Supplementary Information

Engineering sulfated polysaccharide and silk fibroin based injectable IPN hydrogels with stiffening and growth factor presentation abilities for cartilage tissue engineering

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1. Supplementary Methods

1.1 Rheology

Rheology was performed in oscillatory mode at room temperature utilising a rheometer (AR-G2, TA Instruments) fitted with a Peltier bottom plate and a 25 mm parallel upper plate to investigate the viscoelastic characteristics of hydrogels. The hydrogels were placed on the lower plate while maintaining 2mm space between both the plates. Within the linear viscoelastic zone, frequency sweeps were performed at angular frequencies ranging from 0.1 to 50 rad/sec with a constant strain of 0.1 %. For CMC/t-CMC/Silk and s-CMC/t-CMC/Silk hydrogels, the storage modulus (G′), loss modulus (G″), and tan δ (G′/ G″) were recorded for day 1 and day 28.

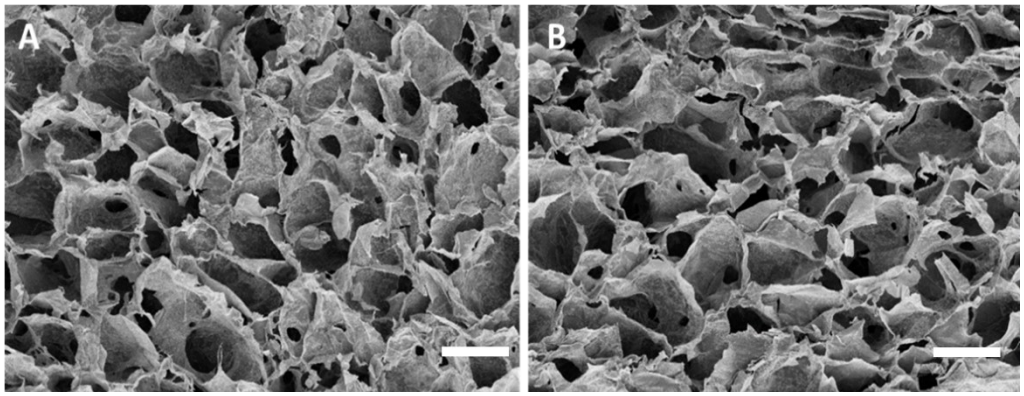
1.2 Hydrogel degradation

The CMC/t-CMC/Silk and s-CMC/t-CMC/Silk hydrogels were incubated in phosphate buffer saline (PBS) with 0 μg/mL, 50 μg/mL and 200 μg/mL collagenase type II for 14 and 28 days. Following this, the hydrogels were freeze dried. Hydrogel degradation was inferred by the analysis of dry weight of the hydrogels from each group at the aforementioned time points.

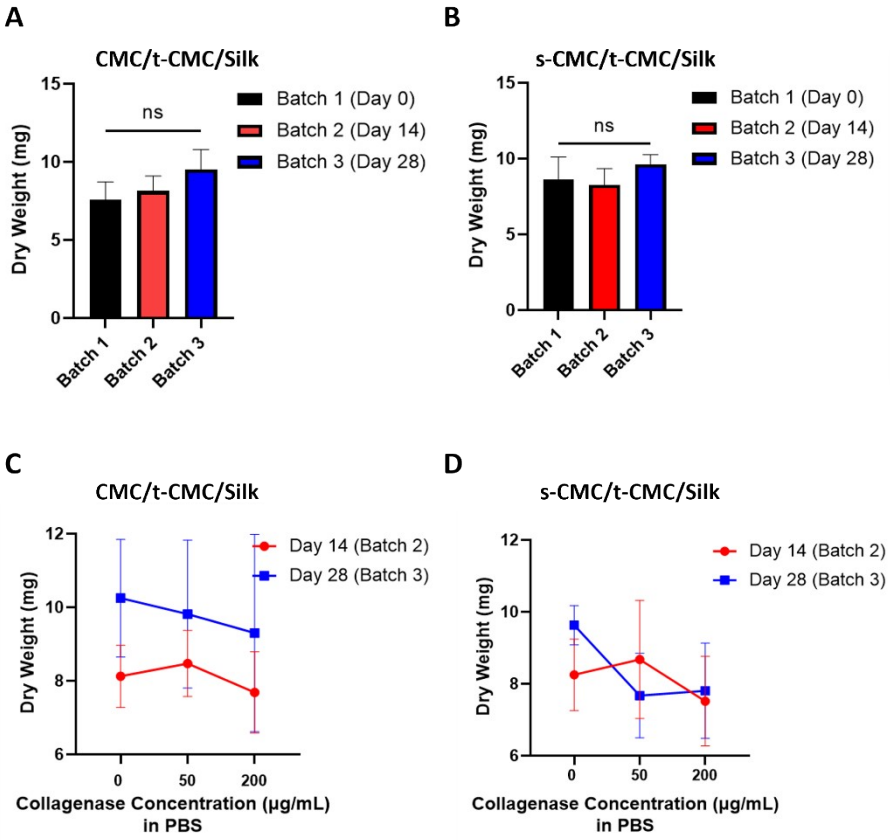
1.3 Fourier Transformed Infrared (FTIR) spectroscopy

The changes in the secondary structure of silk fibroin present within hydrogels were recorded using a Bruker Tensor 27 IR spectrometer (Bruker Optik, Germany) equipped with a mercury–cadmium–telluride (MCT) detector. After incubation in PBS the hydrogels were placed on the zinc–selenide crystal of the attenuated total reflectance (ATR) unit at Day 1, Day 14 and Day 28. Each sample was scanned from 4000 to 850 cm^{-1} with a resolution of 2 cm^{-1}. The atmospheric compensation program was used to remove spectral changes due to CO_2 present in the surrounding environment (Opus software, Build 7.2, Bruker Optik, Germany). The amide I area ranging from 1700 to 1600 cm^{-1} was selected, with spectra baseline-corrected (hence referred to as the original spectra). The second derivative of this spectra was utilised for peak identification. Curve fitting was employed with the peaks (cm^{-1}) found from the second derivative to determine the locations and intensities of the peaks in the amide I region of the original spectra.

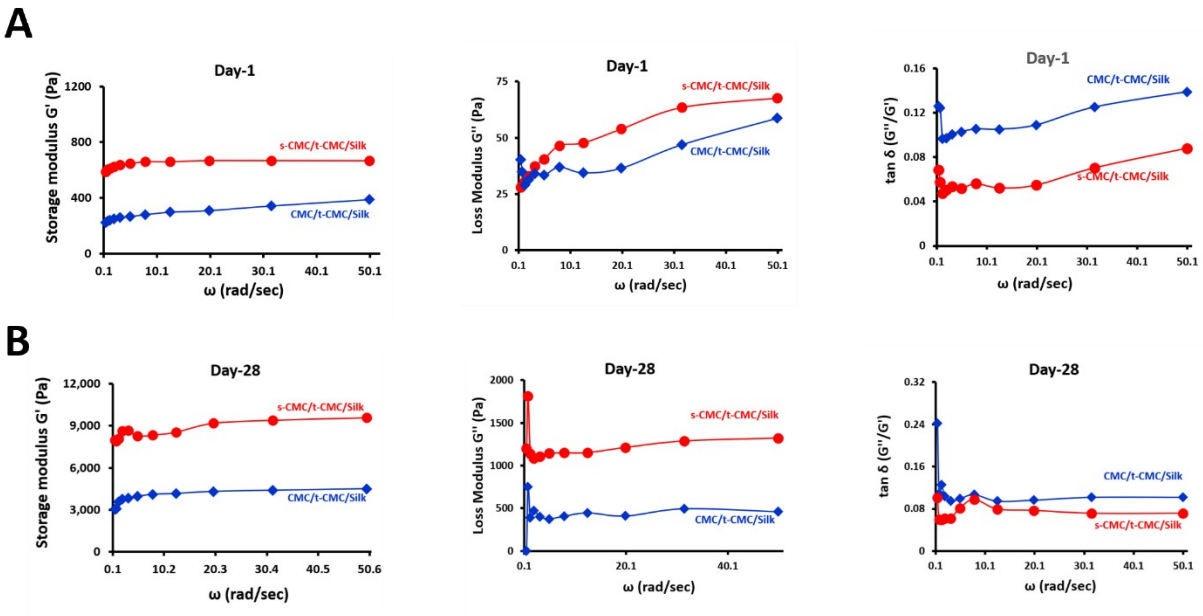
2.Supplementary Results:



Supplementary figure 1. The scanning electron micrographs depicting micromorphology of the (A) CMC/t-CMC/Silk and, (B) s-CMC/t-CMC/Silk hydrogels (scale bar - 200 μm).



Supplementary figure 2. Degradation analysis of IPN hydrogels. Dry weight determined immediately after fabrication of different batches of (A) CMC/t-CMC/ Silk and (B) s-CMC/t-CMC/Silk hydrogels (Batch 1, 2 and 3 were used for day 0, day 14 and day 28 experiments respectively). Dry weight of (C) CMC/t-CMC/Silk and (D) s-CMC/t-CMC/Silk hydrogels after incubation in phosphate buffer saline containing 0 µg/mL, 50 µg/mL and 200 µg/mL collagenase at day 14 and day 28.



Supplementary figure 3. Rheological characterisation of CMC/t-CMC/Silk (blue) and s-CMC/t-CMC/Silk (red) IPN hydrogels. Frequency sweep performed from 0 to 50 rad/s angular velocity at a constant shear rate of 0.1% for the determination of storage modulus, loss modulus and tan δ at (A) Day 1 and (B) Day 28.

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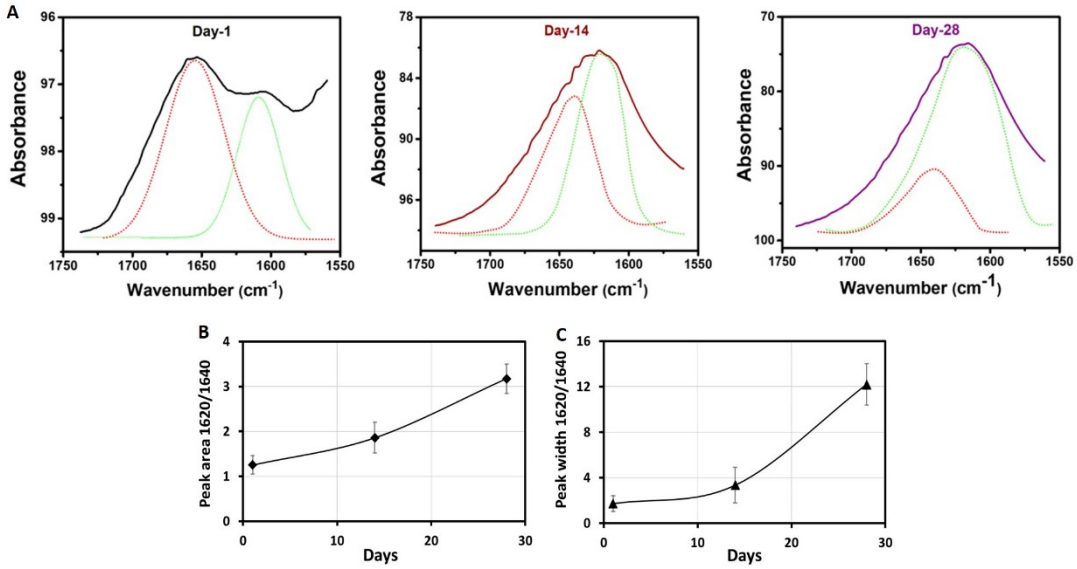
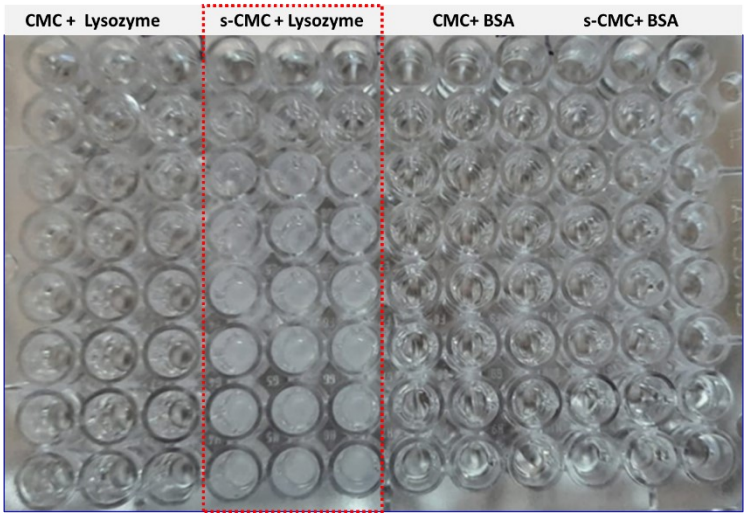
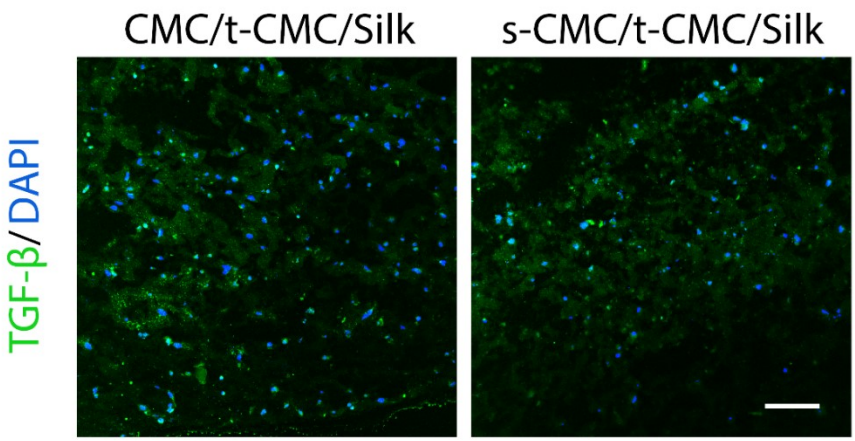


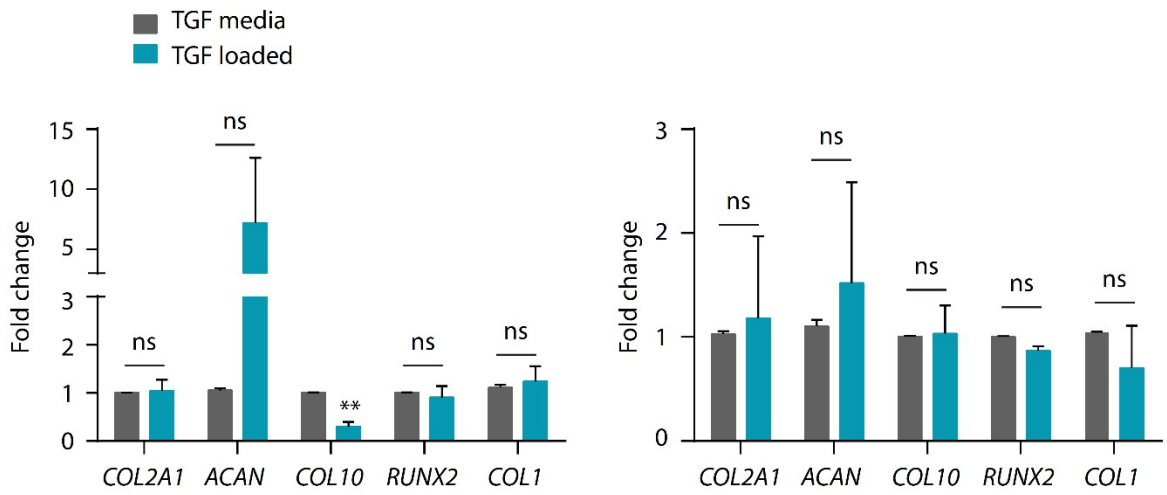
Supplementary figure 4. Dynamics of β-sheet transition as a function time in the s-CMC/t-CMC/Silk hydrogels using Fourier Transform Infrared (FTIR) Spectroscopy. (A) Deconvoluted spectra illustrating the progressive shift of the peak from 1660-1640 cm^{-1} (red) to 1620 cm^{-1} (green) at distinct time points (Day-1, Day-14, and Day-28). Quantification of the β-sheet transition through the ratio of (B) Area under the curve at 1660-1640 cm^{-1} and 1620 cm^{-1}, (C) Peak widths at 1660-1640 cm^{-1} and 1620 cm^{-1}.



Supplementary figure 5. Gross image of turbidimetric assay for model protein binding. The marked region in red shows an increase in turbidity due to interaction of s-CMC with different concentrations of lysozyme.



Supplementary figure 6. Interaction of sCMC with cationic proteins. Immunofluorescence micrographs of anti-TGF-β stained sections of TGF-β1 loaded CMC/t-CMC/Silk and s-CMC/t-CMC/Silk IPN hydrogel after 14 days of culture (Background green staining depicts loaded TGF-β) (scale bar: 100 μm)



Supplementary figure 7. Gene expression analysis. Relative mRNA levels of chondrogenic (*COL2A1* and *ACAN*), hypertrophic (*COL10* and *RUNX2*) and osteogenic markers (*COL1*) after chondrogenic differentiation of IFP-MSCs encapsulated within s-CMC/t-CMC/Silk hydrogels with TGF-β present either in media or loaded in hydrogels for 14 and 28 days. Error bar depicts standard error of mean (n=3), ** ($P < 0.01$) represents significant difference (calculated using t-test) with respect to samples treated with TGF-β in media. 'ns' represents non-significant difference between the groups.

Table S1. List of primer sequences

Name	Sequence
HPRT (Housekeeping gene)	F: 5' CCCTCGAAGTGTTGGATATAGAC 3' R: 5' AGGGCATATCCCACAACAAA 3'
COL2A1	F: 5' GGACGTTCTGGCGAAACT 3' R: 5' CAAAGGCGGACATGTCAATG 3'
ACAN	F: 5' CACGATGCCTTTCACCACGAC 3' R: 5' TGCGGGTCAACAGTGCCTATC 3'
COL10	F: 5' TGCTTTCACTGTTATCCTCTCC 3' R: 5' TCCAGTTCTTGGGTCGTAGTAATG 3'
RUNX2	F: 5' CTTCACAAATCCTCCCCAAGTAGCTACC 3' R: 5' GGTTTAGAGTCATCAAGCTTCTGTCTGTG 3'
COL1	F: 5' AGGGCCAAGACGAAGACATC 3' R: 5' AGATCACGTCATCGCACAACA 3'

Table S2: Tabulated representation of peak stress and compressive modulus values (at 10%, 20% and 30% strain) of CMC/t-CMC/Silk and s-CMC/t-CMC/Silk IPN hydrogels.

Sample		**Stress (kPa)**	**Modulus (kPa)**		
			10%	**20%**	**30%**
Day-1	CMC/t-CMC/Silk	6.36±2.89	1.75±0.78	2.92±0.85	5.06±1.86
	s-CMC/t-CMC/Silk	5.27±0.98	1.99±0.48	3.75±0.81	5.16±0.63
Day-14	CMC/t-CMC/Silk	29.14±6.31	11.2±4.31	17.66±5.27	35.59±10.46
	s-CMC/t-CMC/Silk	26.24±9.26	11.45±5.39	17.47±6.10	33.49±19.17
Day-28	CMC/t-CMC/Silk	63.68±16.68	26.21±12.21	59.70±25.50	96.81±22.12
	s-CMC/t-CMC/Silk	59.42±22.97	20.44±4.47	53.22±16.01	97.39±46.22