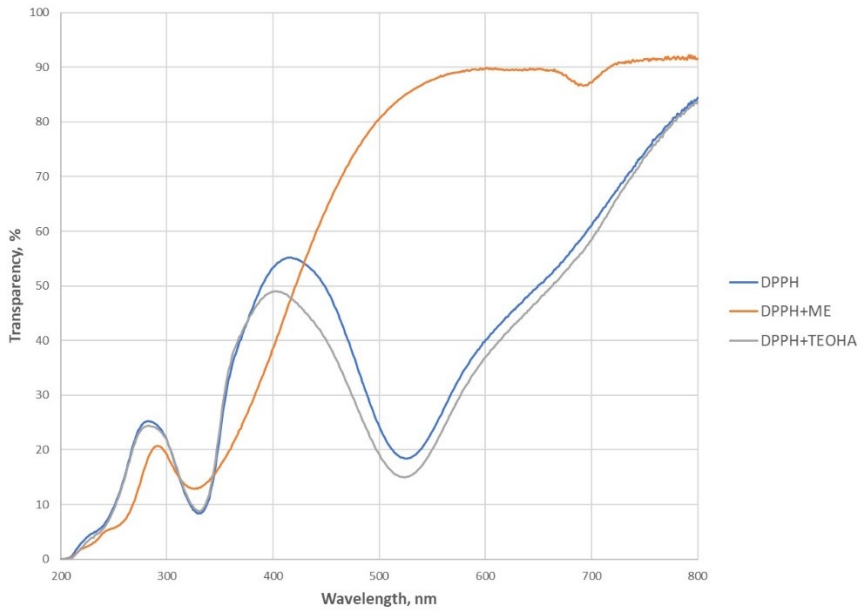

Figure S6. DPPH test with amine coinitiator (TEOHA) and thiol coinitiator (ME).

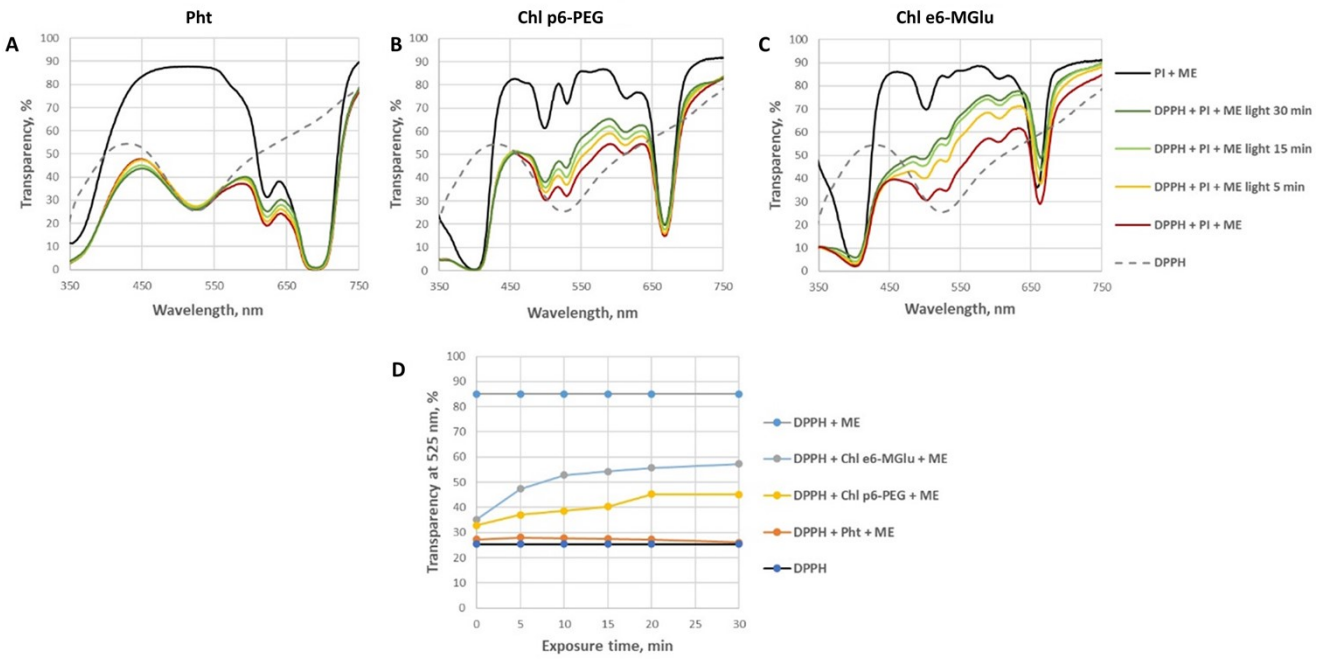

Figure S7. DPPH test of photoinitiating systems: Pht + ME (A), Chl p6-PEG + ME (B), Chl e6-MGlu + ME (C). Comparative graph of all potential PIs interaction with DPPH (D).

SECTION 5

Cell viability in presence of Chl e6-MGlu, Pht, and Chl p6-PEG was studied using murine L-929 fibroblasts, human WI-26 fibroblasts and human breast adenocarcinoma MCF-7 cells (**Fig. S8**).

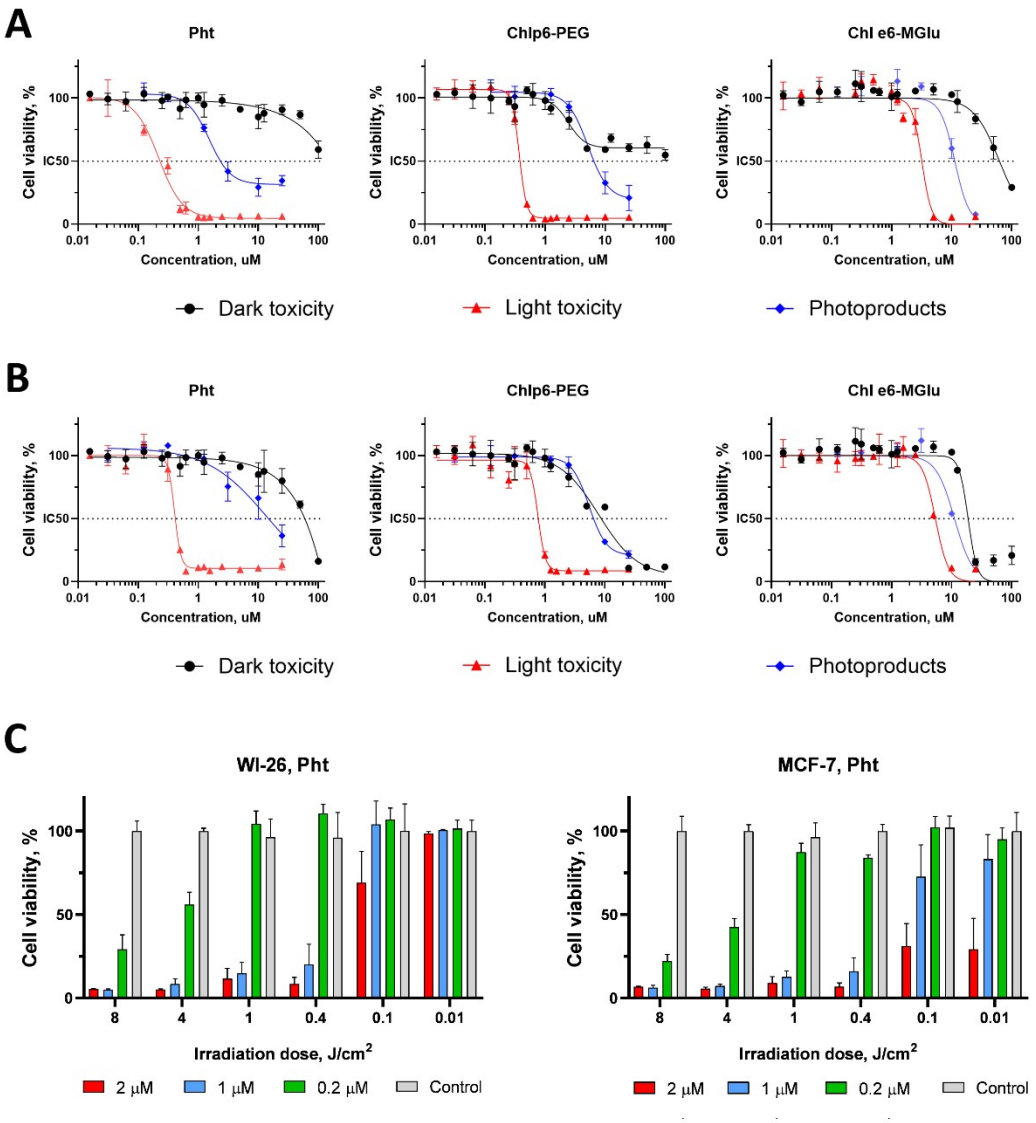

Figure S8. The cell viability of human breast adenocarcinoma MCF-7 (A) and human immortalized fibroblasts (B) in the dark and light (660 nm, 3.5 J/cm^2) conditions, 72 h, MTT assay data. The data are presented as mean ± SD. The cell viability of human WI-26 fibroblasts and human MCF-7 breast adenocarcinoma cells for Pht under the different light doses at 660 nm irradiation (C), 72 h, MTT assay data.

SECTION 6

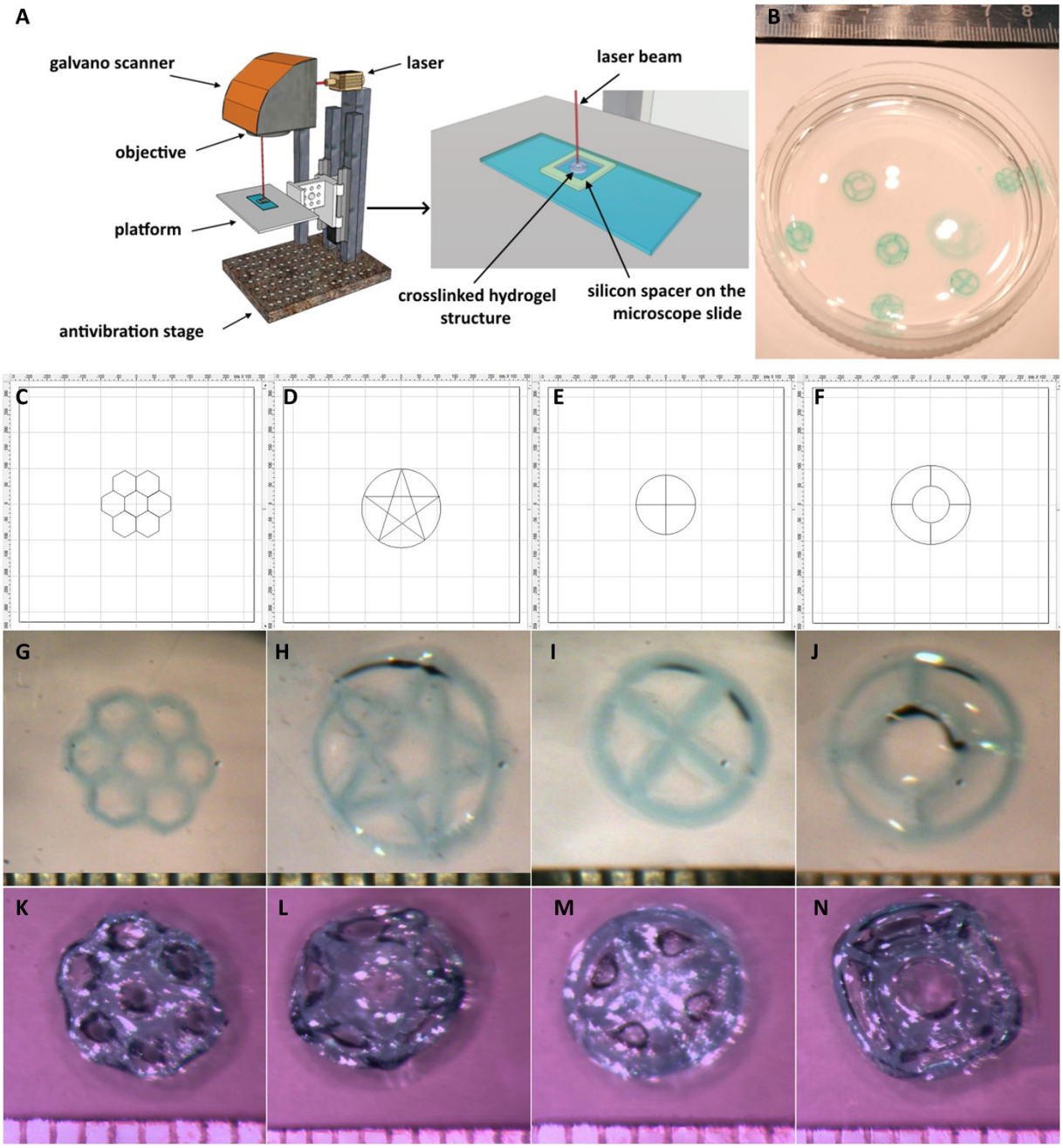

Figure S9. Fabrication process (A). Developing of samples in water (B). Computer model (C-F), zoomed images of hydrogel structures in distilled water (F-I) and partially dried samples on the microscope slide (K-N) with 1 mm graduation scale marks.

SECTION 7

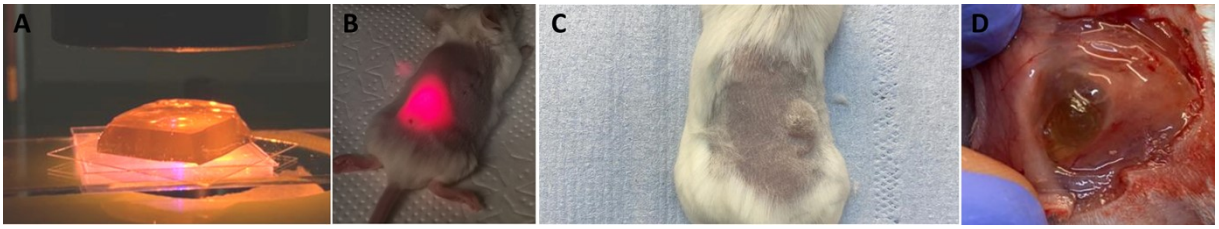

Figure S10. Photocrosslinking of HAGM hydrogel under phantom under irradiation at 660 nm wavelength (A). Gelation process *in vivo* (B). Place of injection after exposure (C).Photo of crosslinked hydrogel (D).

Typically, systems in which PS is utilized to initiate free radical polymerization under red light irradiation are highly complex systems, such as a reversible addition-fragmentation chain transfer free radical polymerization [11], systems using a special solvent [12] or photoinitiator II incorporated in PS macrocycle [13], as well as a system required pre-irradiation [14], prolonged irradiation [11] or multiple components [15]. We demonstrate the Pht and chlorins application as a type II photoinitiator (PI), consisting only of a PS and a co-initiator, in HAGM photocrosslinking.

We have performed a comparative study to better highlight the potential of red light for photocrosslinking of hydrogels in comparison to UV or blue light. Lithium phenyl phosphinate (LAP) was chosen as a widely used photoinitiator for biological applications due to its increased water solubility and high polymerization rates with 365 nm light. This traditional photoinitiator was compared with our photoinitiation system. Since LAP is a type I photoinitiator (LAP molecule splits into 2 radicals under light irradiation), HAGM-based PCC was prepared with 3.2 mM LAP concentration (1/2 of ME concentration). The samples were placed in 1 mm silicon spacers with 5 mm holes in the center for subsequent photocrosslinking. Short duration (~3 s) exposure of PCC containing LAP to 365 nm UV 220 mW/cm^2 light results in the formation of a fully crosslinked hydrogel.

In the course of the experiment, the intensities of the 365 nm and 660 nm light sources were adjusted to be equal (220 mW/cm^2), while the exposure time was fixed at 10 min. Irradiation of PCCs with LAP and Pht/ME under 3 mm agarose phantoms showed complete photocrosslinking of the samples. Meanwhile, increasing the phantom thickness up to 10 mm leads to impossibility of photocrosslinking the PCC with LAP. A similar result was obtained using 10 mm chicken breast instead of the phantom. In contrast, PCCs with Pht as photoinitiator and ME as coinitiator were successfully photocrosslinked in all experiments (**Fig S11**).

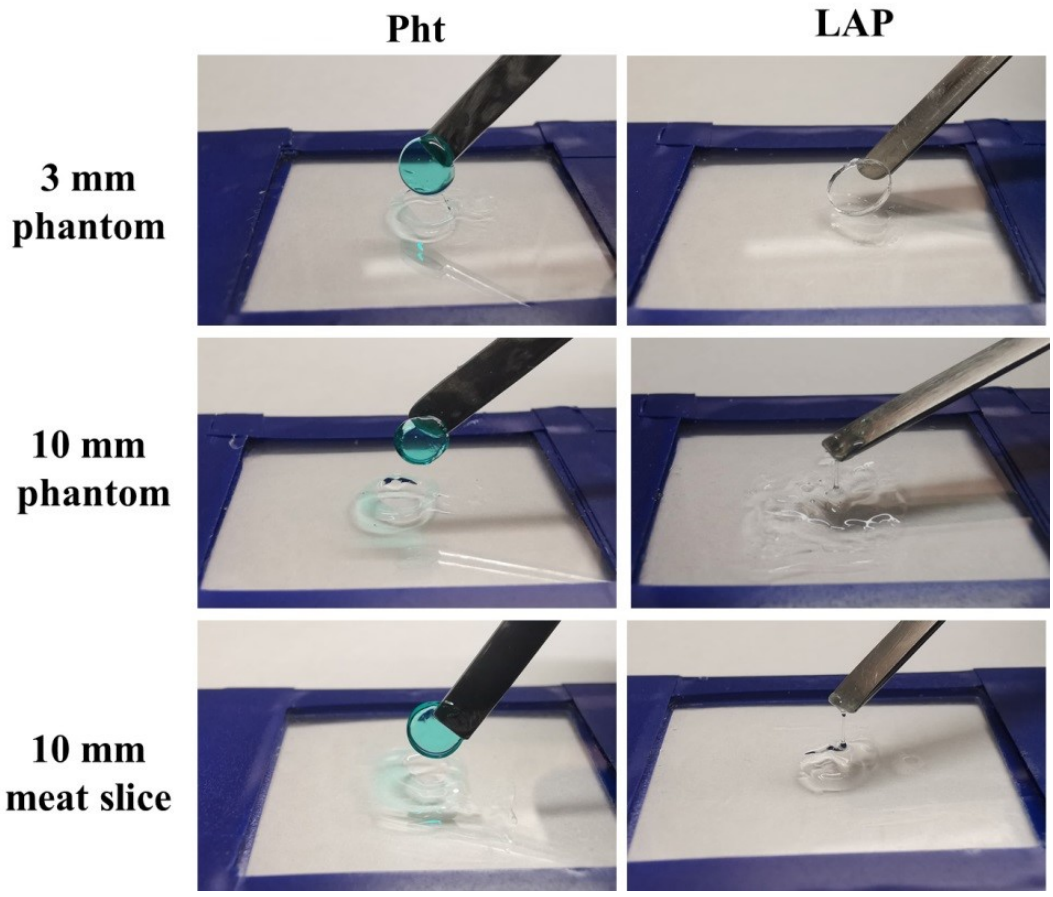

Figure S11. Photocrosslinking of HAGM-based PCCs containing 200 μM Pht with 6.4 mM ME or 3.2 mM LAP under 3 and 10 mm phantoms and 10 mm chicken breast slice.

In order to demonstrate influence of irradiation time on degree of crosslinking, we performed photocrosslinking of PCC under 1.5 mm agarose phantom at 800 mW/cm^2 at different exposure times as follows: 30 seconds, 1 minute, 2.5 minutes, 5 minutes, 10 minutes, 15 minutes (**Fig S12**). At 30 seconds, PCC becomes more viscous, but the entire crosslinked structure is not formed; at 1 minute, the gel forms, but it can be easily deformed and does not hold its shape; at 2.5 minutes, the gel holds its shape better, while being able to stretch; at 5 minutes, the gel loses its ability to stretch, but still has plasticity; at 10 minutes, the hydrogel becomes more rigid. After 15 minutes the hydrogel samples achieve almost maximum rigidity. A further increase in the irradiation time slightly changes the characteristics of the gel.

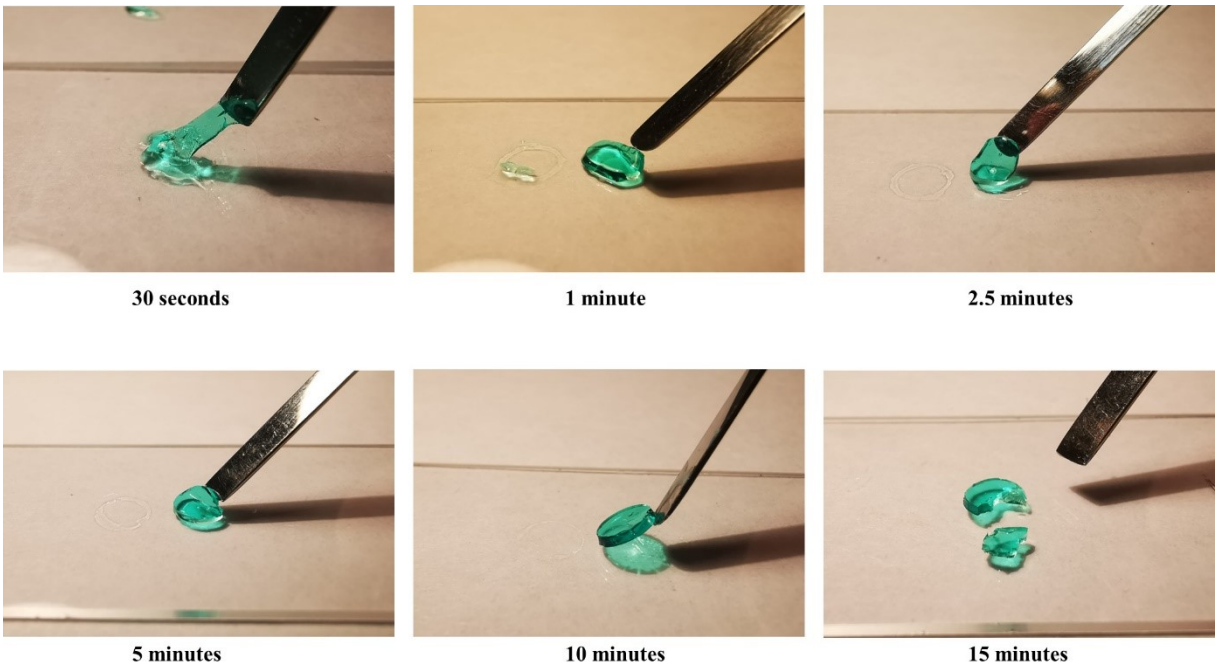

Figure S12. Appearance of hydrogels with Pht and ME depending on exposure time.

Swelling properties of hydrogels at equilibrium point is an informative parameter for assessment of crosslinking degree [16], so they were numerically evaluated here. Decreasing swelling ratio at increasing exposure time clearly demonstrates correlation between irradiation duration and degree of crosslinking (**Table S1**)

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Table S1. Dependence of gel's swelling ratio from exposure time.

Exposure duration, min	1	2.5	5	10	15
Swelling ratio, %	466.2 ± 12.7	420.3 ± 10.5	124.4 ± 5.1	174.6 ± 6.0	76.2 ± 3.1

SECTION 8

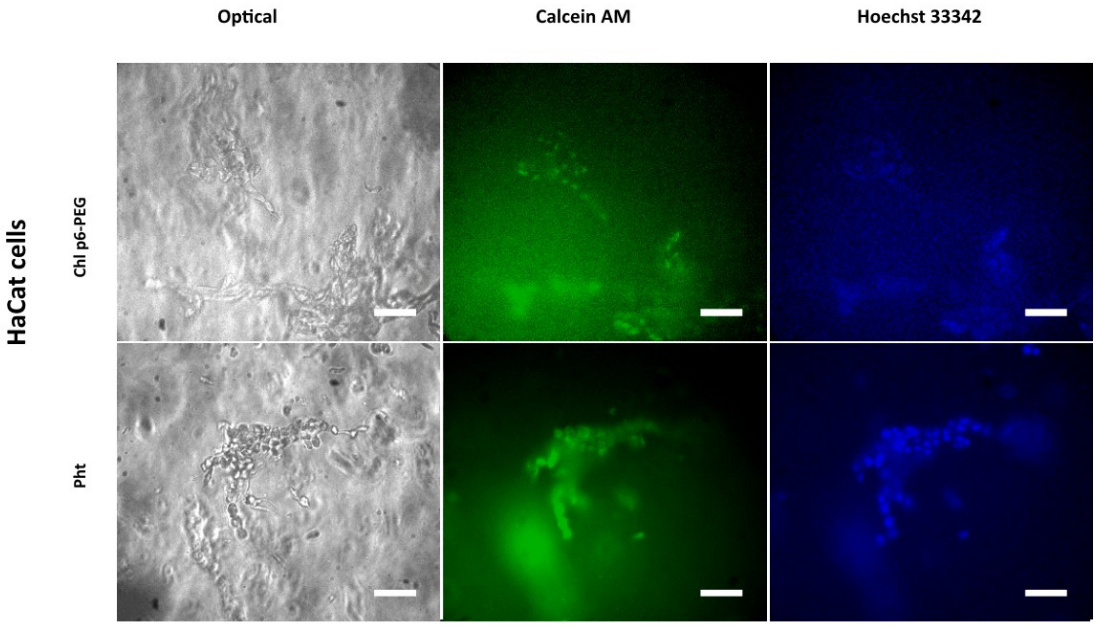

Figure S13 Human keratinocytes HaCaT after polymerization in HA-based hydrogels: optical and fluorescent microscopy. Green is for Calcein AM (alive cells), blue is for cell Hoechst 33342 (cell nuclei), 24 h incubation. Scale bar 50 μm.

SECTION 9

Visualization of scaffolds was demonstrated *in vivo* (**Fig. S12**) followed by *ex vivo* study of luminescent signal in organs (**Fig. S13**).

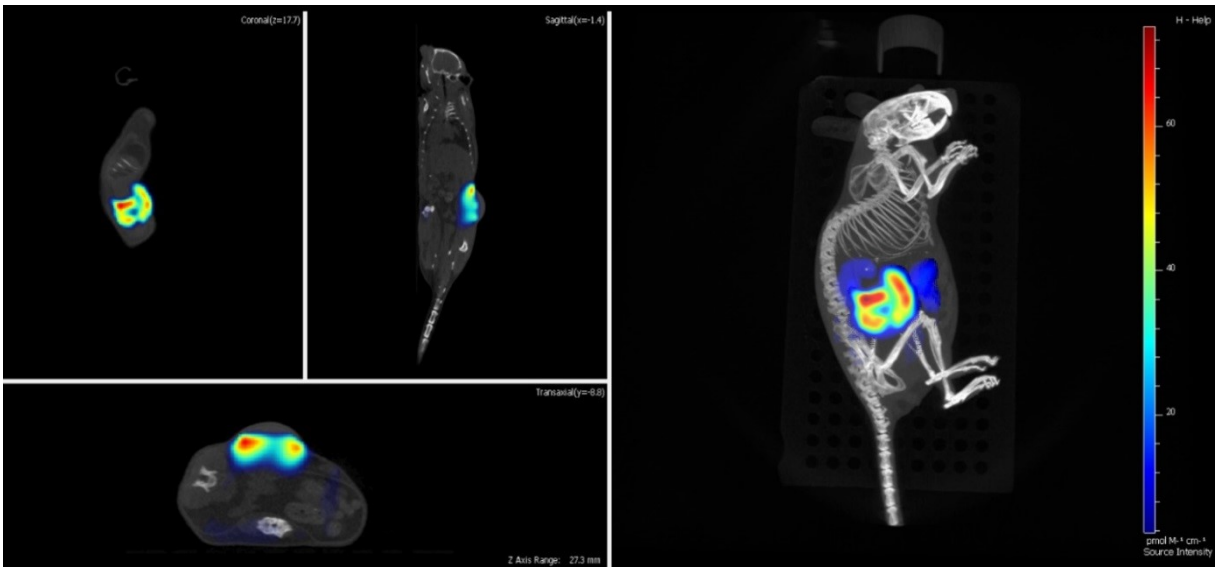

Figure S14. Fluorescent tomography of a mouse with *in situ* photocrosslinked scaffold. On the left side coronal, sagittal and trans-axial views are shown, on the right side – 3D reconstruction.

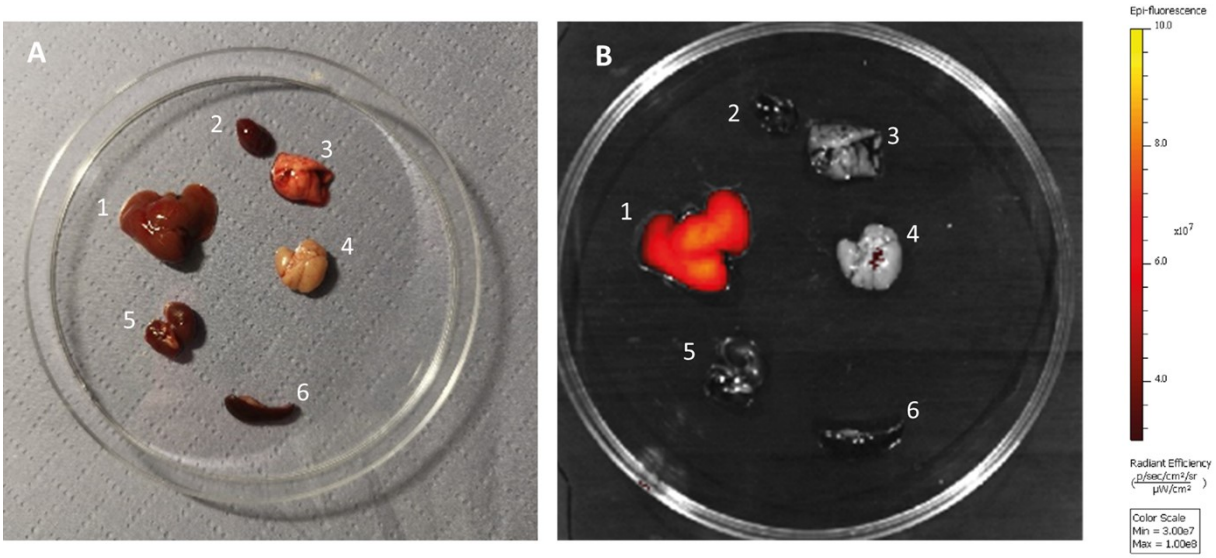

Figure S15. A) Photo images of isolated internal organs and B) their fluorescence. 1 – liver, 2- heart, 3 – lungs, 4 – brain, 5 – kidneys, 6 – pancreas.

References

1 X. Hu, G. Szczepaniak, A. Lewandowska-Andralojc, J. Jeong, B. Li, H. Murata, R. Yin, A. M. Jazani, S. R. Das and K. Matyjaszewski, *J Am Chem Soc*, 2023, **145**, 24315–24327.

2 N. Corrigan, J. Xu and C. Boyer, *Macromolecules*, 2016, **49**, 3274–3285.

3 S. E. Korkut, G. Temel, D. K. Balta, N. Arsu and M. K. Şener, *J Lumin*, 2013, **136**, 389–394.

4 Y. Wang, B. Han, R. Shi, L. Pan, H. Zhang, Y. Shen, C. Li, F. Huang and A. Xie, *J Mater Chem B*, 2013, **1**, 6411.

5 Z. Wu, W. Fang, C. Wu, N. Corrigan, T. Zhang, S. Xu and C. Boyer, *Chem Sci*, 2022, **13**, 11519–11532.

6 S. Dadashi-Silab, S. Doran and Y. Yagci, *Chem Rev*, 2016, **116**, 10212–10275.

7 B. Groitl and U. Jakob, *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 2014, **1844**, 1335–1343.

8 A. N. Generalova, P. A. Demina, R. A. Akasov and E. V. Khaydukov, *Russian Chemical Reviews*, 2023, **92**, RCR5068.

9 W. Tomal and J. Ortyl, *Polymers (Basel)*, 2020, **12**, 1073.

10 L. Breloy, O. Yavuz, I. Yilmaz, Y. Yagci and D.-L. Versace, *Polym Chem*, 2021, **12**, 4291–4316.

11 Z. Wu, W. Fang, C. Wu, N. Corrigan, T. Zhang, S. Xu and C. Boyer, *Chem Sci*, 2022, **13**, 11519–11532.

12 N. Corrigan, J. Xu and C. Boyer, *Macromolecules*, 2016, **49**, 3274–3285.

13 S. E. Korkut, G. Temel, D. K. Balta, N. Arsu and M. K. Şener, *J Lumin*, 2013, **136**, 389–394.

14 Y. Wang, B. Han, R. Shi, L. Pan, H. Zhang, Y. Shen, C. Li, F. Huang and A. Xie, *J Mater Chem B*, 2013, **1**, 6411.

15 H. Mokbel, G. Noirbent, D. Gigmes, F. Dumur and J. Lalevée, *Beilstein Journal of Organic Chemistry*, 2021, **17**, 2067–2076.

16 Z. Li, C. Yu, H. Kumar, X. He, Q. Lu, H. Bai, K. Kim and J. Hu, *Gels*, 2022, **8**, 682.