SUPPORTING INFORMATION

Using thiol-ene click chemistry to engineer 3D printed plasmonic hydrogel scaffolds for SERS biosensing

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1. Characterization of the functionalized polymers

1.1. Functionalization of alginate

Norbornene-bearing alginate: ALGNB

The presence of the norbornene units was confirmed by the appearance of a new vibrational band around 1730 cm^{-1} in the FTIR spectrum (***Figure S1A***). This signal can be attributed to the new amide carbonyl group stretching.[1] Also, the ^{1}H-NMR spectrum revealed the appearance of new peaks in the range of 5.90-6.30 ppm, that can be attributed to the norbornene ring (***Figure S1B***).[2] The integral of these peaks was used to calculate the substitution degree of the polymer. In the particular case of alginate, it needs to be considered that the reaction is more likely to happen in the guluronic units, thus, its guluronic content needs to be determined first accordingly to EQ1. Subsequently, the percentage of norbornenes in the alginate backbone was calculated using EQ2, resulting that 60% of repetition units were functionalized (30 mg NB/g alginate).

$$G(\%) = \frac{H_g}{H_m + H_g} = \frac{I_{4.97}}{I_{4.38} + I_{4.97}} \cdot 100 \qquad \textbf{EQ1}$$

$$DS\ (\%) = \frac{H_a + H_b}{2\ x\ H_g} \cdot G(\%) = \frac{I_{6.31-6.17} + I_{6.05-5.95}}{2\ x\ I_{4.97}} \cdot G(\%) \qquad \textbf{EQ2}$$

Where $\boldsymbol{H_g}$ is the anomeric proton of the guluronic unit while $\boldsymbol{H_m}$ corresponds to the anomeric proton of the mannuronic unit. $\boldsymbol{H_a}$ and $\boldsymbol{H_b}$ are the protons of the alkene belonging to the norbornene unit and ***I*** refers to the integral of the area under the peak.

Thiolated alginate: ALGSH

The presence cysteine units in alginate were determined by the appearance of a new vibrational band around 1730 cm^{-1} in the FTIR spectrum (***Figure S1A***). This signal can be attributed to the new amide carbonyl group stretching vibrational mode.[1] In this case, the ^{1}H-NMR spectra did not offer any particular signal belonging to the thiol group, as it might exchange the proton with the solvent (***Figure S1B***). Therefore, the Ellman's assay was used instead (*further details in Section 2*). Accordingly, the obtained alginate showed 30% of repetition units functionalized with cysteine (20 mg SH/g alginate).

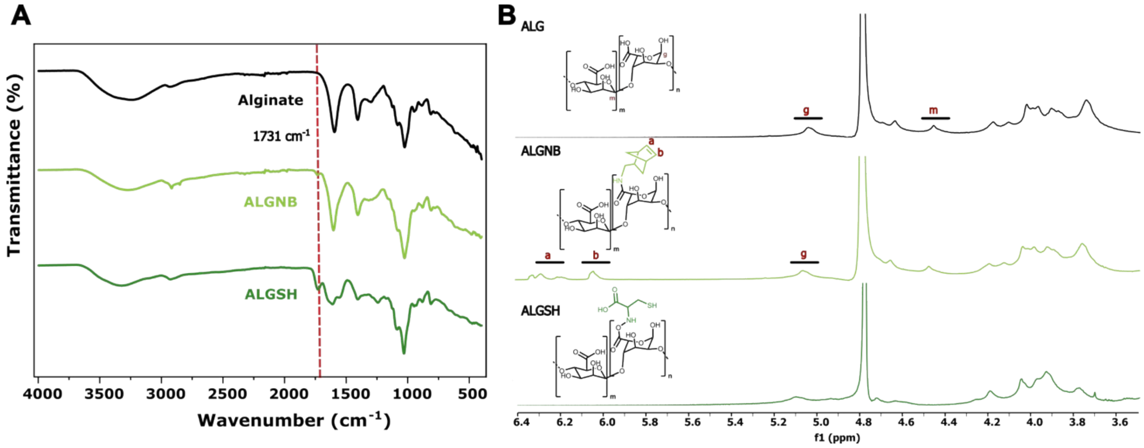


Figure S1 (A) FTIR and (B) ^{1}H-NMR spectra of alginate, ALGNB and ALGSH.

1.2. Functionalization of gelatin

Norbornene-bearing gelatin: GELNB

In the FTIR spectrum of gelatin, two characteristic vibrational bands can be observed at 1521 cm^{-1} and 1627 cm^{-1} corresponding with the amide I and amide II vibrational modes. In norbornene-functionalized gelatin, these bands respectively shifted to 1535 cm^{-1} and 1639 cm^{-1} (***Figure S2A***).[1] Also, the ^{1}H-NMR spectrum revealed the appearance of new peaks in the range of 5.90-6.30 ppm, that can be attributed to the norbornene ring (***Figure S2B***).[3] In the particular case of gelatin, the reaction is more likely to happen in the amino groups of lysine residues. Therefore, the success of the functionalization could be assessed through the change in the ratio between the signal of the protons belonging to the phenylalanine ring ($\delta_{H\text{-}Phe}$= 7.5 ppm) and those of the amino group of lysine ($\delta_{H\text{-}Lys}$= 2.8 ppm), accordingly to EQ3. The degree of substitution achieved for GELNB was 100% (50 mg NB/g polymer).

$$DS\,(\%) = 1 - \frac{H_{Lys}}{H_{Phe}} \cdot 100 = \frac{I_{3.00-2.90}}{I_{7.35-7.15}} \cdot 100 \qquad \textbf{EQ3}$$

Where $\boldsymbol{H_{Lys}}$ corresponds to the protons belonging to the -NH$_2$ group of lysine residues while $\boldsymbol{H_{Phe}}$ refers to the protons of the aromatic ring for phenylalanine residues. In all cases, ***I*** refers to the integral of the area under the peak.

Thiolated gelatin: GELSH

In the FTIR spectrum of gelatin, two characteristic vibrational bands can be observed at 1521 cm^{-1} and 1627 cm^{-1} corresponding with the amide I and amide II vibrational modes, respectively. In thiolated gelatin, these bands respectively shifted to 1535 cm^{-1} and 1639 cm^{-1} (***Figure S2A***).[1] In this case, the ^{1}H-NMR spectra did not offer any particular signal belonging to the thiol group, as it might exchange the proton with the solvent (***Figure S2B***). However, the degree of substitution was assessed through the change in the ratio between the signal of the protons belonging to the phenylalanine ring ($\delta_{H\text{-}Phe}$= 7.5 ppm) and those of the amino group of lysine ($\delta_{H\text{-}Lys}$= 2.8 ppm), accordingly to EQ3. The degree of substitution achieved for GELSH was 65% (39 mg SH/g polymer).

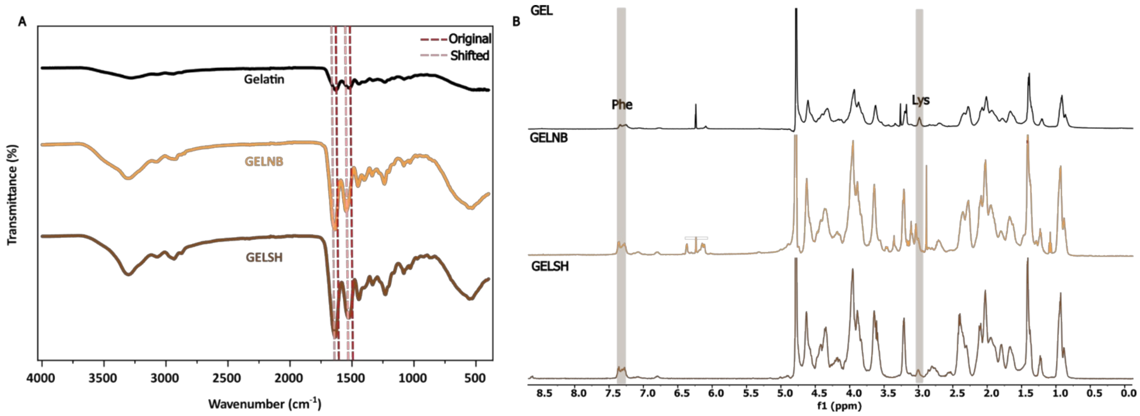


Figure S2. (A) FTIR and (B) ^{1}H-NMR spectra of gelatin, GELNB and GELSH.

1.3. Functionalization of carboxymethyl cellulose

Norbornene-bearing carboxymethyl cellulose: CMCNB

The presence of the norbornene units was confirmed by the appearance of a new vibrational band around 1730 cm^{-1} in the FTIR spectrum (***Figure S3A***).[1] This signal can be attributed to the new amide carbonyl group stretching. Moreover, the ^{1}H-NMR spectrum revealed the appearance of new peaks in the range of 5.90-6.30 ppm, that can be attributed to the norbornene ring (***Figure S3B***).[4] The integral of these peaks was used to calculate the substitution degree of the polymer. The carboxymethyl cellulose exhibits an average of 8.4 protons per anhydroglucose repeat unit as each unit might have either 7 or 9 protons depending on the number of carboxymethyl units. The % of carboxymethyl units equals 70%, thus, 70% of units have 7 protons and 30% 9 protons, leading to an average of 8.4 protons. Taking this into account, the degree of norbornene substitution achieved can be calculated according to EQ4, resulting in 18% (11.7 mg NB/g polymer).

$$DS\,(\%) = \frac{H_a + H_b}{H_{CMC}} \cdot 100 = \frac{I_{6.31-6.19} + I_{6.05-5.95}}{I_{4.97-3.0}} \cdot 4.2 \cdot 100 \qquad \textbf{EQ4}$$

Where $\boldsymbol{H_a}$ and $\boldsymbol{H_b}$ are the protons of the alkene belonging to the norbornene moiety while $\boldsymbol{H_{CMC}}$ refers to the average protons in the glucopyranose unit. In all cases, ***I*** refers to the integral of the area under the peak.

Thiolated carboxymethyl cellulose: CMCSH

The presence of cysteine units in CMC was determined by the appearance of two vibrational bands around 1730 cm^{-1} in the FTIR spectrum (***Figure S3A***).[1] This signal can be attributed to the new amide carbonyl group stretching vibrational mode. In this case, the ^{1}H-NMR spectra did not offer any particular signal belonging to the thiol group, as it might exchange the proton with the solvent (***Figure S3B***). Therefore, the Ellman's assay was used instead (*further details in Section 2*). Accordingly, the obtained CMC showed 30% of repetition units functionalized with cysteine (20 mg SH/g alginate).

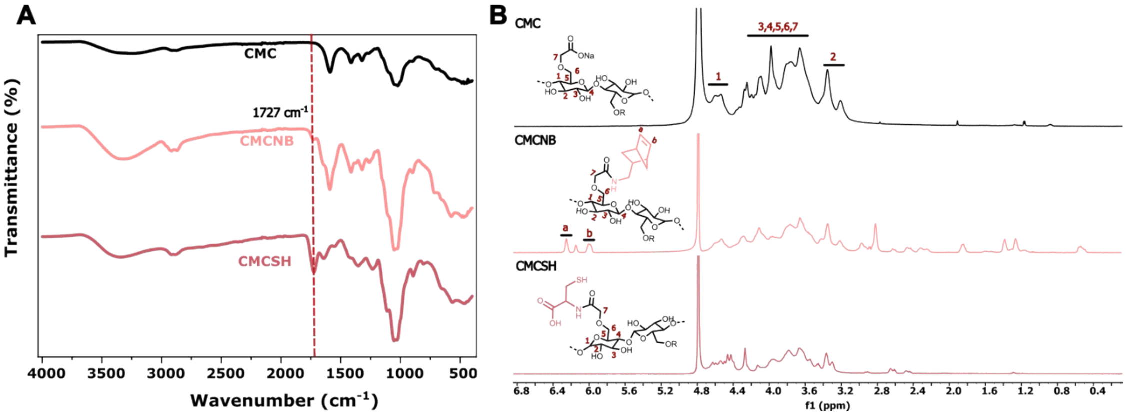


Figure S3. (A) FTIR and (B) ^{1}H-NMR spectra of carboxymethyl cellulose, CMCNB and CMCSH.

2. Characterization and properties of the synthesized AuNRs

For the use of hydrogels as SERS substrates, plasmonic nanoparticles must be incorporated to the hydrogel forming polymers. In the current work, AuNRs were the particles of choice due to their excellent biostability and SERS enhancing properties under near infrared irradiation. The UV-Vis spectrum of the AuNRs used in this study showed characteristic transversal (530 nm) and longitudinal (785 nm) bands (**Figure S4A**). Moreover, TEM images revealed these AuNRs were 56.5 ± 4.5 nm in length and 17.5 ± 3.8 nm in width (**Figure S4B**).

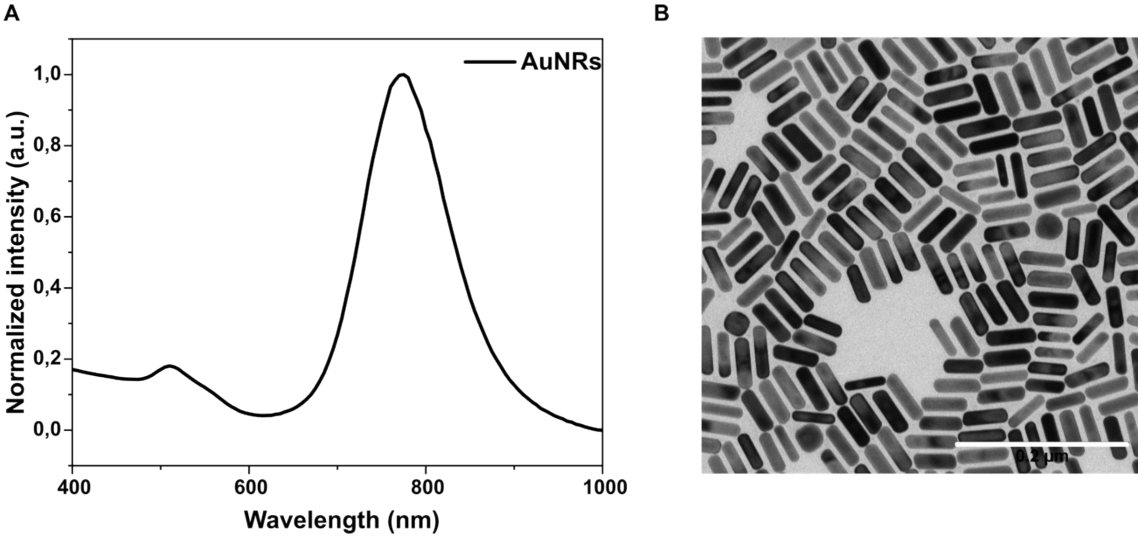
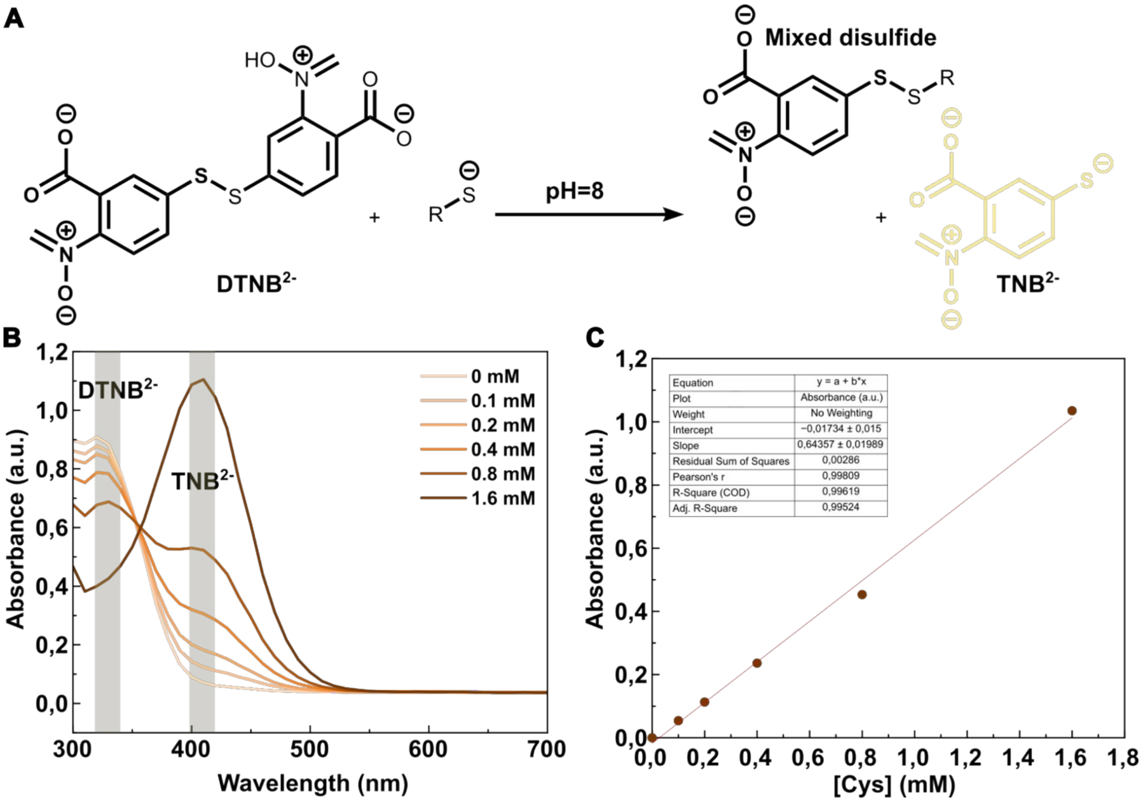


Figure S4. (A) UV-Vis spectrum of the resulting AuNRs. (B) TEM image of AuNRs. Scale bar = 200 nm.

3. Ellman’s test

The Ellman’s assay is a colorimetric method for the quantification of thiol groups. The method is based on the reaction between DTNB^{2-} and free thiol groups present in the samples. The assay is conducted under basic pH causing free thiols to form the corresponding thiolate group that cleaves the dithiol bond of DTNB^{2-} resulting in the formation of TNB^{2-}, a yellow-coloured compound (***Figure S5A***).[5] The intensity of the yellow colour, measured at 412 nm using a spectrophotometer, is directly proportional to the concentration of thiols in the sample, according to the Lambert-Beer law. In order to quantify the number of free thiols, a set of cysteine standards was prepared with concentrations ranging from 1.6 mM to 0.1 mM. Then, 250 µL of the solutions were mixed with 50 µL of Ellman’s reagent (4 mg DTNB^{2-}/ mL of reaction buffer) and they were diluted to a final volume of 2.5 mL of reaction buffer (0.1 M sodium phosphate buffer, pH = 8.0). The reaction mixtures were incubated over 15 min at room temperature before measuring the absorbance by taking 200 µL into a 96-well plate. The resulting absorbance spectra are shown in ***Figure S4B*** and the absorbance at 412 nm was used to build the calibrate showed in ***Figure S4C***. The regression equation was used to determine free thiols in the functionalized polymers and hydrogels by running the Ellman’s reaction and measuring of the absorbance at 412 nm on test tubes containing known amounts of the samples.



Equation	y = a + b*x
Plot	Absorbance (a.u.)
Weight	No Weighting
Intercept	−0,01734 ± 0,015
Slope	0,64357 ± 0,01989
Residual Sum of Squares	0,00286
Pearson's r	0,99809
R-Square (COD)	0,99619
Adj. R-Square	0,99524

Figure S5. (A) Ellman’s reaction for the determination of free thiols. (B) Absorbance spectra of cysteine at different concentrations. (C) Correlation between the absorbance and the concentration of free thiols for cysteine.

4. Photo-rheology: control experiments

Control experiments were carried out without AuNRs and it could be observed that the nanoparticles enhance the mechanical properties of the bioinks. Also, experiments carried out without LAP did not lead to any increase in the moduli.

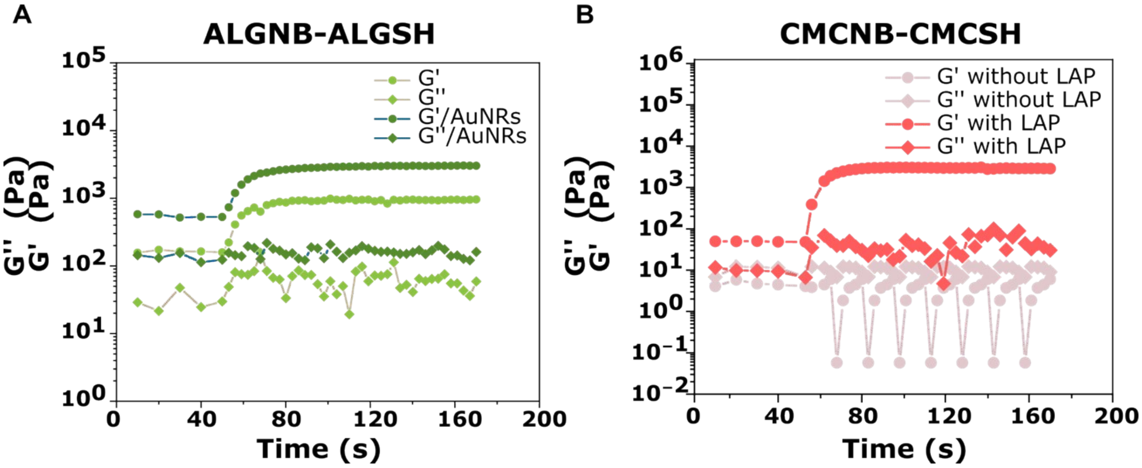


Figure S6 (A) In situ photo-rheology of ALGNB-ALGSH (equimolar) hydrogel formed through thiol-ene crosslinking chemistry using 0.1% (w/v) LAP as photoinitiator with (dark green) and without (light green) AuNRs. (B) In situ photo-rheology of CMCNB-CMCSH (equimolar) plasmonic hydrogel formed through thiol-ene crosslinking chemistry with (red) and without (pink) 0.1% (w/v) LAP used as photoinitiator.

5. SEM: AuNRs

SEM micrographs were taken to capture AuNRs on the surface of these hydrogels, as showed in the following figure for all the studied compositions.

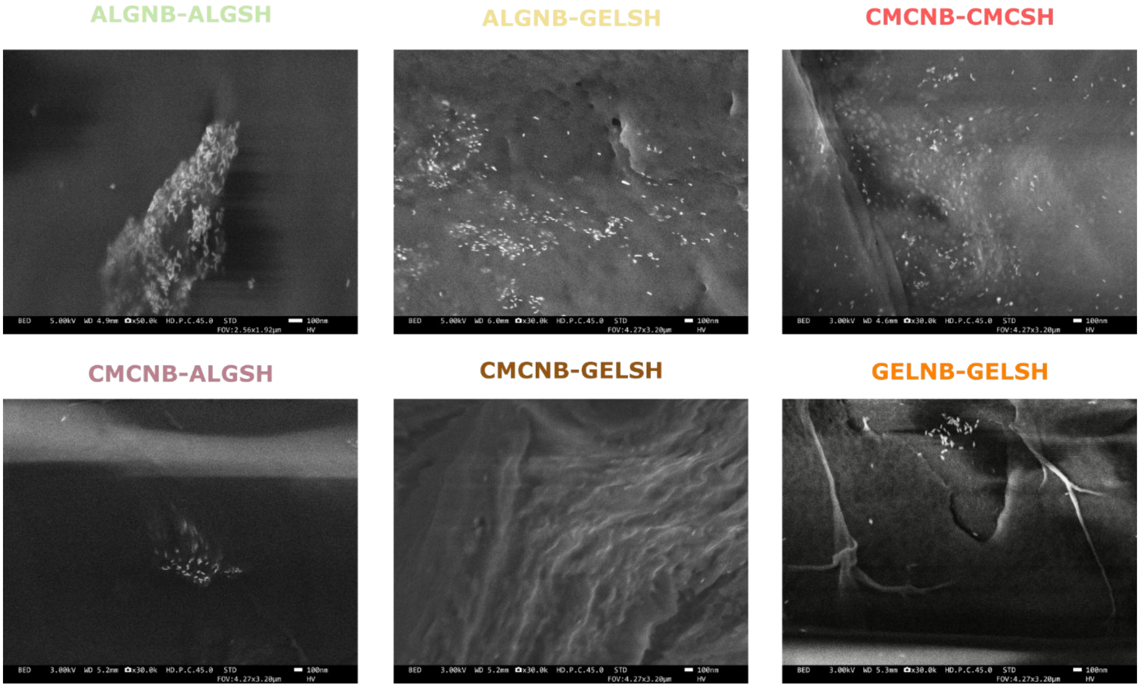


Figure S7. SEM images of freeze-dried hydrogels formed through thiol-ene crosslinking at 3% (w/v) concentration of the highest functionalized polymer. Scale bar = 100 nm.

6. Swelling test

The swelling ratio (SR) of the hydrogels is calculated accordingly to EQ5, being W_{WET} the weight of the hydrogel in the hydrated state and W_{DRY} in the dried state. The swelling test was also carried out for those hydrogels without AuNRs and the results showed the presence of the nanoparticles also enhances the swelling ratio.

$$SR\ (\%) = \frac{W_{WET} - W_{DRY}}{W_{DRY}} \cdot 100 \qquad \textbf{EQ4}$$

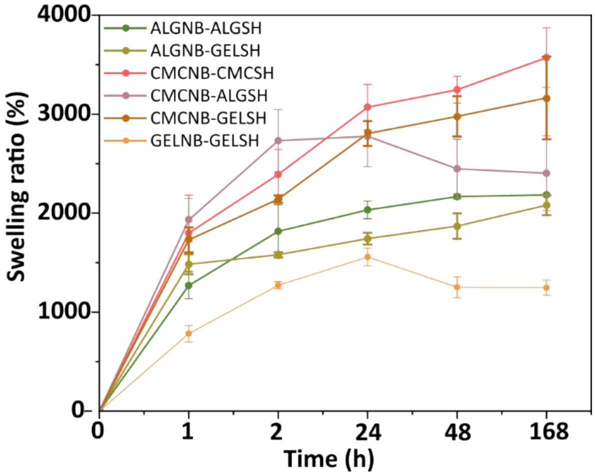


Figure S8. Swelling and degradation ratio for all combinations of hydrogels at 3% (w/v) concentration of the highest functionalized polymer, without AuNRs, measured at different time points over a week of incubation at 37 °C.

7. SERS studies

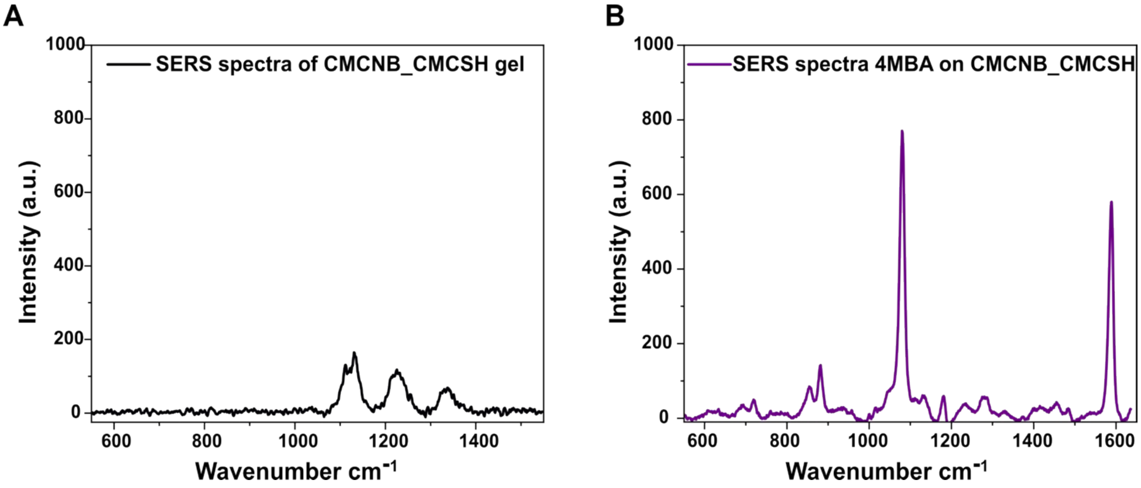


Figure S9. SERS spectra of the CMCNB-CMCSH hydrogel before (A) and after (B) incubation with 4-MBA.

The average intensity of the peak at 1080 cm^{-1} was compared with the swelling ratio for each hydrogel resulting in a monotonic relationship between both variables.

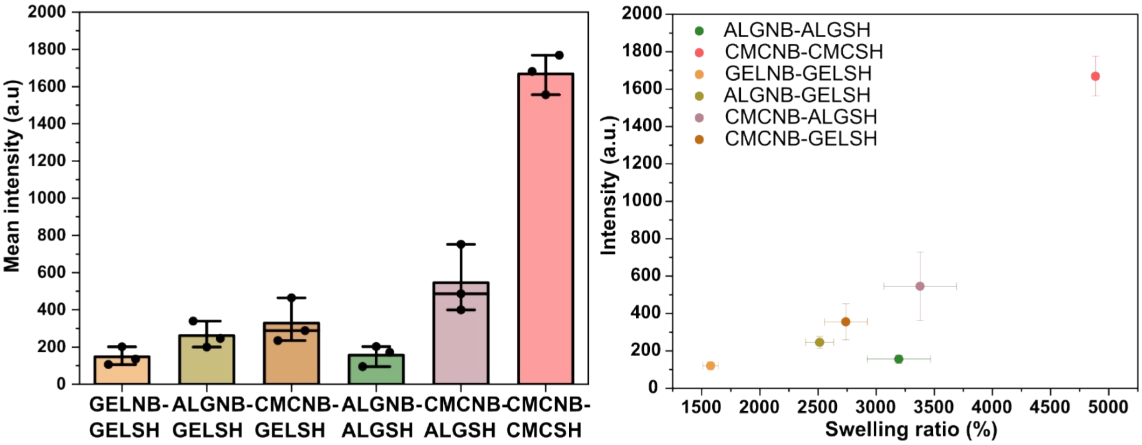


Figure S10. (A) Reproducibility of SERS measurements for 4-MBA embedded in different hydrogels. (B) SERS intensity for 4-MBA vs. swelling ratio for each hydrogel.

8. Biocompatibility of 1.5% (w/v) hydrogels

To use 1.5% (w/v) as plasmonic bioinks for the obtention of plasmonic-assisted 3D printed materials for cell culture, the biocompatibility was assessed and all the combinations offered values above 70% (standard for cell-friendly biomaterials).

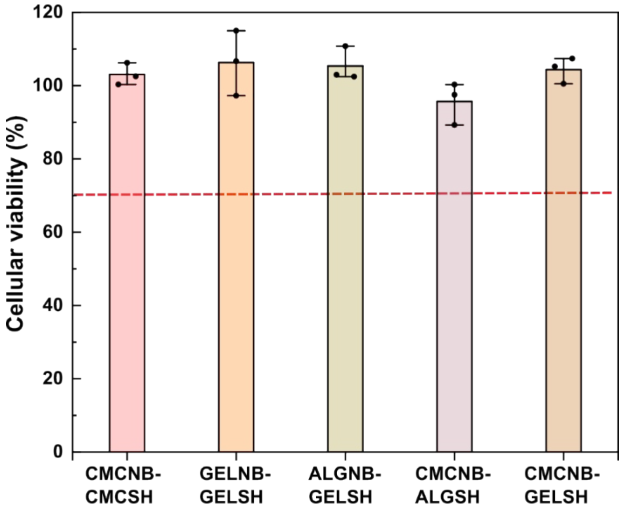


Figure S11 Biocompatibility of the 1.5% (w/v) hydrogels.

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