# *Crosslinkable Gelatins with Superior Mechanical Properties Through Carboxylic Acid Modification: Increasing the Two-Photon Polymerization Potential.*

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**Supporting information**

## Methacrylation of the gel-MOD carboxylic acids: reaction condition study

### GPC data

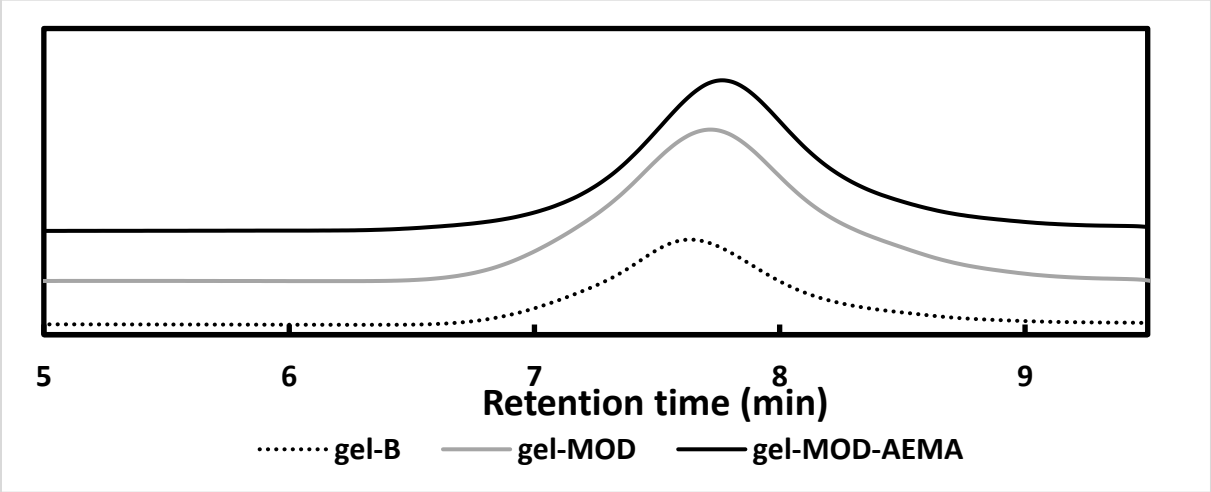



**Figure S1: GPC Chromatograms obtained for gelatin type B, gel-MOD and gel-MOD-AEMA**

The GPC chromatogram indicates that no peak broadening occurs throughout the course of the modification. As a consequence it can be concluded that no crosslinking nor chain extension takes place between residual primary amines and carboxylic acids present in gelatin during the EDC/NHS assisted modification. Furthermore, a fluoraldehyde assay performed on the gelatin derivatives (data not shown) indicates that the first modification was quantitative, as no primary amines could be retrieved both for gel-MOD as well as for gel-MOD-AEMA.

## Determination of the mechanical properties of hydrogels based on functionalized gelatins via rheology

When comparing the reported gelatin derivatives to the state-of-the-art (see **Fig. S2** and **Table S1),** it becomes clear that the gel-MOD-AEMA derivative is a material suitable for a range of applications when considering mechanical properties. For instance, the obtained range of storage moduli (7724-147000 Pa) corresponds to the mechanical properties typically observed for brain (3000 – 12000 Pa), prostate (6600 – 22000 Pa), intervertebral discs (8400 – 94000 Pa), and comes close to the mechanical properties of nasal cartilage (234000 Pa). Additionally, when comparing the obtained mechanical properties with those of gelatin-containing formulations characterized by a similar concentration range, gel-MOD-AEMA outperforms all currently reported gelatin derivatives applied in the absence of alternative crosslinkers. (eg. Gel-SH: 2000 – 23000 Pa)[1] (see **Fig. S2** and **Table S1**) However, when gelatin is used as part of a co-network, higher storage moduli have been reported at similar gelatin concentrations. It is however anticipated that a similar phenomenon is likely to occur for the herein reported gel-MOD-AEMA. Furthermore, by using gel-MOD-AEMA, it is possible to obtain shear moduli close to values reported for crosslinked collagen gels, despite the less pronounced physical interactions present in gelatin in comparison to collagen.

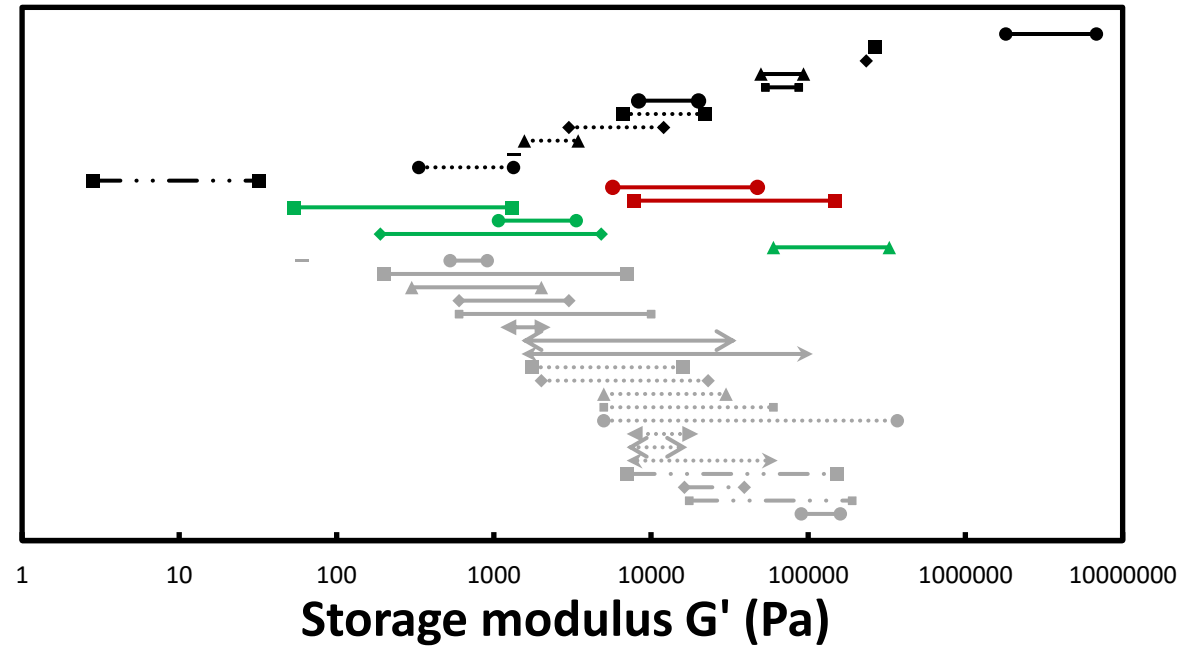
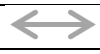
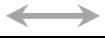



**Figure S2:** Overview of storage moduli of different types of tissue (black), and different biomaterials, including collagen (green) and gelatin containing formulations (grey). Mechanical properties of the materials reported in the present paper (red). *(legend of the figure can be found together with the references and numerical data in Table S1.)*

**Table S1:** Overview of the storage moduli of different types of tissues, different biomaterials and the reported gelatin derivatives based on literature reports.

| Tissue | min compressive storage modulus E' (Pa) | max compressive storage modulus E" (Pa) | min shear storage modulus G' (Pa) *(when indicated with*, calculated using $E' = 2G'(1+\mu))^2$* | max shear storage modulus G' (Pa) *(when indicated with*, calculated using $E' = 2G'(1+\mu))^2$* | Symbol |
|---|---|---|---|---|---|
| Vitreous fluid[3] | | | 2.8 | 32 | |
| adipose tissue[45] | 1000 | 4000 | 333.3333* | 1333.333* | |
| Dermis[6] | 4000 | 4000 | 1333.333* | 1333.333* | |
| Cervix[7] | 4700 | 10300 | 1566.667* | 3433.333* | |
| brain tissue[8] | | | 3000 | 12000 | |
| prostate[9] | 19800 | 65600 | 6600* | 21866.67* | |

| | | | | | |
|---|---|---|---|---|---|
| *IVD*[10] | | | | | |
| *Nucleus Pulposus*[10] | 25000 | 60000 | 8333.333* | 20000* |  |
| *Annulus fibrosus*[10] | 160000 | 260000 | 53333.33* | 86666.67* |  |
| *fibrous tissue*[10] | 150000 | 280000 | 50000* | 93333.33* |  |
| human nasal cartilage[11] | | | | 234000 |  |
| hyaline cartilage[1112] | 794000 | 800000 | 264666.7* | 266666.7* |  |
| Cornea[13] | 5300000 | 20000000 | 1800000 | 6800000 |  |
| **Presented Material** | | | | | |
| gel-MOD (5-15 w/v%) | | | 5698 | 47450 |  |
| gel-MOD-AEMA (5-15 w/v%) | | | 7724 | 147100 |  |
| **Biomaterial** | | | | | |
| porcine atelocollagen - 4S star PEG[14] | 160 | 3909 | 53.33333* | 1303* |  |
| Collagen[1510] | 3210 | 10000 | 1070* | 3333.333* |  |
| thiol-ene photo-click collagen-PEG[16] | | | 190 | 4810 |  |
| lysine crosslinked collagen[17] | 180000 | 980000 | 60000* | 326666.7* |  |
| gelatin (5w/v%) physical[18] | | | 60 | |  |
| gelatin-NB - 4 arm thiolated PEG (5 wt%)[19] | | | 200 | 7000 |  |
| physically crosslinked porcine gelatin Mn 43 kDa[20] | | | 300 | 2000 |  |
| gelatin (5w/v%) enzymatically crosslinked[18] | | | 527 | 907 |  |
| gel-MA/UV labile crosslinker (6W/V%)[21] | | | 600 | 3000 |  |
| gel-MA (DS 49)[22] | | | 600 | 10000 |  |
| gel-MA hydrosylate + crosslinkers[23] | | | 1100 | 2300 |  |
| gel-MA (DS 66) (5-15W/V%)[2425] | | | 1500 | 35000 |  |
| gel-MA (DS 66) (5-20w/v%)[24] | | | 1500 | 107000 |  |
| gelatin EDC crosslinked[5] | | | 1760 | 16000 |  |
| gel-SH (15w/v%)[1] | | | 2000 | 23000 |  |
| thiol-ene PEG; gel-MA interpenetrating networks[26] | | | 5000 | 30000 |  |
| gel-MOD (DS 100) (5 to 15wt%)[1125] | | | 5000 | 60000 |  |
| gel-MOD (DS 100) (5 to 30wt%)[11] | | | 5000 | 368000 |  |
| hyaluronic acid/gelatin[27] | | | 7000 | 20000 |  |
| chitosan-alginate-hyaluronate[12] | | | 7000 | 17000 |  |
| gel-acrylamide (5-15w/v%)[24] | | | 7000 | 64000 |  |
| gel-acrylamide (5-20w/v%)[24] | | | 7000 | 152000 |  |
| purified gelatin, EDC crosslinking[28] | | | 16300 | 39200 |  |
| gel-MOD/chondroitin sulfate-MOD (5-15w/v%)[29] | | | 17500 | 190000 |  |
| gelatin/hyaluronic acid cryogels crosslinked using EDC/NHS (10%)[6] | 270000 | 480000 | 90000* | 160000* |  |

Gelatin is a material characterized by UCST behavior. This means that below this temperature, the material will be present under the form of triple helices thereby forming a physical hydrogel. When the material is irradiated in this state, the functional groups will be in close proximity,

thereby enabling an efficient crosslinking reaction. Additionally, these crosslinks make sure that the triple helix structure is "locked", thereby providing additional structural integrity, even when surpassing the UCST.[1830] However, when the gelatin is heated above its UCST, the material is present as random polymer chains. When the crosslinking is induces, random bridges will be formed inside this random network, thereby making it insoluble. Additionally, when afterwards decreasing the temperature below the UCST, the triple helix formation will be hampered, and the increase in mechanical properties due to this phase transition will be very limited. [1830] (see **Fig. S3)**

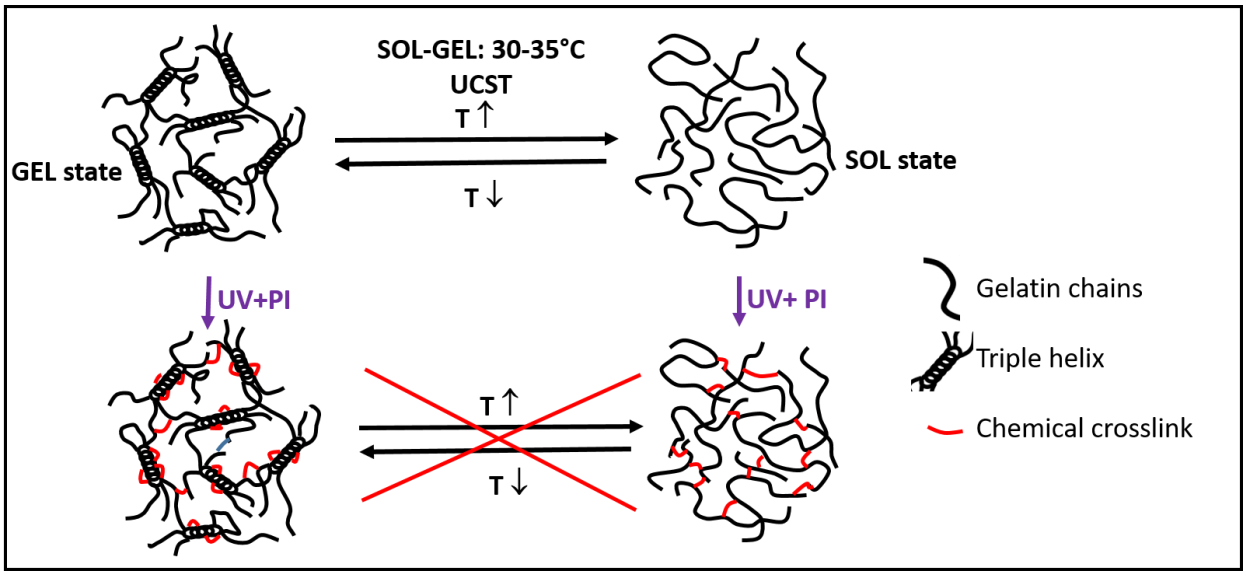



**Figure S3.** Influence of triple helix formation during physical gelation on the final mechanical properties.

## Effect of gelatin functionalization and -concentration on the hydrogel gel fraction, water uptake capacity & network density

### Rubber Elasticity theory

The network density of a hydrogel can be calculated using the average molecular weight, the equilibrium swelling ratio and the mechanical properties using the rubber elasticity theory.[24,31,32] This theory allows to calculate an estimation of several important parameters including the polymer volume fraction in the swollen state ($v_{2,s}$), the volumetric swelling ratio

(Q), the average molecular weight between crosslinks ($\overline{Mc}$), the network mesh size (ξ) and the crosslink density ($\rho_x$).

Q and $v_{2,s}$ are both indications for the amount of liquid that can be imbibed inside a hydrogel which can be calculated starting from the mass swelling ratio q:[24,33]

$$v_{2,s} = \frac{V_p}{V_g} = \frac{1}{Q} = \frac{\left(\frac{1}{\rho_{gelatin}}\right)}{\left(\frac{q}{\rho_{H_2O}}\right) + \left(\frac{1}{\rho_{gelatin}}\right)} \quad (4)$$

Herein, $V_p$ and $V_g$ represent respectively the polymer volume and the hydrogel volume at equilibrium swelling, while $\rho_{H_2O}$ and $\rho_{gelatin}$ represent the density of water and gelatin respectively. The density of water is 1 g/cm³ while the density of gelatin was estimated to be around 1.36 g/cm³ based on previous reports from literature.[22,24,34,35]Since all network chains within the characterized hydrogels follow the Gaussian statistics model (**Fig. S4**), the obtained volumetric swelling ratio could be applied to determine $\overline{Mc}$ using the following equation:[36, 32]

$$G = \left(\frac{cRT}{\overline{Mc}}\right) * \left(1 - \frac{2\,\overline{Mc}}{M_n}\right) * \left(\frac{1}{Q^{1/3}}\right) \quad (5)$$

in which G is the shear modulus (atm), c is the concentration of gelatin in the solution, R is the universal gas constant ($L*atm*K^{-1}*mol^{-1}$), T is the temperature (K) and $\overline{Mc}$ is the average molecular weight between crosslinks (Da). Literature states that the shear modulus of hydrogels can be derived from the mean peak value of the storage modulus G‘, since the contribution of the loss modulus G“ to the shear modulus can be considered negligible for all analyzed hydrogel samples.[24,37,38]

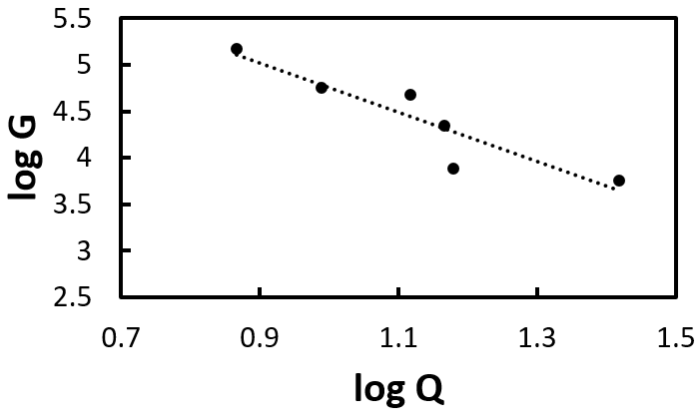



**Figure S4.** Plot of log G vs log Q for all analyzed hydrogel films used in the rubber elasticity theory calculations (Eq (5)).

To obtain the average weight between crosslinks ($\overline{Mc}$), equation (5) can be rewritten as:

$$\overline{Mc} = \frac{1}{\left(\frac{G}{cRTQ^{-1/3}}\right)+\left(\frac{2}{M_n}\right)} \tag{6}$$

Once the average molecular weight between crosslinks ($\overline{Mc}$) is known, an estimation of the average mesh size on the equilibrium swelling (ξ) can be obtained using the following equation:[30]

$$\xi = \left(\frac{2C_n\,\overline{Mc}}{M_r}\right)^{(1/2)} * l * Q^{(1/3)} \tag{7}$$

with $C_n$ being the Flory characteristic ratio which corresponds to 8.26 for gelatin based on reports from literature[30] , $M_r$ is the average molecular weight of one repeating unit or one amino acid (assumed to be around 94.7 g/mol).[30,39] and l is the length of a bond along the polymer backbone. Furthermore, it should be noted that equation (7) is derived from the Flory-Rehner theory which is only strictly valid for simple systems like vinyl polymers. Therefore, the factor 2 has to be replaced by a factor 3 since the repetitive unit contains 2 bonds in contrast to 1 bond

in vinyl polymers.[30] For the same reason, the bond length along the polymer backbone was approximated as the average bond length of one bond along the polymer backbone, taken as the arithmetic mean of one carbonyl C-C bond (1.53 Å) one C-N bond next to the carbonyl (1.32 Å) and a C-N bond (1.47 Å).[30,40]

The crosslink density $\rho_x$ is a measure for the number of crosslinks present per unit of volume and can be calculated from $\overline{Mc}$ and $\bar{\nu}$, where $\bar{\nu}$ corresponds to the specific volume of gelatin, which was determined to be 0.735 cm³/g according to a previous study.[24]

$$\rho_x = \frac{1}{\bar{\nu}\,\overline{Mc}} \tag{8}$$

## Voxel size calculations

The voxel size was calculated by approximating the illumination point spread function based by a three dimensional gaussian volume. To calculate this Gaussian volume the 1/e width in the lateral $(\omega_{xy})$ and axial $(\omega_z)$ dimension was calculated using the following formulas as described in literature.[41]

$$\omega_{xy} = \frac{0.325\,\lambda}{\sqrt{2}\,NA^{0.91}}\ NA\ (if\ NA > 0.7) \tag{9}$$

$$\omega_z = \frac{0.532\,\lambda}{\sqrt{2}}\left(\frac{1}{n-\sqrt{n^2-NA^2}}\right) \tag{10}$$

The numerical aperture (NA) corresponds to 0.85 as provided by Zeiss. The refractive index was estimated to be 1.33 as the solutions consist primarily out of water..

## Structuring defects at low precursor concentrations


**Figure S5.** Incomplete structures recorded in 7.5 w/v% gel-MOD-AEMA solutions in the presence of 2 mol% P2CK at 100 mm/s and 100 mW laser power. *(The PBM logo has been used with permission from the Polymer Chemistry and Biomaterials research group of Ghent University.)*

When structuring in low concentrations of gel-MOD-AEMA, structuring is visible. Even after development, structures remain present in contrast to structures produced in 7.5w/v% gel-MOD solutions. However, the gel-MOD-AEMA structures are incomplete due to poor mechanical properties, resulting in partial washing away of the structure during development.

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