Supporting Information

Modular Functionalization of Laminarin to Create Value-Added Naturally-Derived macromolecules

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1. Materials and Methods

1.1. Materials:

Laminarin *from Eisenia Bicyclis* (LAM**,** CAS: 9008-22-4, Mw≈6762 g/mol, PDI=1.7 determined by GPC against PEG standards) was acquired from Carbosynth. Allyl bromide (99%, stabilized), sodium hydroxide (NaOH) pellets, glacial acetic acid (>99%) and hydrochloric acid (HCl, ≈37% w/w) were obtained from Thermo Fisher Scientific, while ethanol (≥99.8%) was purchased from VWR. Cysteamine hydrochloride (≥97.0%), 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (I2959, 98%), tris(2-carboxyethyl) phosphine hydrochloride (TCEP, ≥99.9%), potassium persulfate (KPS, 99%), sodium periodate ($NaIO_4$, ≥98%), thioacetic acid (96%), ethylene glycol (≥99%), dopamine hydrochloride and thiolactic acid (95%) were purchased from Sigma-Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, >98%), N-hydroxysuccinimide (NHS, >98%), 3,4-dihydroxybenzoic acid (>98%) were obtained from TCI Chemicals. Spectra/Por dialysis membranes with a molecular weight cut-off of 3.5 kDa were supplied from Spectrum Laboratories. All reagents used were of analytical grade and, hence, no further purification was performed. Unless otherwise stated, water purified in an Elix® Advantage 3 system (Merck) was used throughout.

1.2. ^{1}H and ^{13}C Nuclear Magnetic (NMR) spectroscopy:

^{1}H and ^{13}C NMR spectra were recorded on a *Bruker Avance-300* spectrometer at 300.13 MHz and 75.47 MHz, respectively, in D_2O and at 298 K. Chemical shifts are reported as parts per million (ppm) downfield from the internal standard trimethylsilane (TMS).

1.3. Fourier Transform Infrared (FTIR) spectroscopy:

IR spectra of the freeze-dried samples were recorded with an attenuated total reflectance (ATR) accessory on a Mattsson 7000 galaxy series spectrometer at room temperature (RT). For each sample, 256 scans were collected with a resolution of 4 cm^{-1} in the spectral width region of 4000–400 cm^{-1}.

1.4. Zeta potential:

The zeta potentials of the synthesized compounds in water were obtained using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd.) under an applied electric field and at 25 °C. All determinations were repeated five times.

1.5. Gel Permeation Chromatography (GPC)/Size-exclusion chromatography (SEC):

GPC/SEC measurements were performed on a 390-MDS Multi-Detector GPC/SEC System from Agilent Technologies fitted with RI and UV detectors (λ = 280 nm), 0.2 M $NaNO_3$ with 0.01 % NaN_3 solution was used as eluent at a flow rate of 1.0 mL min^{-1} and at 40 °C. SEC data were calibrated against PEG standards.

1.6. Ultraviolet–visible (UV-Vis) spectroscopy:

A microplate reader (Synergy HTX, Biotek) was used to measure the absorbance of the catechol derivatives in the range of 200 to 650 nm.

1.7. Synthesis of LAM-OH functionalized with allyl groups (LAM-C=C) via nucleophilic substitution:

LAM-C=C polymer was obtained by nucleophilic substitution based on a previously reported work with some modifications.[1-3] The schematic representation of this chemical reaction was depicted in Figure 1B.

First, LAM-OH (1 g, 1.54 mmol) was dissolved in 5 mL of deionized water. After complete dissolution, 7 mL of a 2 M NaOH solution was added, and the mixture was stirred for 30 minutes at 40 ºC. Afterwards, allyl bromide was added (500 µL, 5.78 mmol), the glass vial was closed, and the reaction left for 24 hours at RT. The previous solution was neutralized by adding dropwise an aqueous solution of 5 M HCl. Finally, LAM-C=C compound was purified by three cycles of precipitation in ethanol and resuspension in deionized water. The precipitate (Mw≈7144 g/mol, PDI=2.1) was dried in an oven at 50 ºC for 2 days in dark (802 mg, 83% yield). The substitution degree (SD) was calculated as the ^{1}H-NMR integral ratio between the allyl-proton (5.82–6.12 ppm) and the proton at 4.40–4.59 ppm, according to the literature[1-3] and was found to be SD≈47%.

^{1}H NMR (D_2O, in ppm): δ 3.28–3.91 (19H), 4.08 (1H), 4.14–4.33 (4H), 4.47–4.67 (4H), 5.24–5.36 (2H, CH_2-Allyl), 5.97 (1H, CH-Allyl). **^{13}C NMR** (D_2O, in ppm): δ 57.4, 60.7, 68.3, 69.6, 73.9, 75.6, 102.7, 118.5 (CH_2 Allyl), 133.7 (CH Allyl).

1.8. Synthesis of LAM-C=C derivatives, namely LAM-NH_2, LAM-COOH and LAM-SH, by thiol Michael addition:

As shown in Figure 1B, an excess of equivalents of the chosen thiol-compound (either 1.13 mmol of cysteamine hydrochloride, 1.46 mmol of thiolatic acid or 1.71 mmol of thioacetic acid for obtaining LAM-NH_2, LAM-COOH or LAM-SH derivatives, respectively) and 4 mL of a 3 M aqueous solution of HCl were added to a LAM-(C=C) polymer suspension (100 mg in 1 mL of deionized water), under stirring, followed by the degassing with N_2 gas (to minimize thiol oxidation) for 5 min at RT. The acid addition is important to maintain the thioacetic acid protonated during LAM-SH synthesis and, consequently, resulting in higher rates of double bond conversion. KPS (6 mg, 0.02 mmol) was dissolved in 2 mL of water and added to the previous polymeric solution, which was left for 1 hour at RT, under vigorous stirring. To obtain the LAM-NH_2 and LAM-COOH derivatives, the reaction mixture was carefully neutralized by adding dropwise an aqueous solution of 5 M NaOH and immediately after transferred to dialysis bags (MWCO 3.5 kDa). The purification was carried out for 5 days under stirring against distilled water, which was changed twice a day. Purified, dried LAM-OH derivatives (104 mg, 89% yield, Mw≈7608 g/mol, PDI=2.1 for LAM-NH_2 and 109 mg, 91%yield, Mw≈8182 g/mol, PDI=2.7 for LAM-COOH derivatives, respectively) were obtained by lyophilization (LyoQuest Plus, Telstar)

and stored in a dry place protected from light. In the case of LAM-SH polymer, the removal of the acetyl group was made in basic conditions. To this end, the compound obtained after incorporating the thioacetic acid (0.30 g) molecule was dissolved in 6 mL of 1 M NaOH for 2 hours under a N_2 atmosphere. The pH was set to 7.0 by adding dropwise an aqueous solution of 1 M HCl. Later, TCEP reducing agent (100 mg, 0.35 mmol) was added to this solution and left for incubation, under stirring, overnight at RT. LAM-SH derivative (93 mg, 85% yield, Mw≈7364 g/mol, PDI=2.4) was obtained after dialysis and lyophilization (LyoQuest Plus, Telstar).

1.8.1. LAM-NH_2: ^{1}H NMR (D_2O, in ppm): δ 1.91 (2H, O-CH_2), 2.67–2.69 (2H, CH_2-CH_2), 2.85 (2H, S-CH_2), 3.21 (2H, CH_2-NH_2), 3.30–3.92 (19H), 4.18–4.20 (2H), 4.52 (2H), **^{13}C NMR** (D_2O, in ppm): δ 27.2 (CH_2-CH2), 28.1 (-S-CH_2), 29.0 (CH_2-S), 38.3 (-CH_2-NH_2), 60.7, 68.2, 69.6, 73.1, 75.6, 102.9.

1.8.2. LAM-COOH: ^{1}H NMR (D_2O, in ppm): δ 1.38 (3H, CH_3), 1.88 (2H, O-CH2), 2.68 (2H, CH_2-CH2), 3.30–3.92 (14H), 4.18 (1H), 4.52 (1H), **^{13}C NMR** (D_2O, in ppm): δ 17.7 (CH_3), 27.5 (CH_2-CH2), 28.5 (CH_2-S), 44.4 (S-$CHCH_3$COOH), 60.7, 68.2, 69.6, 73.1, 75.5, 102.7, 180.6 (COOH).

1.8.3. LAM-SH: ^{1}H NMR (D_2O, in ppm): δ 1.88 (2H, O-CH_2), 2.64 (2H,CH_2-CH2), 3.35–3.93 (17H), 4.18 (2H), 4.52 (2H), **^{13}C NMR** (D_2O, in ppm): δ 27.3 (CH_2-CH2), 28.2 (CH_2-S), 60.7, 68.2, 69.6, 73.1, 75.5, 102.8.

1.9. Synthesis of LAM-C=O derivative through an oxidation process:

LAM-C=O was prepared as described in Figure 1B and was based on a previously reported work[4-5] with few modifications. LAM-OH (1.0 g, 1.54 mmol) was first dissolved in 5 mL of deionized water in a glass vial. Sodium periodate (0.66 g, 3.09 mmol) was added and the reaction mixture was kept protected from light, under stirring, for 24 hours at RT. After this, the oxidation process of LAM-OH was quenched by adding an excess of ethylene glycol (514 µL, 9.25 mmol) and the resulting mixture was transferred to dialysis bags (MWCO 3.5 kDa) and dialyzed for 3 days, under stirring, against distilled water. Purified and dried LAM-C=O derivative was obtained (722 mg, 76% yield) after freeze-drying (LyoQuest Plus, Telstar) (Mw≈5972 g/mol, PDI=2.4). The SD was calculated following a previous method described in literature[4-5] and was found to be ≈ 44%.

^{1}H NMR (D_2O, in ppm): δ 3.30–3.92 (17H), 4.18–4.21 (2H), 4.51 (2H). **^{13}C NMR** (D_2O, in ppm): δ 58.1, 60.7, 68.1, 68.5, 72.4, 88.9, 96.3, 102.5 (C=O).

1.10. Synthesis of LAM-DOPA polymer via EDC/NHS coupling using either LAMCOOH or LAM-NH_2 derivatives as starting materials:

First, LAM-NH_2 or LAM-COOH (0.500 g) was dissolved in deionized water (30 mL). Afterwards, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; 0.276 g, 1.44 mmol) was also dissolved in water (5 mL) and slowly added to the previous solution, which was kept under a N_2 atmosphere. Additionally, *N*-hydroxysuccinimide (0.166 g, 1.44 mmol) was dissolved in 5 mL of deionized water and was slowly incorporated in the resulting mixture. After stirring for 15

minutes, 3,4-dihydroxybenzoic acid (0.222 g, 1.44 mmol) or dopamine hydrochloride (0.166 g, 1.44 mmol) was added to the mixture containing the LAM-NH_2 or LAM-COOH derivatives, respectively, and the reaction was kept, under stirring, overnight at RT (see Figure 1B for a schematic representation of this reaction). The reaction solution was transferred to dialysis bags (MWCO = 3.5 kDa), purified by dialysis against water for 4 days and then lyophilized (455 mg, 77% yield for LAM-NH_2-DOPA and 420 mg, 70% yield for LAM-COOH-DOPA). The correct incorporation of catechol moieties was assessed by ^{1}H-NMR, which resulted in a SD≈10%.[6-7]

1.10.1. LAM-NH_2-DOPA: ^{1}H NMR (D_2O, in ppm): δ 1.37 (2H, CH_2-Catechol), 1.91 (2H, CH_2-S), 2.67–2.69 (2H, O-CH_2), 2.85 (2H, CH_2-NH_2), 3.21 (2H, S-CH_2), 3.30–3.92 (19H), 4.18–4.20 (2H), 4.52 (2H), 6.57–7.28 (3H, catechol group).

1.10.2. LAM-COOH-DOPA: ^{1}H NMR (D_2O, in ppm): δ 0.91–1.21 (2H, CH2-S), 1.38 (3H, CH_3), 1.88 (2H, CH_2-S), 2.68 (2H, O-CH_2), 2.85 (2H, CH_2-NH_2), 3.30-3.92 (14H), 4.18 (1H), 4.52 (1H), 6.77–7.71 (3H, catechol group).

1.11. Hydrogel Preparation through:

1.11.1. Chemical crosslinking between LAM-NH_2 and LAM-C=O derivatives:

Equal volumes of LAM-C=O (10% (w/v) in dPBS; pH7.4) and LAM-NH_2 (20% w/v in dPBS; pH7.4) derivatives were mixed together on a vortex mixer for 5 seconds. This procedure was performed to evaluate the ability of these produced functional macromolecules to form hydrogels *via* the formation of Schiff Bases (thereafter refereed as Chem Condition).

1.11.2. Photocrosslinking between LAM-SH and LAM-C=C derivatives:

A 1:1 mass ratio of LAM-C=O (10% (w/v) in dPBS; pH=7.4) to LAM-SH (10%(w/v) in dPBS; pH=7.4) was used for obtaining hydrogels by thiol-ene coupling in the presence of photoinitiator (I2959) upon UV-light exposure (365 nm, 11.4 mW/cm^{-2}) (thereafter refereed as Photo Condition).

1.11.3. Crosslinking promoted by coordination bonds between Fe^{3+} ions and the catechol groups on LAM-DOPA derivative:

LAM-DOPA biopolymer (30% (w/v) in water) was mixed with Fe^{3+} ions in a 1:3 molar ratio. Afterwards, a strong base, namely NaOH, was added to promote the formation of coordination bonds between the Fe^{3+} ions and the catechol groups on the LAM-DOPA backbone.

1.12. Rheological measurements:

Oscillatory rheology measurements were performed at 37 ºC keeping the normal force constant at 0 N on a Kinexus Lab+ (Malvern Panalytical) rheometer fitted with a parallel plate configuration. The evolution of storage modulus (G') and loss modulus (G'') was recorder over time to infer about the gelation kinetics. To this end, the hydrogel polymeric precursor solution was mixed and immediately after transferred to the pre-heated lower plate (37 ºC) and the

measurement started. A frequency of 1 Hz and a shear strain of 1% was applied to minimize interference with the gelation process and keep the measurement within the linear viscoelastic region. The gelation point was determined by the cross-over between the G' and G" moduli. Regarding the hydrogel formed by combining LAM-SH with LAM-C=C polymers, the photocrosslinking process was followed by connecting the rheometer to a UV curing system.

1.13. Hydrogel cytotoxicity:

The cytotoxicity of the produced hydrogels was assessed by performing an extract test according to the guidelines described in ISO 10993-5 (2009). Briefly, an immortalized mouse lung fibroblast cell line (L929, European Collection of Cell Cultures) was cultured in complete DMEM medium supplemented with 3.7 g/L sodium bicarbonate, 10% FBS and 1% penicillin-streptomycin (pH was adjusted to 7.4). L929 cells were grown in 150 cm^2 tissue culture flasks and incubated at 37 ºC in a humidified air atmosphere containing 5% CO_2. The culture medium was refreshed every 3 days. Upon reaching 90% of confluence, L929 cells were washed with PBS and chemically detached from tissue culture flasks using 0.05% Tryple Express solution for approximately 5 min, in a humidified air atmosphere of 5% CO_2 at 37 ºC. Later, fresh medium was added to inactivate the Tryple Express effect and the cells were centrifuged at 200g for 3 min. Then, the medium was decanted followed by cells re-suspension in complete culture medium at density of 1×10^5 cells per mL. 100 µL of the former cell suspension were dispensed in each well of a 96 well-culture plate and incubated at 37 ºC in a humidified air atmosphere with 5% CO_2. Simultaneously, each of the produced hydrogels (≈0.4 mg in 2 mL of DMEM) were incubated at 37 ºC and 60 rpm to extract possible leachables for either 24 h (Day 1) or 72 h (Day3). Hydrogels were prepared in a similar fashion as described above but using filtered sterile polymeric solutions instead. After 24 h, the cell culture medium in 96-well plates was replaced by 100 µL of the different extraction fluids and left in incubation for 24 h. Both positive (C+; latex rubber) and negative (C-; tissue culture polystyrene plates (TCPS)) controls were extracted under the same conditions. The extract cytotoxicity was quantified by measuring the activity of mitochondrial dehydrogenases of viable cells. This parameter was assessed using a tetrazolium salt, namely MTS,[8] which yields a water-soluble brown formazan product in the presence of mitochondrial dehydrogenase enzymes. The amount of formazan generated is directly proportional to the total mitochondria activity per cell. Briefly, the extracts were removed and 100 µL of 1:5 MTS/DMEM (without phenol red) was added to each well containing L929 cells. The samples were cultured for 3 h at 37 ºC in dark. After that, the absorbance was measured at a wavelength of 490 nm using a microplate reader (Sinergy HT, BioTek). Experiments were performed in triplicate.

1.14. Statistical Analysis:

The level of significant differences between the average MTS values for each condition was assessed using one-way ANOVA (SPSS software) with Tukey's post hoc test, after verifying homogeneity of variances and normality distribution criteria. The homogeneity of variances

criterion was tested using Levene's test, while the Shapiro–Wilk test was used to study the data normality. P-values lower than 0.05 were considered statistically significant. The results are presented as mean ± standard deviation.

2. Supplementary Data

2.1. Tuning the SD of LAM-(C=C) derivative

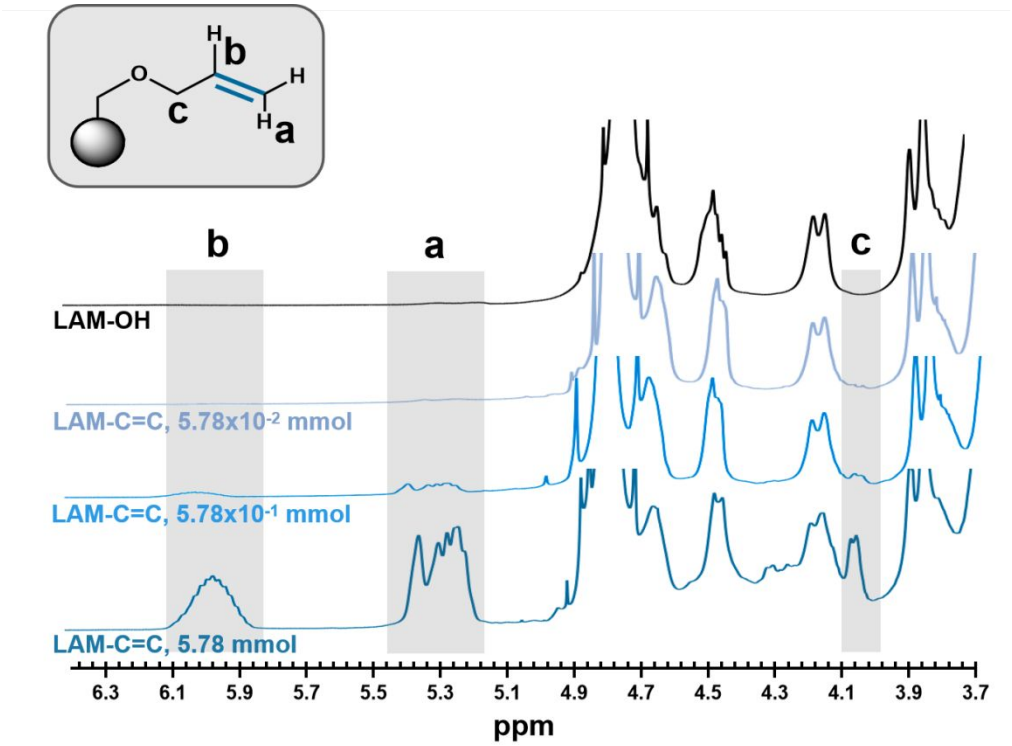

Figure S1. ^{1}H-NMR spectra of LAM-(C=C), in D_2O, with distinct SD values obtained by controlling the amount of allyl bromide added to the reaction mixture. As expected, higher amounts of allyl bromide resulted in higher SD values.

By tuning the stoichiometric ratio of allyl bromide to hydroxyl groups of LAM-OH, the incorporation of allyl moieties in laminarin monomers can be precisely controlled, yielding allyl-functionalized LAM-OH (LAM-C=C) with distinct SD. Interestingly, the intensity of the peaks labeled in Figure S1 as *a, b* and *c* increased with an increasing allyl bromide content. The molar amount of added allyl bromide and the respective ^{1}H-NMR spectrum can be observed in Figure S1.

2.2. Removal of acetyl groups from the intermediate compound of the LAM-SH

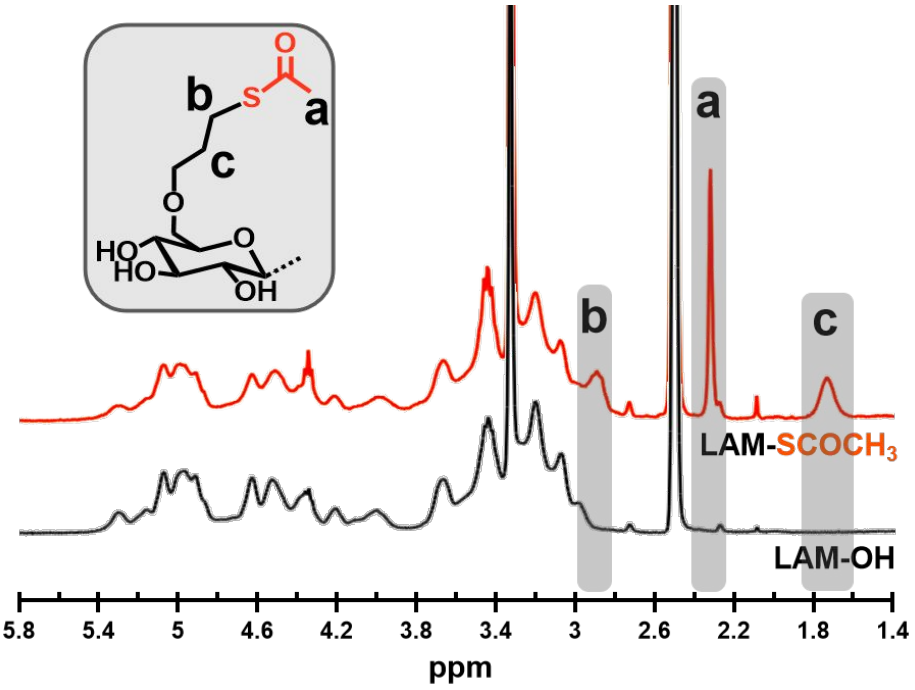

Figure S2. ^{1}H-NMR spectra of LAM-OH polymer and the intermediate compound obtained during LAM-SH synthesis via Michael-addition (LAM-$SCOCH_3$; reaction iv in Figure 1B). Both spectra were acquired using DMSO-d_6 as solvent since LAM-$SCOCH_3$ intermediate is not water-soluble.

Acetyl groups were removed from the intermediate of LAM-SH polymer (Figure 1B; reaction iv; LAM-$SCOCH_3$) after dissolving this compound in an aqueous solution containing NaOH, under a N_2 atmosphere. Visually, it was noted that 1 h after adding the former strong base, the solution became completely clear and homogeneous, suggesting the successful conversion of –$SCOCH_3$ (non-water soluble) into –SH free (water soluble) groups. The complete deprotection of the thiol moiety during hydrolysis can also be confirmed by the disappearance of the acetyl group in the ^{1}H-NMR spectrum of LAM-SH (Figure 2A) when comparing with Figure S2.

2.3. FTIR spectroscopy of LAM-OH, LAM-C=C, LAM-NH_2 and LAM-COOH and colorimetric assays

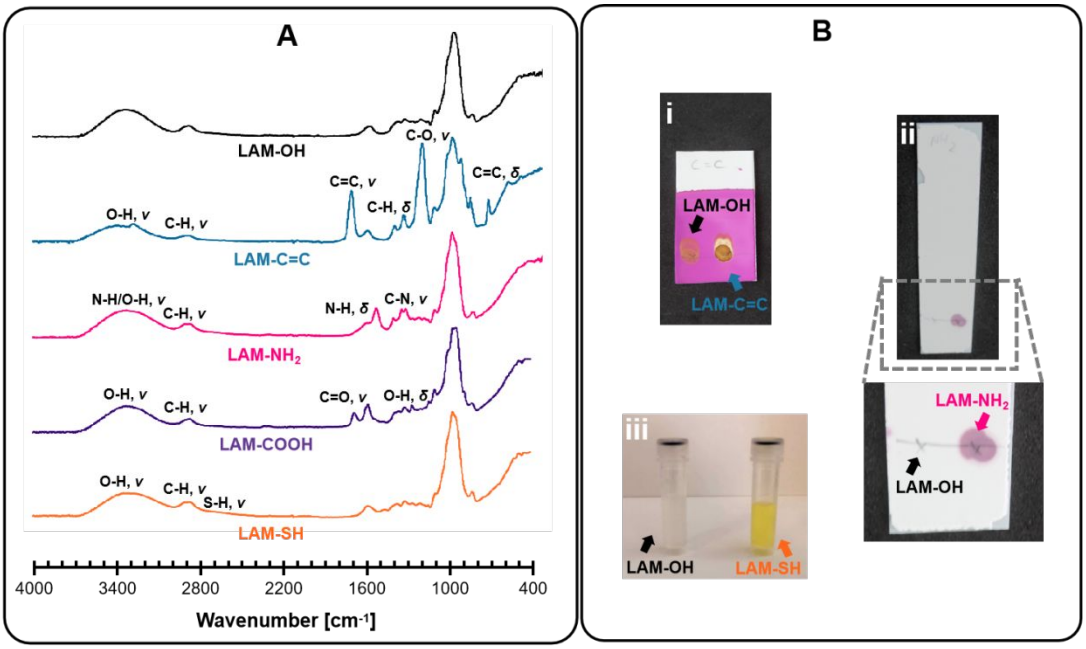

Figure S3. Chemical characterization of the synthesized biopolymers. **A)** FTIR spectra of the native LAM-OH polymer and its derivatives as well as the corresponding peak assignment.[9-12] **B)** Colorimetric assays to detect the presence of the following chemical moeities: i) allyl (potassium permanganate); amine (ninhydrin test) ; iii) thiol (ellman's assay).

2.4. Detailed characterization of LAM-C=O derivative by ^{1}H-NMR, ^{13}C-NMR and FTIR spectroscopy

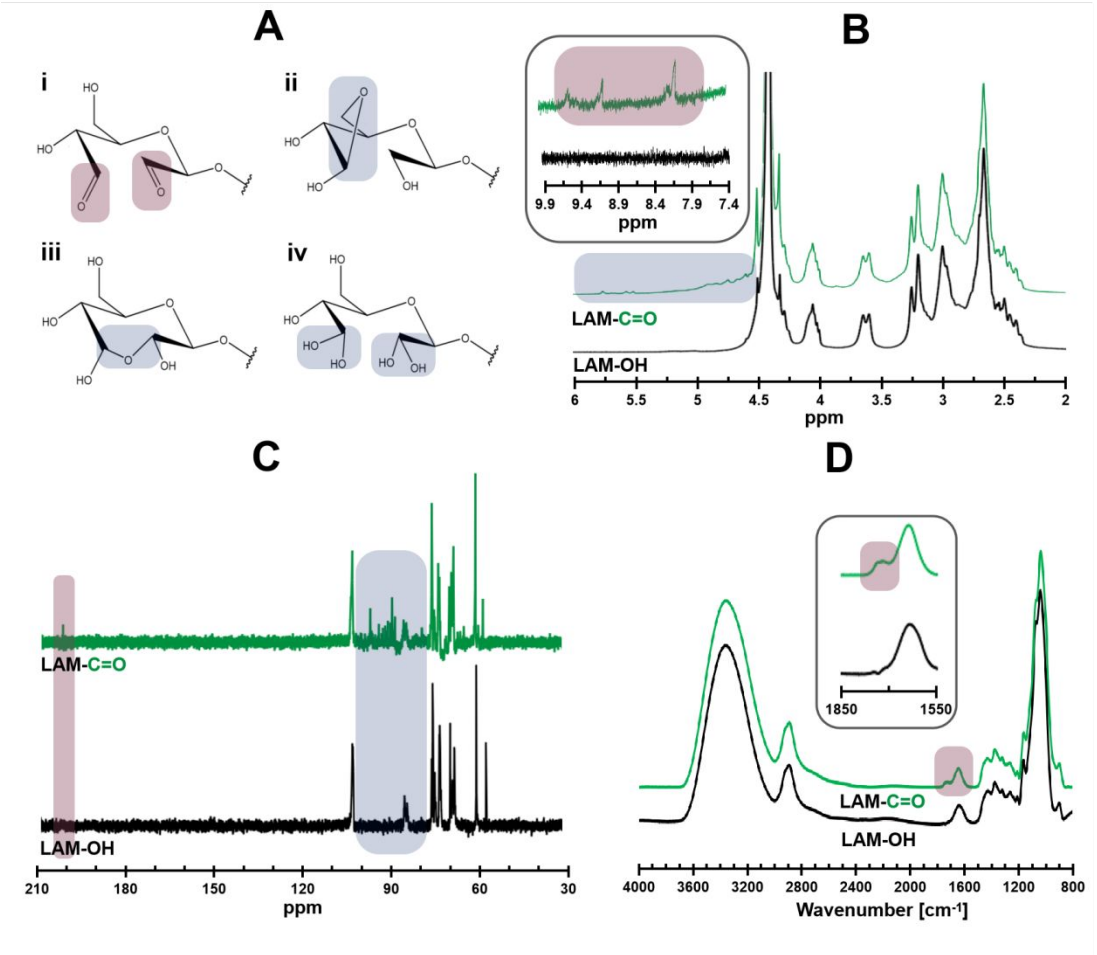

Figure S4. A) Examples of possible structures of LAM-C=O derivative as result of the oxidation process: i) free aldehyde (highlighted in blue); ii) intramolecular hemiacetal (highlighted in violet); 3) hemialdol (highlighted in violet); 4) hydrated aldehydes (highlighted in violet). **B)** ^{1}H-NMR, **C)** ^{13}C-NMR and **D)** ATR-FTIR spectra of native LAM-OH vs LAM-C=O derivative.

3. References:

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