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Functionalization of casein and its use for preparing self-crosslinking protein-based materials

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Supplementary Note 1 - Materials

Technical grade casein from bovine milk (Sigma), adipic acid hydrazide (ADH, Sigma), levulinic acid 98% (Sigma), *N*-hydroxysuccinimide 98% (Sigma), *N,N'*-Dicyclohexylcarbodiimide 99% (Sigma), *o*-phthalaldehyde (Merck), DMSO-d_6 (98%) (Sigma Aldrich), dimethylformamide (DMF, Cicarelli, stored over molecular sieves), sodium tetraborate (BioPack), 2-mercaptoethanol (Cicarelli), methyl methacrylate (MMA) and butyl acrylate (BA) monomers containing traces of mono methyl ether hydro-quinone as a polymerization inhibitor (Aldrich), potassium persulfate (KPS, $K_2S_2O_8$, Mallinckrodt), and sodium bicarbonate ($NaHCO_3$, Cicarelli) were used. All reagents were used as received without further purification. Distilled and deionized water was used throughout this work.

Supplementary Note 2 - Synthesis of levulinic acid NHS-ester

Levulinic acid NHS-ester was obtained following a reported method \cite{Lee1999}, with some modifications. Briefly, levulinic acid (0.580 g, 0.510 mL, 5.0 mmol) was dissolved in dry DMF (5 mL), under an inert atmosphere. *N*-hydroxysuccinimide (0.600 g, 5.2 mmol) and DCC (1.050 g, 5.1 mmol) were added and the solution was stirred overnight at room temperature. The reaction mixture was filtered and DMF was eliminated by distillation under reduced pressure. The residue was washed with ethyl ether affording the crude product. Crystallization from ethyl acetate afforded pure LA-NHS ester as yellowish solid (0.850 g, 80%). Spectral data (Figure S1) agree with the bibliography.[1]

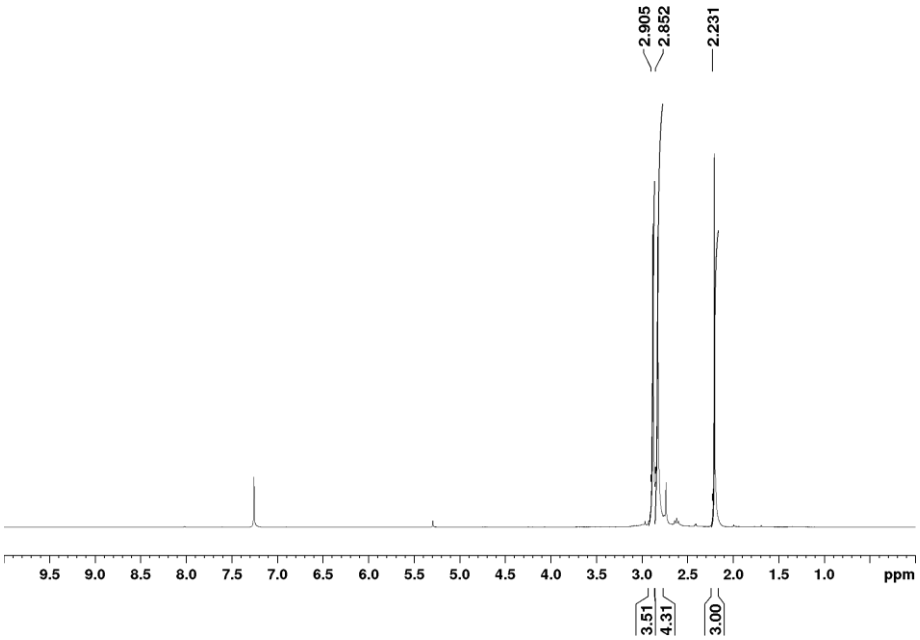

Figure S1. ^{1}H NMR Levulinic acid NHS-ester in $CDCl_3$ (300 MHz): 2.23 (s, 3H); 2.85 (bs, 4H); 2.91 (bs, 4H).

[1] J. H. Lee, T. J. Baker, L. K. Mahal, J. Zabner, C. R. Bertozzi, D. F. Wiemer, M. J. Welsh, *Journal of Biological Chemistry* **1999**, 274, 31 21878.

Supplementary Note 3 - Functionalization of casein

Pristine casein was dissolved in phosphate buffer (pH=8), while LA-NHS ester was dissolved in DMF. The reaction proceeded at room temperature under magnetic stirring for 24 h. Once the reaction was complete, the modified casein (M-Cas) was precipitated reducing the pH (pH=4) by the addition of HCl 1M. Then, the modified protein was washed with water. Next, after centrifugation and lyophilization, M-Cas was obtained as a powder.

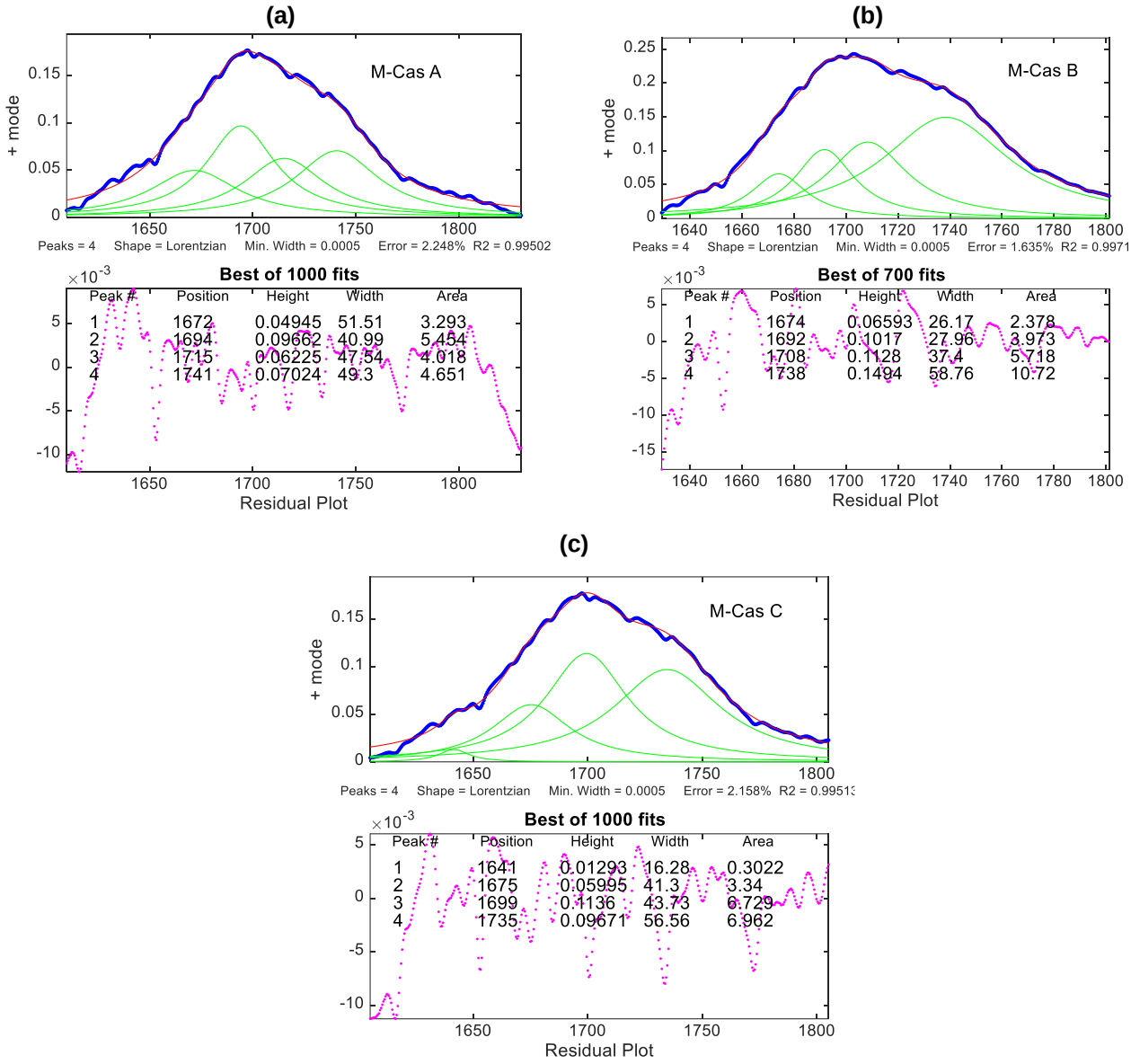

Figure S2. Deconvolution of FTIR signal at 1700 cm^{-1} for M-Cas A, M-Cas B, and M-Cas C powder samples (a, b, c, respectively). The deconvoluted peak and its components are shown at the top, while the constituents' peaks position, height, width, area, and fit error (pink dots) are shown at the bottom.

Table S1. Heights and height ratios of the FTIR signals from peak deconvolution of Fig. S1, corresponding to ketone carbonyl and reference.

	M-Cas A		*M-Cas B*		*M-Cas C*	
	λ (cm^{-1})	*Height*	*λ (cm^{-1})*	*Height*	*λ (cm^{-1})*	*Height*
Ketone carbonyl (-CH_2COCH_3)	1741	0.0702	1738	0.1494	1735	0.0967
Amide II band	1547	0.1896	1564	0.0900	1563	0.0540
Height ratio	0.3703		1.6600		1.7907	

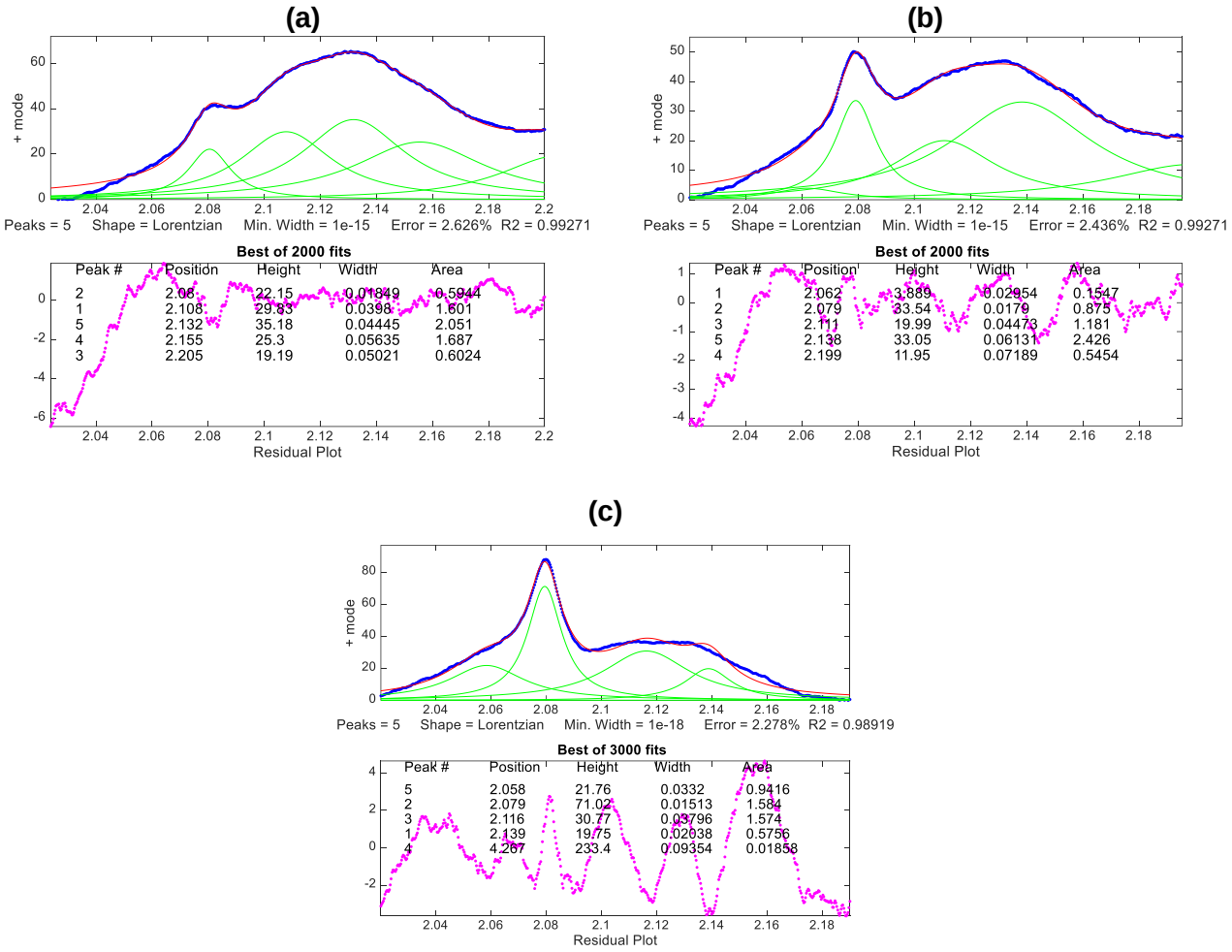

Figure S3. Deconvolution of ^{1}H-NMR signal LA- I(s) for M-Cas A, M-Cas B, and M-Cas C samples (a, b, c, respectively). The deconvoluted peak and its components are shown at the top, while the constituents' peaks position, height, width, area, and fit error (pink dots) are shown at the bottom.

Table S2. Heights and height ratios of the H-NMR signals from peak deconvolution of Fig. S3, corresponding to signal I(s) of levulinic acid and reference.

	M-Cas A		*M-Cas B*		*M-Cas C*	
	δ [ppm]	*Area*	*δ [ppm]*	*Area*	*δ [ppm]*	*Area*
LA - I (s)	2.080	0.5944	2.079	0.875	2.079	1.5840
Reference signal	0.738	34.7266	0.738	13.3621	0.737	35.3089
Height/area ratio	0.0171		0.0655		0.04486	

Supplementary Note 4 - Model reaction

The reaction between ADH and MeLev was performed in methanol followed by the addition of acetic acid (catalytic). The mixture was stirred for 24 hours, at room temperature. After that, the solvent was removed under vacuum pressure, and a white solid was obtained. Conditions: Levulinic acid methyl ester (0.0895 g, 0.69 mmol) and adipic acid hydrazide (0.0669 mg, 0.219 mmol) were dissolved in dry MeOH (5 mL). One drop of acetic acid was added and the mixture was stirred at room temperature for 24 hours. After that, the solvent was evaporated and the crude reaction was dry under vacuum conditions. The mixture was analyzed by FTIR (Figure S5) and NMR (Figure S6), without any purification step.

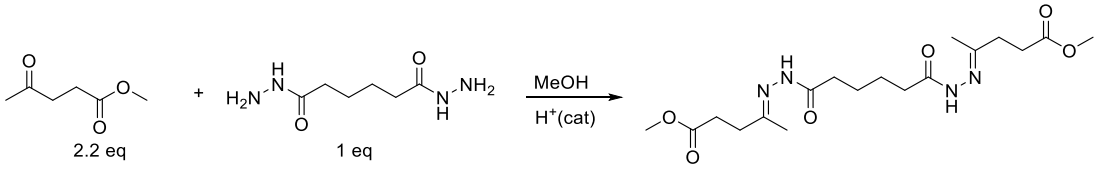

Figure S4 Condensation reaction between MeLev and ADH.

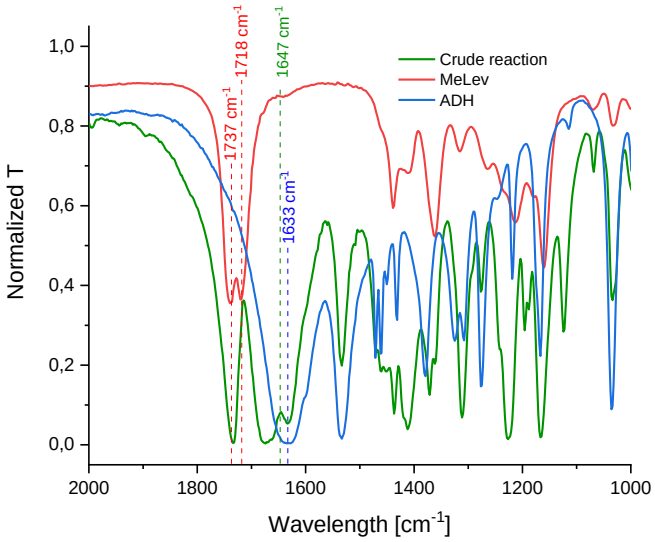

Figure S5. FTIR spectra of MeLev, ADH, and their condensation product.

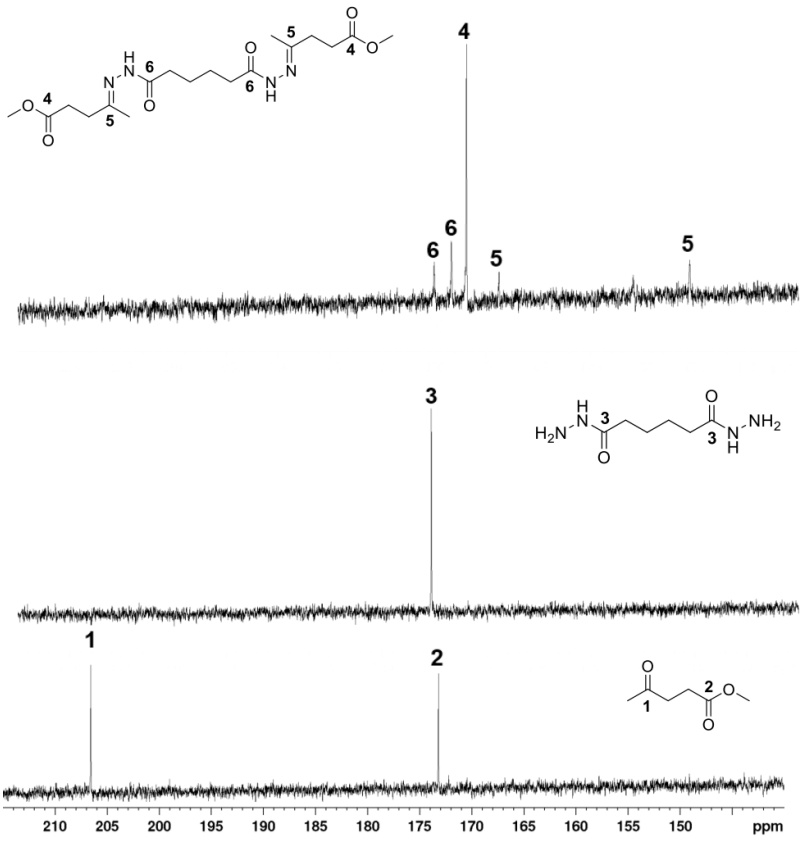

Figure S6. ^{13}C NMR (DMSO-*d6)* spectra of MeLev, ADH, and condensation product. In spectrum of condensation product, it is observed a full depleting of carbonyl (1) from ketone, whereas four new peaks are detected in the region 175-170 ppm (amide bond 6) and in region 165-150 ppm (imide bond 5).

Supplementary Note 5 - Synthesis and characterization of acrylic/protein latexes and film formation

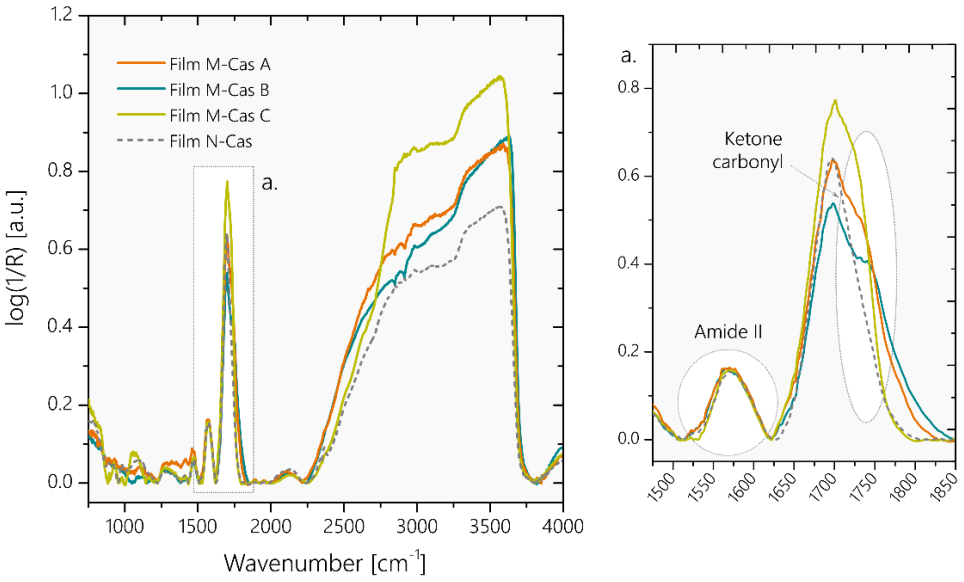

Figure S7. FTIR spectra of films of M-Cas and film of P-Cas formulated with ADH as a function of wavenumber, and (a) zoom in the range of ketone carbonyl characteristic signal.

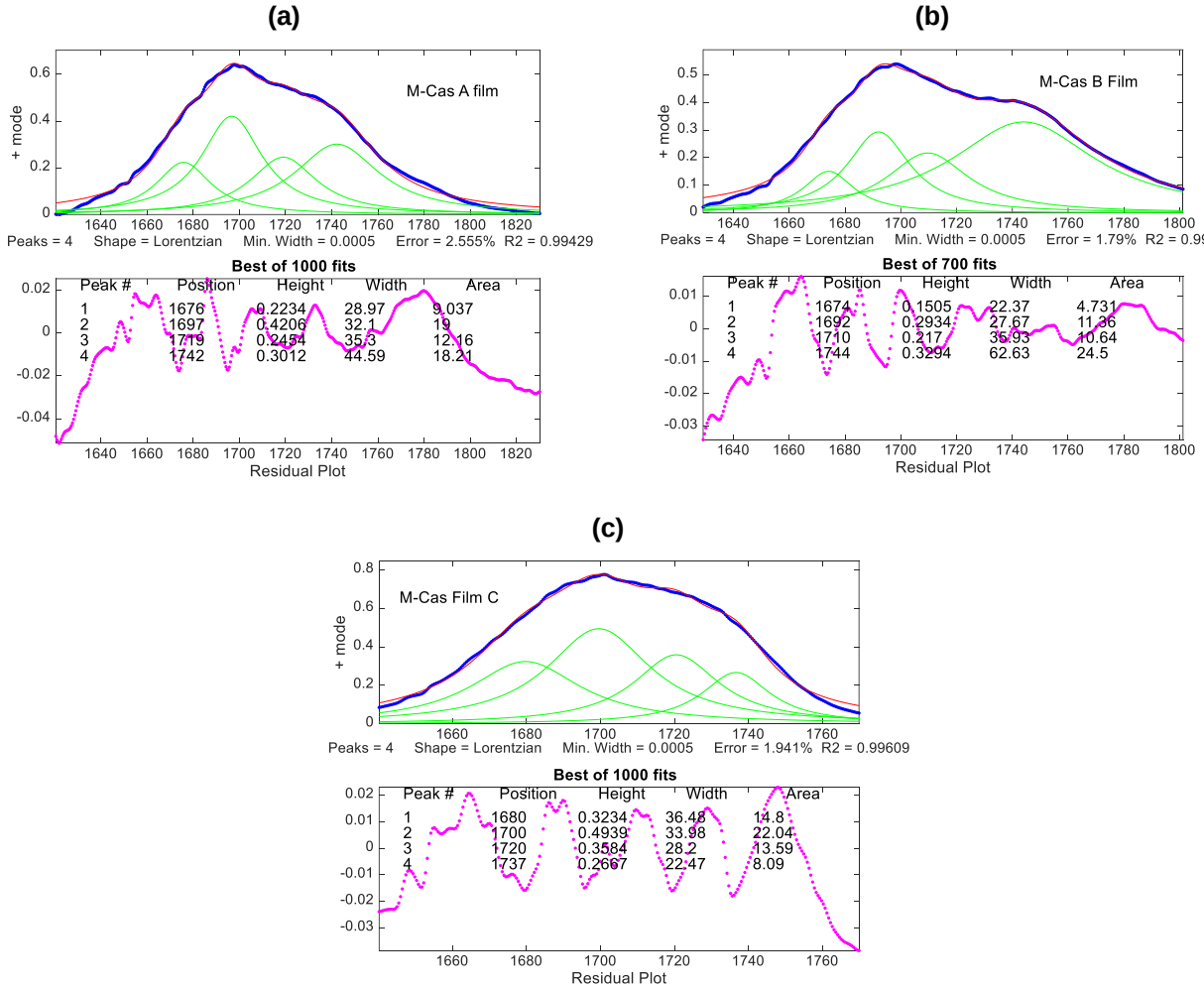

Figure S8. Deconvolution of FTIR signal at 1700 cm^{-1} for M-Cas A, M-Cas B, and M-Cas C film samples (a, b, c, respectively). The deconvoluted peak and its components are shown at the top, while the constituents' peaks position, height, width, area, and fit error (pink dots) are shown at the bottom.

Table S3. Heights and height ratios of the FTIR signals from peak deconvolution of Fig. S11, corresponding to ketone carbonyl and reference.

	Film M-Cas A		*Film M-Cas B*		*Film M-Cas C*	
	λ (cm^{-1})	*Height*	*λ (cm^{-1})*	*Height*	*λ (cm^{-1})*	*Height*
Ketone carbonyl (-CH_2COCH_3)	1742	0.301	1744	0.329	1737	0.267
Amide II band	1570	0.164	1566	0.162	1571	0.158
Height ratio	1.835		2.031		1.690	

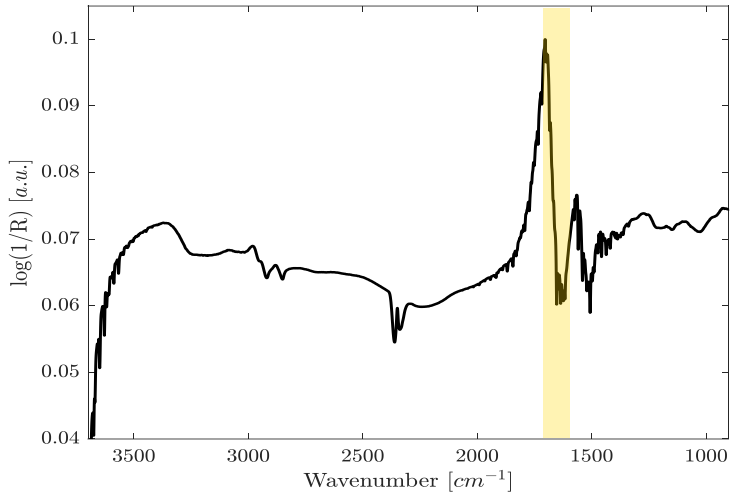

Figure S9. FTIR spectrum of pristine casein. The shadowed area indicates the region where the double bond C=N should appear.

Supplementary Note 6 - Elemental analysis

A CHN628 Series Elemental Analyzer (Leco) was used to perform the EA of the modified and pristine protein samples. The content of carbon (%C) and nitrogen (%N) was determined by duplicate. The C/N ratio of P-Cas was considered as a characteristic value of the protein and the calculations of Table **S4** were performed using equations 1 and 2. Equation 2 considers that the carbon contribution of LA groups added to the protein is 60 g/mol.

$$[added\ \%C] = \%C(M\text{-}Cas) - \%N(M\text{-}Cas)\frac{\%C(P\text{-}Cas)}{\%N(P\text{-}Cas)} \quad (1)$$

$$\frac{mol\ carbonyl\ group}{mol\ M\text{-}Cas} = [added\ \%C]\frac{mol\ LA}{60\ gC}M_w(P\text{-}Cas) \quad (2)$$

Table S4. Results of the elemental analysis for P-Cas and M-Cas samples.

	% average N	*% average C*	*added % C*	*mol FG / mol M-Cas*
P-Cas	13.5	47.1	-	-
M-Cas A	13.2	46.2	0.15	1.2
M-Cas B	12.9	46.5	1.49	6.1
M-Cas C	14.0	51.0	2.16	8.3

Supplementary Note 7 - Degree of protein modification by OPA method

The OPA reagent was prepared essentially as described by Goodno *et al.*[2] The OPA solution was prepared by diluting the following reagents to a final volume of 50 ml with water: 25 ml of 100 mM sodium tetraborate; 2.5 ml of 20% (wt/wt) SDS; 40 mg of OPA (dissolved in 1 ml of methanol); and 100 μL of 2-mercaptoethanol. Protein

[2] C. C. Goodno, H. E. Swaisgood, G. L. Catignani, *Analytical Biochemistry* **1981**, 115, 1 203

amino groups were determined by reacting 10 μL of distilled water containing 5 μg of protein with 3.0 ml of OPA reagent. The resulting solution was incubated for 2 min at room temperature before measuring the absorbance at 340 nm in a Shimadzu UV-2401 PC UV-VIS spectrophotometer. The grade of protein modification was estimated by a function of the reduction of amino group content.

Supplementary Note 8 - FTIR

The chemical structure changes of casein (powders) and films were investigated by attenuated total reflection (ATR) FTIR and Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) FTIR, respectively. To this effect, films were placed inside the ATR cell (Pike Technologies) and pressed toward the internal reflection element (ZnSe). The cell was mounted onto an ATR attachment (Pike Technologies) inside the sample compartment of the FTIR spectrometer (Thermo-Electron, is50 with a cryogenic MCT detector). The bench of the spectrometer was continuously purged with dried air (Parker Balston FTIR purge gas generator) to eliminate CO_2 and water vapor contributions to the spectra. The ATR FTIR spectra (100 scans) were recorded at a resolution of 4 cm^{-1}. The reference spectrum was collected with the empty ATR cell (250 scans).

Supplementary Note 9 - ^{1}H-NMR

^{1}H-NMR experiments were performed in a Bruker Avance II spectrometer (300 MHz) using the residual solvent signal as a reference (H_2O, 4.7 ppm). Chemical shifts are provided in δ units.

Supplementary Note 10 - Percentage of crosslinked protein

The amount of crosslinked casein was determined from equation 3 by correlating the quantity of casein in solution after immersion of the films in water with the quantity of initial casein in each film. For that purpose, the films (ca. 40-50 mg) were immersed in 3 mL of deionized water for 2h at room temperature. Afterward, the samples were centrifuged at 5000 rpm for 15 minutes, and the supernatants were separated and analyzed through UV absorbance spectroscopy. The free protein concentrations were obtained by combining the characteristic peak height at 280 nm with a calibration of P-Cas concentration. The calibration curve was proved to be consistent with different concentrations of M-Cas samples. The spectra and calibration curve can be found in Figure S10.

$$\%\ Crosslinked\ Cas = \left[1 - \frac{Free\ protein}{Loaded\ protein}\right] 100 \qquad (3)$$

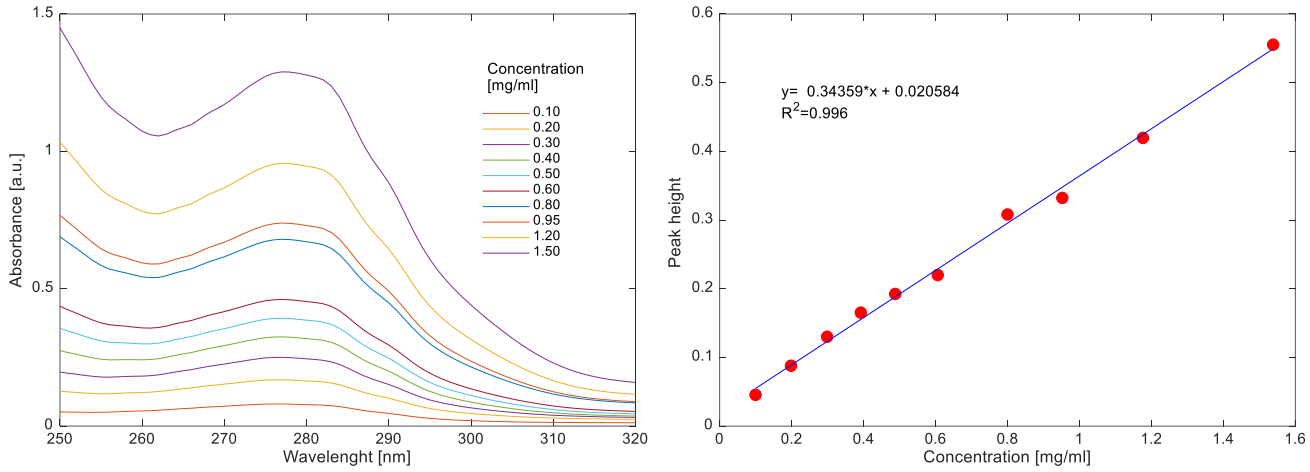

Figure S10. UV absorbance spectra of P-Cas solutions (left). After baseline correction, these spectra were employed to obtain the calibration curve UV peak height Vs. P-Cas concentration (right).

Supplementary Note 11 - Polymerization process

The casein with the highest degree of modification (M-Cas C) was used to obtain a modified casein-based latex (L2-M), containing 20% of protein (weight based on monomers) and 20 wt% of solids. The batch polymerization process was carried out in a 100 mL jacketed glass reactor equipped with a reflux condenser, a sampling device, a magnetic stirrer, and a nitrogen inlet. First, the M-Cas C was dissolved in a buffer solution of $NaHCO_3$ (0.175 % weight based on water phase, pH = 8), at 50 °C. The system was kept under stirring and nitrogen bubbling for 20 min, and then the acrylic monomers mixture (with a BA/MMA ratio of 60/40) was loaded into the reactor. Finally, the reactor temperature was adjusted until the desired reaction temperature (80 °C) was reached, and KPS (0.51 % weight based on monomers) was added as the initiator. A pristine casein-based latex (L1-P) was synthesized as a reference.

Supplementary Note 12 - Film formation

Before obtaining the polymer films, the latexes (L2-M w/ADH and L1-P w/ADH) were prepared by adding ADH (4.15 mol of ADH/mol of casein) as a crosslinking agent, keeping them under constant stirring to ensure their

complete homogenization. Then, polymer films were prepared by casting and drying the latexes in silicone molds at room temperature for a week, to obtain 1 mm thick films. In addition, ADH-free films were prepared for each latex (L2-M wo/ADH and L1-P wo/ADH, respectively).

Supplementary Note 13 - Minimum film formation temperature

The MFFT of the latexes was determined by an optical method[3] involving observation of the clarity of a cast film (120 μm thickness) on a large metal table. A temperature gradient was applied to the table, and the minimum temperature at which the film was judged to be clear was considered the MFFT value.

Supplementary Note 14 - Latex particles diameter

The diameters of latex particles were measured by dynamic light scattering using a Litesizer 500 of Anton Paar at a detection angle of 90° at 25 °C. The reported particles' diameters are the mean value of three measurements.

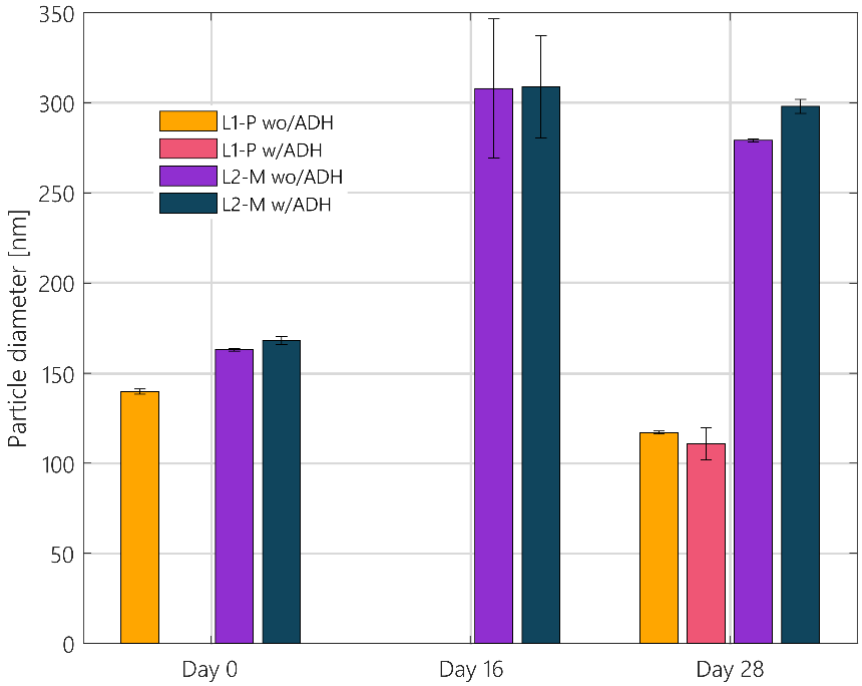

Figure S11. Particle diameter of L1-P and L2-M w/ and wo/ADH at day 0, 16 and 28 after latex's synthesis.

Supplementary Note 15 - Water resistance

Discs of the films (1 mm in thickness and 10 mm in diameter) were immersed in distilled water at room temperature. Each disc was removed from water at regular time intervals, dried with absorbent paper, weighed, and immediately immersed again. The process was repeated for 200 minutes or until the film ruptured. The weight variation was calculated as the percentage of the water mass absorbed with respect to the mass of the initial dried film.

Supplementary Note 16 - Tensile tests

Polymer films were prepared according to ASTM D882 (cut with a dumbbell shape). The tensile test was carried out at an elongation rate of 25 mm/min, employing a universal testing machine (INSTRON 3344), at 25 °C and 50% relative humidity. Three specimens of each sample were tested and average values were reported.

[3] J. Keddie, *Materials Science and Engineering: R: Reports* **1997**, 21, 3 101.