SUPPLEMENTARY INFORMATION

Double-membrane Thermoresponsive Hydrogels from Gelatin and Chondroitin Sulphate with Enhanced Mechanical Properties

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Experimental part

Materials. Gelatin type A from porcine skin (G1890) and chondroitin sulphate A sodium salt from bovine trachea (C9819) were purchased from Sigma Aldrich and used as received. Acetic acid was employed for pH adjustment (Aldrich).

Preparation of gelatin-chondroitin sulphate semi-IPN and IPN.

For the preparation of semiIPN G1-ChS0.5, gelatin and chondroitin sulphate were dissolved in milliQ water in the same recipient for 1 hour at 50 ºC with magnetic stirring. The recipient containing the polymers was sonicated twice for 30 seconds at 50 ºC to remove bubbles. Then, the solution was pipetted fast into Teflon cylindrical moulds with a diameter of 20 mm and capacity for 0.6 ml and left at 4 ºC overnight. The concentration ratio of gelatin to chondroitin sulphate was 2:1. Sample was designated as semiIPN G1-ChS0.5 where numbers denote the concentration of the polymers in % w/v.

For the preparation of IPN G1-ChS0.5, 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) (Aldrich) were weighed and dissolved in 1 ml of milliQ water to facilitate their incorporation into the polymer mixture. For 600 µl G1-ChS0.5 gels, 30 mg EDC and 19.9 mg NHS were weighed and dissolved in 1 ml of milliQ water. Addition of the crosslinkers was performed by carefully pipetting them into the G1-ChS0.5 concentrated solution avoiding bubble formation, and rapidly vortexing from 5 to 10 seconds. Gelification takes place within approximately 1 minute after incorporation of EDC/NHS, therefore pipetting into the Teflon moulds must be quick. Samples were stored at 4 ºC for 24 hours to ensure complete crosslinking and then were washed in deionized water for another 24 hours to remove crosslinking NHS-ester residues. The concentration ratio of gelatin to chondroitin sulphate was 2:1. Chemical hydrogels were designated as IPN G1-ChS0.5 CL where numbers denote the concentration of the polymers in % w/v.

Optimization of the Experimental conditions for the preparation of LbL hydrogels

A study was performed to determine the optimal concentration and pH conditions for generating interactions between gelatin (G) and chondroitin sulphate (ChS), which take place by the formation of a precipitate. Both polymers were dissolved separately in water at three different concentrations: 0.5, 1 and 2.5% w/v. The resulting aqueous

solutions were acidified with 1M acetic acid to pH 3 and 5. 250 mL of each polymer solution were mixed in vials and observed after 3 hours in order to determine the presence of precipitate. Gelatin and ChS solutions with pH 3 and 5 at concentrations 0.5, 1 and 2.5% w/v were mixed in all possible combinations to observe which conditions gave the greatest amount of precipitate and, therefore, higher degree of electrostatic interactions. A total of 36 combinations of gelatin and ChS were considered (figure S1_A). Samples with pH 5 in both ChS and gelatin were transparent after 4 hours, indicating that minimum interactions take place whenever the pH of the solutions is equal to pH 3 and 5. These conditions are similar to the pH of unaltered ChS and gelatin solutions (5.2 and 6.75 respectively), which did not produce observable interactions (P0).

A consistent trend involving an increase in polymer-aggregate precipitation was observed in samples with ChS at pH 3, as a general rule, the amount of precipitate increased with the ChS concentration. Samples with high gelatin concentration gelified after 4 hours and, hence, they were not employed for the LbL method (P9, P10, P12, P22, P23, P32-36).

Taking into account these results, the experimental conditions chosen to carry out the LbL experiments were 1% w/v gelatin concentration, and ChS (1 and 2.5% w/v). The pH of gelatin and ChS solutions were maintained at pH 3 and 5, respectively. These conditions were employed in the preparation of samples labelled as P19 and P31 as shown in figure S1_B. An additional sample prepared with unaltered pH and 1% w/v of every polymer, ChS and gelatin, will also be used in LbL experiments as control (P0 in figure S1_B)

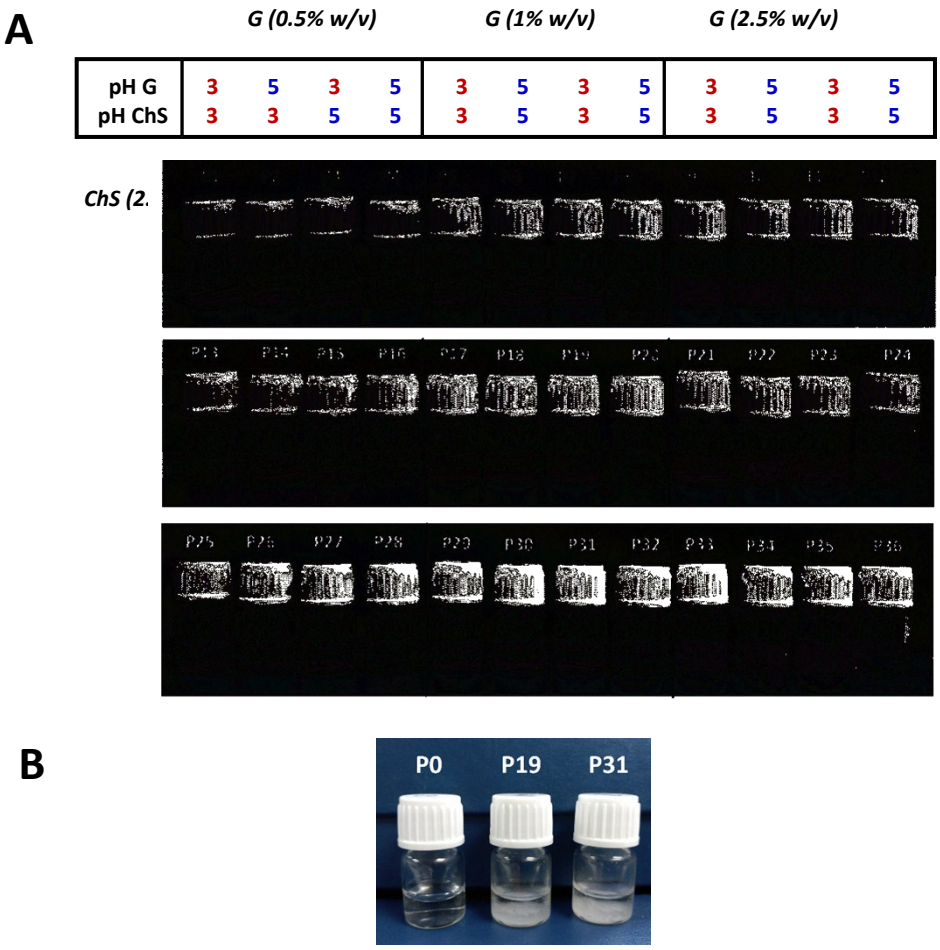

Figure S1. *A. Set-up for optimization of concentration and pH conditions of G-ChS multimembrane gels. B. Selected mixtures for successive experiments.*

FTIR spectroscopy

IR transmittance was carried out in a Perkin Elmer spectrometer in the range 450-4000 cm^{-1} with a resolution of 4 cm^{-1}, performing 4 scans per sample.

The assembly of gelatin and ChS followed by FTIR was carried out on a polystyrene substrate (PS) of 70 µm thickness. For that, PS was immersed in the gelatin solution (1% w/v pH 3) during 15 minutes, then washed in milliQ water for 3 minutes, followed by immersion in the ChS solution (2.5% w/v pH 5) for 15 minutes and a final wash in milliQ water for 3 minutes. The pH of the washing solutions were identical to the preceding polymer solutions in which the films were immersed. In order to follow the deposition process, sequential spectra were acquired at different number of deposited layers.

The FTIR spectra plotted in the range 2000-800 cm^{-1} corresponding to a gelatin film and a chondroitin sulphate film prepared by film casting of aqueous solutions at pH **3** and **5** respectively and the corresponding assignment of the bands are shown in the figure S2. Gelatin shows a characteristic band at 1651 cm^{-1}

corresponding to amide I representing C=O stretching/hydrogen bonding coupled with COO-. The band located at 1550 cm^{-1} assigned to amide II arises from the bending vibration of N–H groups and stretching vibrations of C–N groups. The band corresponding to amide III is located at 1240 cm^{-1} and it is related to vibrations in the plane of C–N and N–H groups of bound amide or vibrations of CH_2 groups of glycine, one of the amino acids that takes part in the polypeptidic structure of gelatin.[1,2] In the FTIR spectrum corresponding to the chondroitin sulphate film, the absorption bands assigned to the bending vibrations of the N–H (*N*-acetylated residues, amide band), C–O–C and C–O stretching, and O–H angular coupling, indicating the existence of free carboxyl groups, were observed at 1630, 1414, and 1053 cm^{-1}, respectively. Notice that the band located at 1630 cm^{-1} shows a shoulder at 1570 cm^{-1} which can be attributed to amide II C-N stretch and N-H bend. The absorption band assigned to the S=O stretching related to the sulfate groups on ChS, appeared at 1240 cm^{-1} and the absorption band assigned to the α-(1,4) glycoside bond is observed at 928 cm^{-1}. [3, 4]

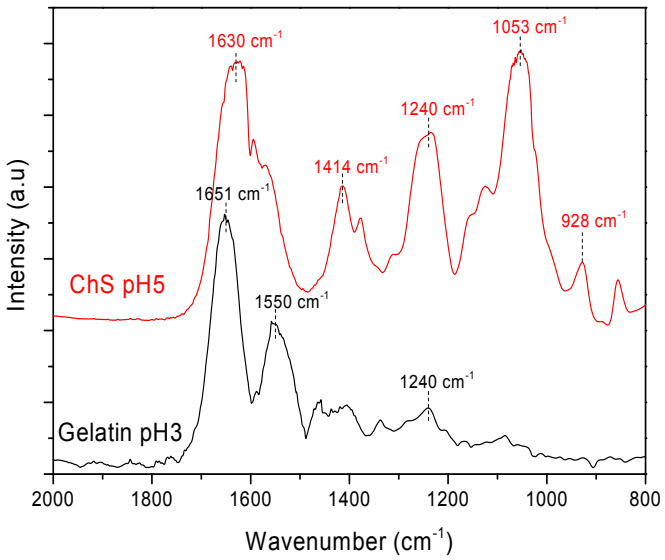

Figure S2. *FTIR spectra of gelatin at pH 3 and chondroitin sulphate at pH 5*

Rheological properties by dynamic oscillatory measurements

The rheological properties of G-ChS hydrogels were evaluated in an AR-G2 rheometer (TA instruments, USA) using parallel steel plates of 20 mm. A solvent trap was used to prevent solvent evaporation during the course of the experiments. Temperature sweeps were carried out from 20 to 90 ºC at 10 ºC/min and at 1Hz frequency. All experiments were performed at a constant strain in the linear viscoelastic region (LVR) of the gels, determined with the aid of strain sweeps carried out at 1 Hz and 20 ºC. Results were visualized using the Rheology Advantage Data Analysis software (TA instruments, USA).

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3. K. T. Mader, M. Peeters, S. E. L. Detiger, M. N. Helder, T. H. Smit, C. L. Le Maitre and C. Sammon, *Faraday Discussions*, 2016, **187**, 393-414.
4. A. R. Fajardo, M. B. Silva, L. C. Lopes, J. F. Piai, A. F. Rubira and E. C. Muniz, *RSC Advances*, 2012, **2**, 11095-11103.