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Highly Robust Hydrogels via a Fast, Simple and Cytocompatible Dual Crosslinking-based Process

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Supporting Information

Materials: Medium molecular weight chitosan (M_{MW}-CHI, 200-800 cP), methacrylic anhydride (MA, ≥92%), sodium tripolyphosphate (TPP, 85%), β-glycerolphosphate disodium salt hydrate BioUltra (β-GF, ≥ 99.0%), 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (I2959, 98%), dialysis tubing cellulose membrane (MWCO 14,000) and phosphate buffer saline (PBS) were from Sigma-Aldrich (U.S.A.). Low molecular weight chitosan 95/20 (L_{MW}-CHI, 16-30 mPa.s) was from Heppe Medical Chitosan GmbH (Germany). Acetic acid (Glacial) and sodium bicarbonate were supplied from VWR (Belgium). Deuterium oxide (D_2O) was purchased from Laborspirit (Portugal). TrypLE Express, fetal bovine serum (FBS), penicillin/streptomycin solution, calcein-AM, propidium iodide (PI) and complete α-MEM culture medium were from Alfagene (Portugal). All materials were used as received. Unless otherwise stated, water purified in an 18 MΩ cm MilliQ Plus water system was used throughout.

S1. L_{MW}-MACHI polymer synthesis and characterization.

L_{MW}-CHI was modified into its photocrosslinkable derivative, L_{MW}-MACHI, by incorporating methacrylic groups on its backbone. [1] Briefly, L_{MW}-CHI (3% (w/v)) was dissolved in 2% (v/v) acetic acid overnight at RT with constant stirring. Once dissolved, MA (at 1 molar equivalents per chitosan repeat unit) was added

dropwise and left in incubation for 5h at RT in dark. The mixture was, then, dialyzed against distilled water for 5 days, changing the water twice a day. Finally, the L_{MW}-MACHI solution was freeze-dried and stored at -20 ºC until use.

^{1}H–Nuclear Magnetic Resonance (^{1}H-NMR, Bruker Avance III, 300 MHz) was used to infer about the correct L_{MW}-CHI methacrylation and to estimate both the substitution (SD) and crosslinking (CD) degrees. To this end, 20 mg of L_{MW}-MACHI were dissolved in 1 mL of deuterium oxide containing 0.25 w/v % I2959 and placed inside a 5 mm diameter NMR test tube (Sigma-Aldrich). On the one hand, the SD was determined by the ratio between the average value of the area of the peaks at 5.7 and 5.4 ppm, [2] and the area at 3.0 ppm (H2 (GluN, glucosamine)). [3] On the other side, to determine the CD degree, the L_{MW}-MACHI solution inside the NMR tubes was photocrosslinked using UV-light (365 nm, 11.4 W/cm^2) for different exposition times ranging from 30 to 900 s. As a result of the UV-light exposure, the photoinitiator (I2959) forms free radicals needed to initiate the polymerization process between the pendant vinyl groups of the L_{MW}-MACHI. As the reaction proceeds, these vinyl groups are consumed. Accordingly, the crosslinking degree was determined as the decrease in the area under the curve of the peaks corresponding to the two hydrogens adjacent to C=C double bond (located at 5.7 and 5.4 ppm) relative to the H2 peak at 3 ppm (GluN). [4] Chemical shifts were expressed in ppm (δ units). Data were processed using the MestReNova software (MestreLab, Spain).

S2. Production and characterization of CHI DN hydrogels.

CHI DN hydrogels were synthesized by first dissolving M_{MW}-CHI in a 2% (v/v) acetic acid solution followed by its neutralization (pH 6.4) using a weak base, β-

GF. Afterwards, the former solution was mixed with L_{MW}-MACHI polymer and a cytocompatible photoinitiator, I2959 (0.25% (w/v)). Different amounts of both CHI derivatives were used by keeping constant the total polymer concentration in 2% (w/v). The resultant polymeric precursor solution was poured into cylindrical PDMS molds with a diameter of 6 mm and height of 4 mm and, then, harden into a hydrogel by employing two different crosslinking processes. In a first step, the L_{MW}-MACHI polymer was photocrosslinked through the reaction between its methacrylic groups, which occurs upon UV-light (365 nm, 11.4 W/cm^2) exposure in the presence of I2959. Various UV-light exposure times were tested in order to assess the minimum value which allowed both high mechanical properties and cell encapsulation with high viability rates. On the other hand, in the second step, the M_{MW}-CHI polymer was ionically crosslinked by immersing the resultant hydrogel in a 2% (w/v) TPP aqueous solution to prepare the soft and ductile structure. Then, the hydrogels were rinsed in deionized water to remove any TPP ion excess and guarantee a homogeneous structure.

The mechanical characterization was carried out on the basis of compression tests employing a Universal Mechanical Testing Machine (INSTRON 5540) equipped with a load cell of 1 kN. To this end, both unidirectional and cyclic compression assays were performed at room temperature on as-prepared cylindrical hydrated hydrogels specimens with a diameter of 6 mm and height of 4 mm. Afterwards, the sample was set on the lower plate and compressed by moving the upper plate at a compression rate of 1 mm per min. It is noted that a pre-load of 0.1 N was applied to ensure the correct contact of hydrogel with the equipment plates. An exception was made for the L_{MW}-MACHI and M_{MW}-CHI single-network hydrogels were a pre-load of 0.01N was used instead. The

nominal stress was obtained by dividing the compressive load by the initial (uncompressed) cross-sectional area of the specimen. From the obtained stress-strain curves, several parameters were determined including compressive modulus (i.e. the average slope of the stress-strain curve in the initial linear region from 0 to 10% strain), fracture stress and strain (i.e. the stress or strain point at which the hydrogel broke or the maximum stress/strain value reached since some of the developed hydrogels did not fracture until a strain of 90%) and energy dissipation (i.e. the area between the loading and unloading curves). All these tests allowed the assessing of the resulting mechanical properties of CHI DN hydrogels as well as to elect the optimal synthesis parameters.

S3. I2959 photoinitiator structure and properties

The photoinitiator used in this work was the 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (I2959), which is radical photoinitiator widely used for the UV curing of unsaturated monomers and prepolymers (Figure S1). This compound cytocompatibility was already assessed in several publications.[5]

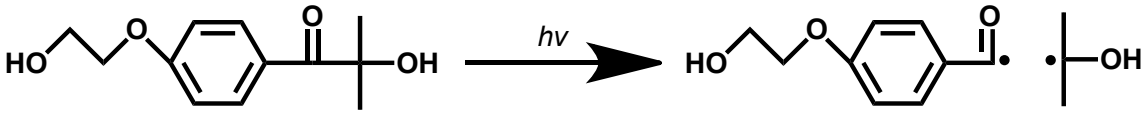

Figure S1. Chemical structure of the I2959 photoinitiator.

S4. Optimization of the crosslinking mechanism of the L_{MW}-MACHI network

L_{MW}-CHI was successfully modified into its photocrosslinkable derivative, L_{MW}-MACHI, through its reaction with MA. Figure S2A depicts ^{1}H-NMR spectra of L_{MW}-MACHI polymer as a function of the UV-light exposure time using D_2O as a solvent. Two new peaks appeared as a consequence of the chemical modification of L_{MW}-CHI at 5.7 and 5.4 ppm corresponding to the protons of the double bond (C=C, Figure S2A Ha and Hb). The MD was calculated by dividing the average

value of the area of the peaks at 5.7 and at 5.4 ppm by the area at 3.0 ppm (Figure S2A H2), which corresponds to the anomeric proton, and was found to be ca. 21%. These results confirm the successful incorporation of methacrylic groups into the L_{MW}-CHI molecular backbone structure, which are needed for the photocrosslinking process.

The NMR spectra was also used to infer about the CD of the L_{MW}-MACHI using different UV-light exposure times. During the photocrosslinking process, the peaks at 5.4 and 5.7 are consumed as can be observed on Figure S2A. Therefore, the obtained CD was defined as % of reacted methacrylic groups and are summarized on Figure S2B. As expected, an increase on the CD was obtained with the increase on the UV-light irradiation time. Taking into account the aforementioned outcomes, a crosslinking time of 150 s was selected for further studies as after this value the crosslinking degree remain almost constant. Moreover, since the final purpose of this device is to encapsulate cells, shorter crosslinking times are of extreme importance to not compromise cell viability.

Freeze dried L_{MW}-MACHI polymer (0.2 g) was first dissolved in 10 mL of distilled water containing 0.25% (w/v) of I2959. Then, the previous solution was transferred into silicon molds (diameter 6 mm and height 4 mm) to covalently crosslink L_{MW}-MACHI polymer upon UV-light (365 nm, 11.4 W/cm^2) exposure. Figure S2C displays the resultant hydrogels after different UV-light exposure times using an optical stereomicroscope.

The effect of the L_{MW}-MACHI crosslinking density on the mechanical properties was assessed by varying the time of crosslinking from 30 s to 900 s. The nominal elastic modulus (E), fracture stress (σ_{max}) and strain (ε_{max}) were determined in compression using a Universal Mechanical Testing Equipment (Model 5543,

INSTRON) equipped with BLUEHILL software. Figure S3A illustrates the increase of the *E* with the increase of the crosslinking time. On the other side, for higher crosslinking times the fracture strain is lower. Regarding the σ_{max}, the results suggest that independently of the UV-light irradiation time the maximum stress sustained by the MA-CHI hydrogels was *ca.* 0.1 MPa (Figure S3B). These results corroborate with other photocrosslinked hydrogels from natural-origin polymers. [2b, 6]

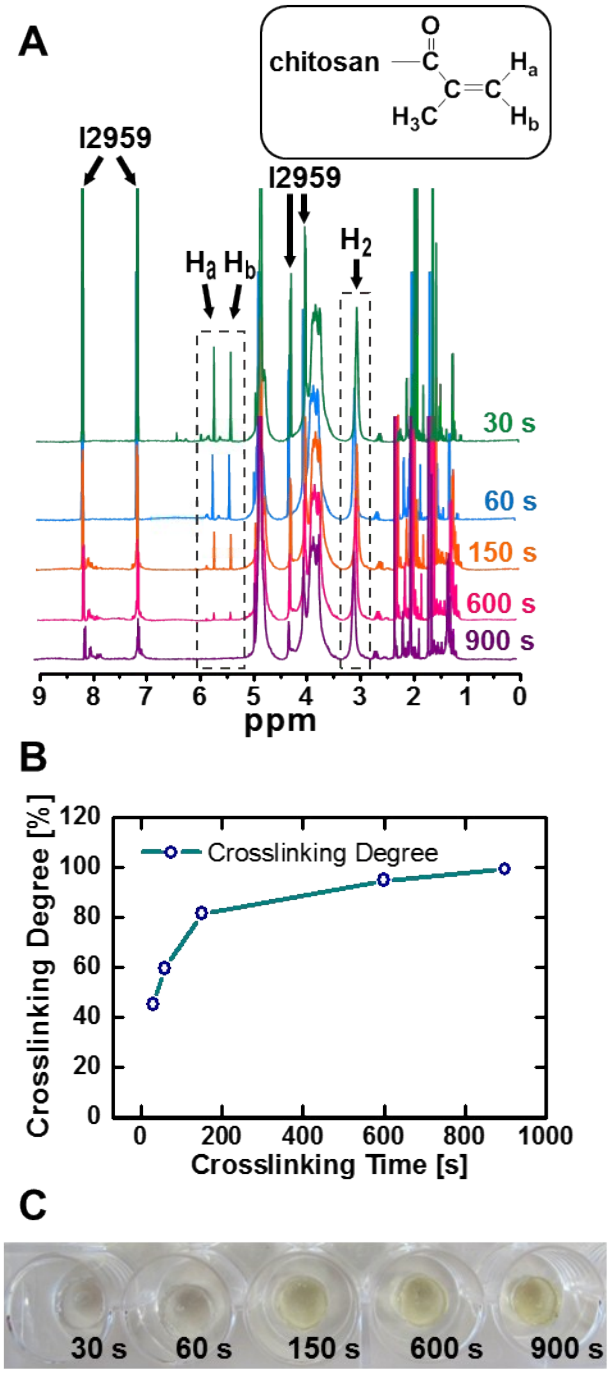

Figure S2. (A) ^{1}H NMR spectra of L_{MW}-MACHI after different crosslinking times. The box on top of the graphs shows the chemical structure of L_{MW}-MACHI. **(B)** Variation of the crosslinking degree (defined as % of reacted methacrylic groups) as a function of the UV-light exposure time. **(C)** Visual inspection of the obtained hydrogels after different UV-light exposure times using an optical stereomicroscope (TR500, VWR, USA) equipped with a digital camera (G12 Olympus, Canon, Japan).

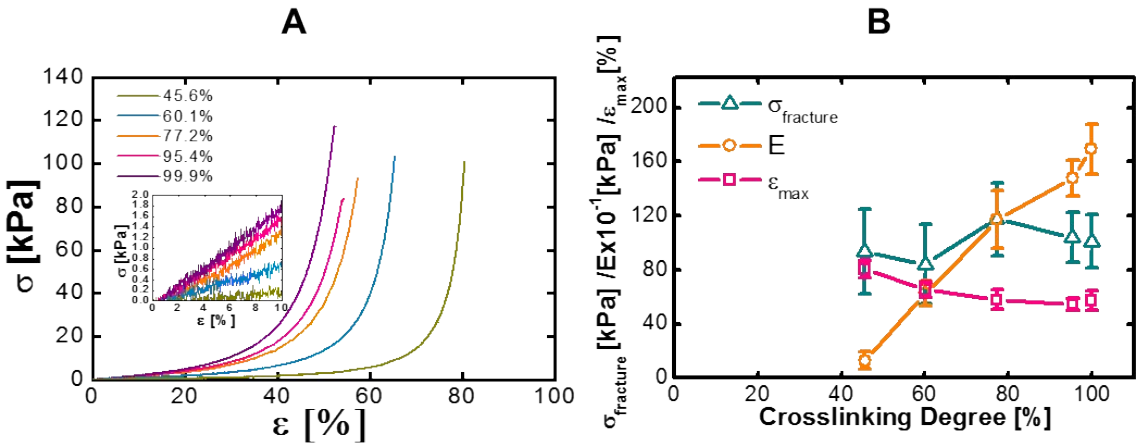

Figure S3. (A) Representative compressive stress-strain curves of L_{MW}-MACHI with different crosslinking degrees. The inset represents a zoom of the region used to determine the compressive modulus (*E*, from 0-10% of strain). **(B)** Variation of fracture stress ($\sigma_{fracture}$), maximum strain (ε_{max}) and *E* as a function of the crosslinking degree of L_{MW}-CHI polymer.

[redacted]

S5. Optimization of the ionic crosslinking of the M_{MW}-CHI network

M_{MW}-CHI was dissolved in acetic acid (2% (v/v), pH = 4.0) followed by the addition of a weak base, β-GF, to achieve a pH equal to 6.4. The resultant solution was transferred to a dialysis membrane and placed into a TPP (2% (w/v)) aqueous solution, resulting in the formation of ionic crosslinks between the positively charged amine groups present on the M_{MW}-CHI backbone and the negatively charged TPP ions. Afterwards, the dialysis membranes were sliced to yield cylinder-shaped hydrogels with a diameter of 6 mm and a height of 4 mm using a biopsy punch. To figure out the time needed for the TPP ions to diffuse into the center of CHI hydrogels (Figure S4A), optical images were periodically collected using an optical stereomicroscope. As result of the formation of ionic crosslinks, the hydrogel structure loses its transparency as easily observed in Figure S4B. These results suggest that after 60 min the TPP ions reach the center of the hydrogel as proved by a homogeneous structure (Figure S4B) and the increase

on the overall amount of phosphorus in hydrogel network (Figure S4C). The presence of phosphorus in the initial structure (time of 0 min) can be explained by the presence of β-GF in the polymer precursor solution. This increase on the amount of phosphorus is easily seen on Figure S4D.

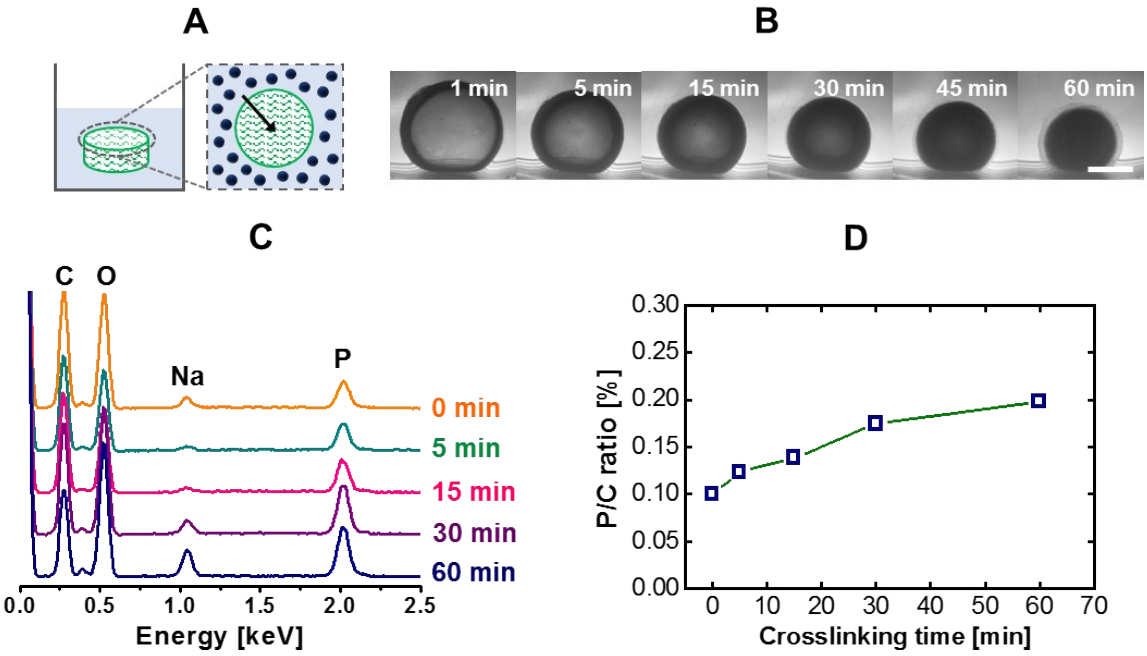

Figure S4. (A) Schematics of the TPP ions diffusion (blue circles) into the center of CHI DN hydrogels after their immersing in an aqueous solution containing these negatively-charged ions. **(B)** Visual inspection of the previous process using an optical stereomicroscope (TR500, VWR, USA) equipped with a digital camera (G12 Olympus, Canon, Japan). The CHI DN hydrogels were cylindrical with 6 mm diameter and 4 mm height. The scale bar corresponds to 3 mm. **(C)** EDS profiles obtained by fixing the analysis area and for different incubation times. **(D)** Phosphorus (P)/Carbon (C) ratio [%] in function of the incubation time in the TPP solution.

Above 3 % (w/v), it is difficult to dissolve M_{MW}-CHI in acetic acid and for concentrations lower than 1 % (w/v) is challenging to obtain a hydrogel structure. Therefore, two M_{MW}-CHI concentrations, 2 and 3 % (w/v), were tested and the results suggest that both CHI networks were able to withstand fracture strains of 90%, however they differ on the fracture stress: 2 % (w/v) M_{MW}-CHI reach values

of *ca.* 100 kPa while 3% (w/v) M_{MW}-CHI reach *ca.* 350 kPa (Figure S5). Moreover, the compressive modulus increased from 3.6 ± 2 to 5.2 ± 2 kPa when the M_{MW}-CHI concentration was increased from 2% to 3% (w/v). This enhancement on the mechanical properties for higher polymer concentrations was already observed in other hydrogel systems and have been ascribed to the increased viscosity of the solution with higher polymer concentration. [7] Although the 3% (w/v) M_{MW}-CHI network has improved mechanical performance, its viscosity is too high. Therefore, the lower M_{MW}-CHI polymer concentration was used in further studies.

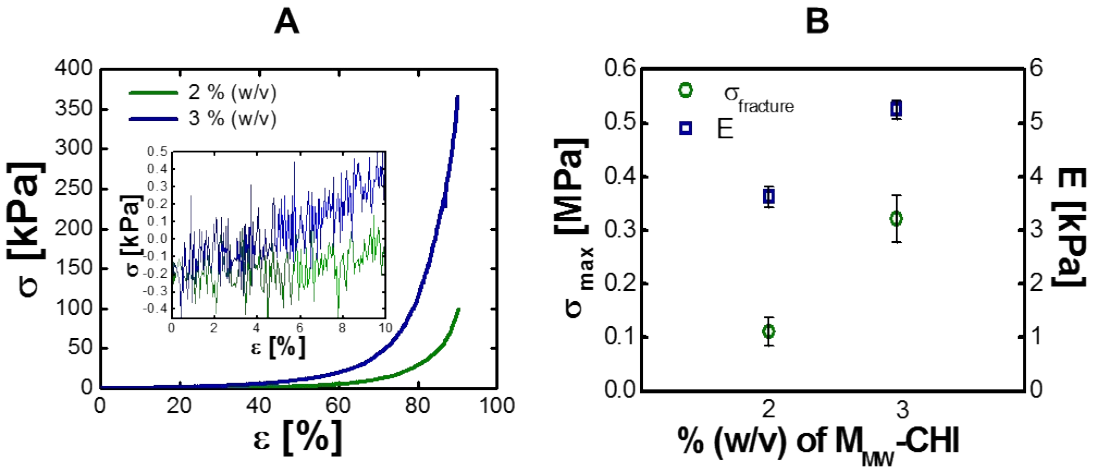

Figure S5. (A) Typical compressive stress-strain curves of M_{MW}-CHI using 2 different polymer concentrations, namely, 2% and 3% (w/v). The inset represents a zoom of the region used to determine the compressive modulus (*E*, from 0-10% of strain). **(B)** Variation of both maximum stress (σ_{max}) and *E* in function of the M_{MW}-CHI polymer concentration. It is worth notice that both structures were able to withstand a compressive strain of at least 90%.

S6. Study of the morphology of the CHI DN hydrogels

The CHI DN hydrogels were characterized for their morphology and elemental composition by Scanning Electron Microscopy (SEM, JSM-6010LV, JEOL, Japan) and Energy-Dispersive X-ray Spectroscopy (EDS), respectively. Briefly,

the CHI DN hydrogels were first dehydrated by immersing them in solutions with an increased amount of ethanol: 20, 50, 70, 90, 95 and 100% and, then, dried using supercritical carbon dioxide (Thar SFE1000-F, Thar Instruments). [8] To this end, the samples were loaded in a high pressure vessel heated at 37 °C and pressurized to 80 bar. In order to ensure the complete removal of the organic solvent, a carbon dioxide stream was passed through the vessel at constant rate for 1 h.

To prepare the samples for SEM analysis, the CHI DN hydrogels were fractured in liquid nitrogen to reveal the cross-sectional surface followed by their sputter-coating with gold. The images were acquired in high-vacuum by tracking the signal of secondary electrons, and employing an accelerating voltage of 10 kV and a working distance of 10 mm. On the other side, the EDS profile was obtained on a non-coated sample after drawing a line in the middle section of the CHI DN hydrogel.

It can be easily observed on Figure S6, that the CHI DN hydrogel presents both a homogeneous structure (A) and composition (B).

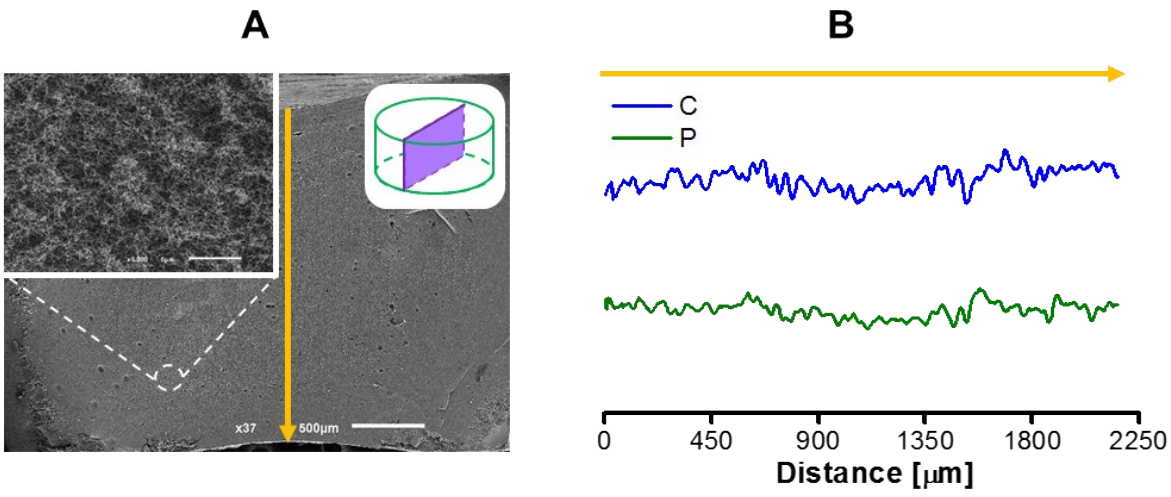

Figure S6. (A) SEM image of the CHI DN hydrogel cross-section after 1h of immersing in a TPP solution and successive washes to remove the excess of ions. The scale bar corresponds to 500 μm. The inset displays a zoom in of a certain region of the former SEM image. The scale bar corresponds to 5 μm. **(B)** Carbon (C) and phosphorus (P) distribution on CHI DN hydrogels along a line (represented with a yellow arrow).

S7. Study of the ability of the three types of hydrogels, L_{MW}-MACHI, M_{MW}-CHI and CHI DN hydrogels, to efficiently dissipate energy

All three types of CHI hydrogels produced on this work, namely CHI DN, L_{MW}-CHI and M_{MW}-CHI, were subjected to five consecutive loading/unloading cycles at 50% of strain to assess their ability to dissipate energy as shown on Figure S7. The first compressive cycle was performed on a fresh sample that has not been previously deformed and between cycles the sample was not allowed to rest. The dissipated energy during a cycle (U_{hist}) was estimated from the area between the loading and unloading curves following the equation 1:

$$U_{hist} = \int_{0\%}^{50\%} \sigma_{loading} d\varepsilon - \int_{0\%}^{50\%} \sigma_{unloading} d\varepsilon \quad \textbf{(1)}$$

The U_{hist} value of the first compressive cycle is 743 ± 68 kJ m^{-3}, 71 ± 9 kJ m^{-3} and 13 ± 1 kJ m^{-3} for the CHI DN, L_{MW}-MACHI and M_{MW}-CHI hydrogels.

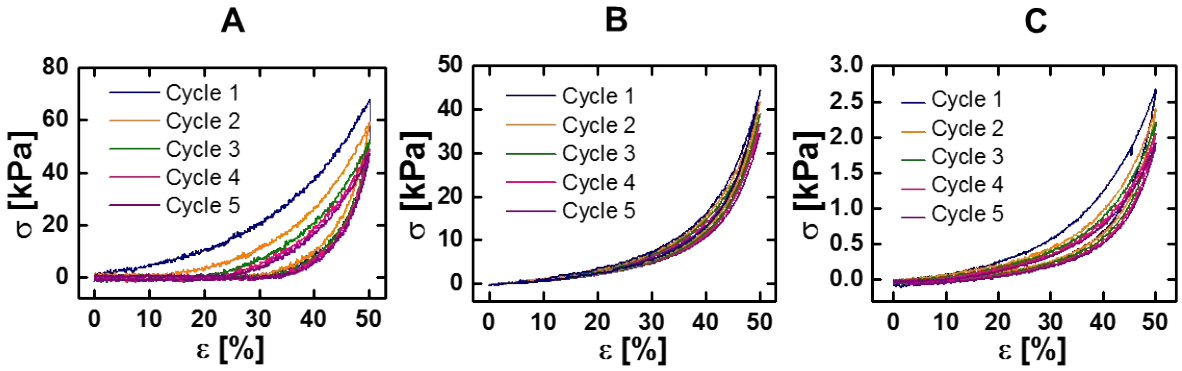

Figure S7. Representative cyclic strain/stress curves of CHI DN hydrogels **(A)**, L_{MW}-MACHI hydrogels **(B)** and M_{MW}-CHI **(C)** hydrogels after applying five successive compression cycles.

S8. Study of the water uptake by the CHI hydrogels with different amounts of M_{MW}-CHI polymer

The amount of water contained in the CHI DN hydrogels was defined as the weight ratio of swollen to dry sample. Dry hydrogels were obtained by drying in vacuum until a constant weight was reached. The CHI DN hydrogels with optimized mechanical properties exhibit a water content of *ca.* 93 ± 2 %.

Moreover, water uptake studies were performed on as-prepared CHI hydrogels with different amounts of M_{MW}-CHI polymer. Briefly, hydrogels were first weighed ($W_{initial}$) and, then, immersed in different liquid environments, namely distilled water (pH = 6.9), culture medium (α-MEM, pH = 7.4) and 0.01 M PBS (pH = 7.4) at 37ºC. After 24 h, the hydrogels were removed from the respective solution, blotted with tissue for removal of the excess of water, and weighed ($W_{equilibrium}$) again. The water uptake (WU) was measured through the following equation (2):

$$WU\ [\%] = \frac{Wequilibrium - Winitial}{Wequilibrium} \times 100 \quad \textbf{(2)}$$

The hydrogels were produced and immediately transferred to the three different solutions tested. With the exception of L_{MW}-MACHI network (0% of M_{MW}-CHI polymer), when the obtained hydrogels were immersed in the previous solutions their mass decreased as suggested on Figure S8. This fact can be ascribed to the presence of water in the hydrogel network, which may be expelled to reach the osmotic equilibrium. Additionally, this decrease on hydrogel weight can also be attributed to the release of non-bonded ions present on the hydrogel initial structure. For the solutions containing ions, the high degree of water release from the CHI hydrogels may be explained to the formation of more crosslinked

networks. This crosslinking mechanism results from the presence of ions in solution, which may lead to the establishment of new junction zones.

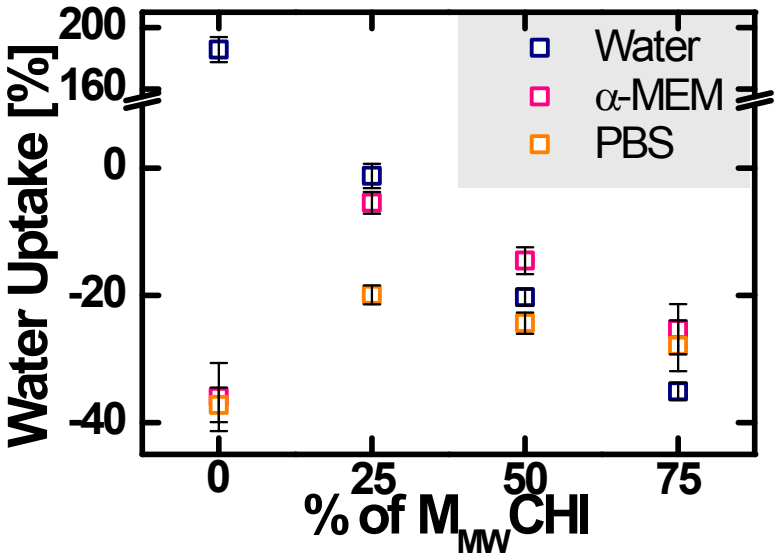

Figure S8. Water uptake by as-prepared CHI hydrogels as a function of the % of the amount of M_{MW}-CHI polymer in the final hydrogel structure using three different media: water, α-MEM and PBS at 37ºC.

S9. Study the effect of the M_{MW}-CHI polymer on the CHI DN hydrogel mechanical properties.

The methacrylation process of the L_{MW}-MACHI polymer was not complete (see section 3 for further information), meaning that this polymer bears free amino groups. Having this in mind, one could think about synthesizing CHI DN hydrogels by employing the two crosslinking mechanisms to only L_{MW}-MACHI polymer as this polymer has both positive amino groups to react with the TPP negative ions and methacrylic groups to create the covalent crosslinks. Therefore, the charge density of the main components involved on CHI DN hydrogel fabrication process was obtained by measuring their zeta potential (Zetasizer, Malvern, United Kingdom). Figure S9A shows that the L_{MW}-MACHI solution exhibits a less positive potential when comparing with the one of the M_{MW}-CHI; additionally, the TPP

solution presented a negative charge. For this reason, both CHI derivatives are able to react with TPP ions, but it is expected that M_{MW}-CHI polymer will be more reactive than L_{MW}-MACHI.

Figure S9B shows the typical compressive curves of L_{MW}-CHI hydrogels obtained using only a photocrosslinking mechanism (line in orange) and by combining the photocrosslinking mechanism with an ionic (line in purple). Although the dual-crosslinked L_{MW}-MACHI hydrogels have higher compressive modulus (*ca.* 35.0 ± 83 kPa) when comparing with the equivalent photocrosslinked structure (*ca.* 11.8 ± 36 kPa), their fracture stress and strain (*ca.* 41 ± 13 kPa and 28 ± 5 %) are much lower than the ones obtained using the DN-based methodology (*ca.* 19 ± 3 MPa and 90%). These results suggest that the improved mechanical behavior of the CHI DN hydrogels is the result of combining two CHI derivatives with contrasting molecular weights as well as two distinct crosslinks.

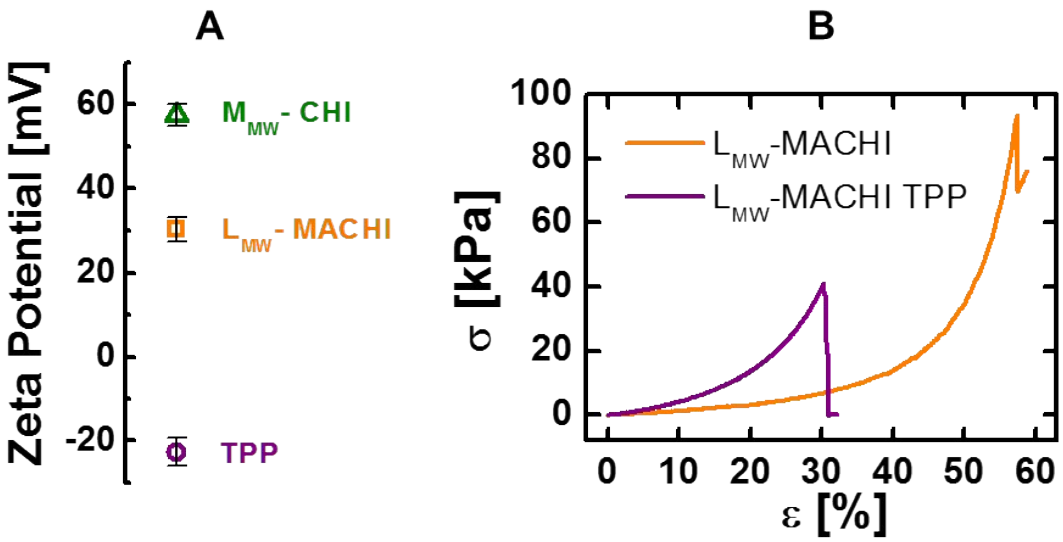

Figure S9. (A) Zeta potential of the L_{MW}-MACHI, TPP and M_{MW}-CHI solutions. Error bars correspond to the S.D. of 5 replicates. **(B)** Representative compressive stress-strain curves of L_{MW}-MACHI hydrogel before and after 1h of incubation in a TPP aqueous solution.

S10. Cells Encapsulation inside the CHI DN hydrogels.

Fibroblasts from an immortalized mouse lung fibroblast cell line (L929, European Collection of Cell Cultures) were grown in 150 cm^2 tissue culture flasks using a complete α-MEM medium supplemented with 3.7 gL^{-1} sodium bicarbonate, 10% FBS and 1% penicillin-streptomycin (pH 7.4) at 37 ºC in a humidified air atmosphere of 5% CO_2. The culture medium was exchanged every 3 days.

Upon reaching 90% of confluence, culture medium was replaced by PBS and L929 cells were chemically detached from the tissue culture flasks using 0.05% Tryple Express solution for 5 min at 37ºC in a humidified air atmosphere of 5% CO_2. To inactivate the Tryple Express effect, fresh medium was added and the cells were centrifuged at 1200 rpm for 5 min. Afterwards, the medium was decanted followed by the cells re-suspension in the optimized polymeric precursor solution (this solution was prepared by mixing L_{MW}-MACHI with M_{MW}-CHI in ratio of 1:1 - final polymer concentration of 2% (w/v) -, 0.25% w/v I2959 and β-GF enough to neutralize the acetic acid needed to dissolve the M_{MW}-CHI and set the pH to 6.4) at density of 1×10^6 cells per mL of this polymer solution. CHI DN hydrogels with cells-enclosed were, then, produced using the method previously described in Scheme 1 and placed in 24-well tissue culture plates at 37ºC with a humidified air atmosphere of 5% CO_2.

To evaluate the effect of the developed methodology to fabricate strong CHI DN hydrogels on the cell viability, the LIVE/DEAD assay was performed. Briefly, this assay uses two dyes, calcein-AM (membrane permeant dye), which is hydrolyzed into calcein (green fluorescent dye) by estereases on viable cells, and propidium iodide (PI, red fluorescent dye), which, in turn, binds to DNA of only disrupted

cells due to its membrane impermeant character. Therefore, the viable cells will be stained in green whereas the disrupted cells will appear in red. After 24h, the culture medium was replaced by 1 mL of PBS containing 2 µL of calcein-AM and 1 µL of PI and the samples were incubated at 37ºC for 10 min. To remove excess of dye, the samples were washed three times with PBS and immediately after visualized using a confocal microscope (TCS SP8, Leica) with an excitation and emission wavelengths of 494 and 535 nm and 517 and 617 nm for calcein-AM and PI, respectively.

Movie S1. Compression assay until ε=50% performed on the L_{MW}-MACHI hydrogels;

Movie S2. Compression assay until ε=50% performed on the CHI DN hydrogels.

References:

[1] L. M. Y. Yu, K. Kazazian, M. S. Shoichet, *J. Biomed. Mater. Res., part A* **2007**, *82A*, 243-255.

[2] a) S. H. Kim, C. C. Chu, *J. Biomed. Mater. Res.* **2000**, *49*, 517-527; b) O. Jeon, K. H. Bouhadir, J. M. Mansour, E. Alsberg, *Biomaterials* **2009**, *30*, 2724-2734.

[3] E. Fernandez-Megia, R. Novoa-Carballal, E. Quiñoá, R. Riguera, *Carbohydr. Polym.* **2005**, *61*, 155-161.

[4] A. M. Costa, M. Alatorre-Meda, N. M. Oliveira, J. F. Mano, *Langmuir* **2014**, *30*, 4535-4539.

[5] Stephanie J. Bryant, Charles R. Nuttelman, Kristi S. Anseth, J. Biomater. Appl. **2000**, 439.

[6] J. W. Nichol, S. T. Koshy, H. Bae, C. M. Hwang, S. Yamanlar, A. Khademhosseini, *Biomaterials* **2010**, *31*, 5536-5544.

[7] N. Annabi, S. M. Mithieux, P. Zorlutuna, G. Camci-Unal, A. S. Weiss, A. Khademhosseini, *Biomaterials* **2013**, *34*, 5496-5505.

[8] S. S. Silva, A. Motta, M. T. Rodrigues, A. F. M. Pinheiro, M. E. Gomes, J. F. Mano, R. L. Reis, C. Migliaresi, *Biomacromolecules* **2008**, *9*, 2764-2774.