Synthesis and Biological Evaluation of a Bioinspired, Tissue-Adhesive Gellan Gum-Based Hydrogel Designed for Minimally Invasive Delivery and Retention of Chondrogenic Cells

David A. Learmonth*, Pedro M. Costa, Tiago R. Veloso, Cristiana B. Cunha, Mafalda P. Cautela, Cristina Correia, Mariana C. Vallejo and Rui A. Sousa.

*Corresponding author: David A. Learmonth (dalearmonth@stemmatters.com).

Stemmatters, Biotecnologia e Medicina Regenerativa SA, Parque de Ciência e Tecnologia Avepark, Zona Industrial da Gandra, 4805-017 Barco, Guimarães, Portugal

Supplementary Methods

Isolation of ovine knee chondrocytes (OCHs): OCHs were obtained from the knees of healthy sheep during arthroplasty for preclinical assessment of STM-148B efficacy, a procedure performed in accordance with Portuguese National Authorities (DGAV) authorization 0421/000/000/2019 and the EU Directive 2010/63/EU for animal experiments. Briefly, after collection in a 50 mL falcon tube containing decontamination solution (Base 128, Alchimia srl, Italy), the cartilage fragments were washed twice with PBS supplemented with 10 % antibiotic-antimycotic for 10 min. After centrifugation, the fragments were digested overnight in collagenase at a ratio of 0.5 mL per 1 mm of diameter of lesion (0.8 U/mL collagenase) at 37 ºC in the orbital shaker with agitation (~100 rpm). The cell suspension was then filtered through a 70 µm cell strainer, washed twice with PBS and platted at 15,000 cells/cm^2 for subsequent cell expansion.

Supplementary Figures

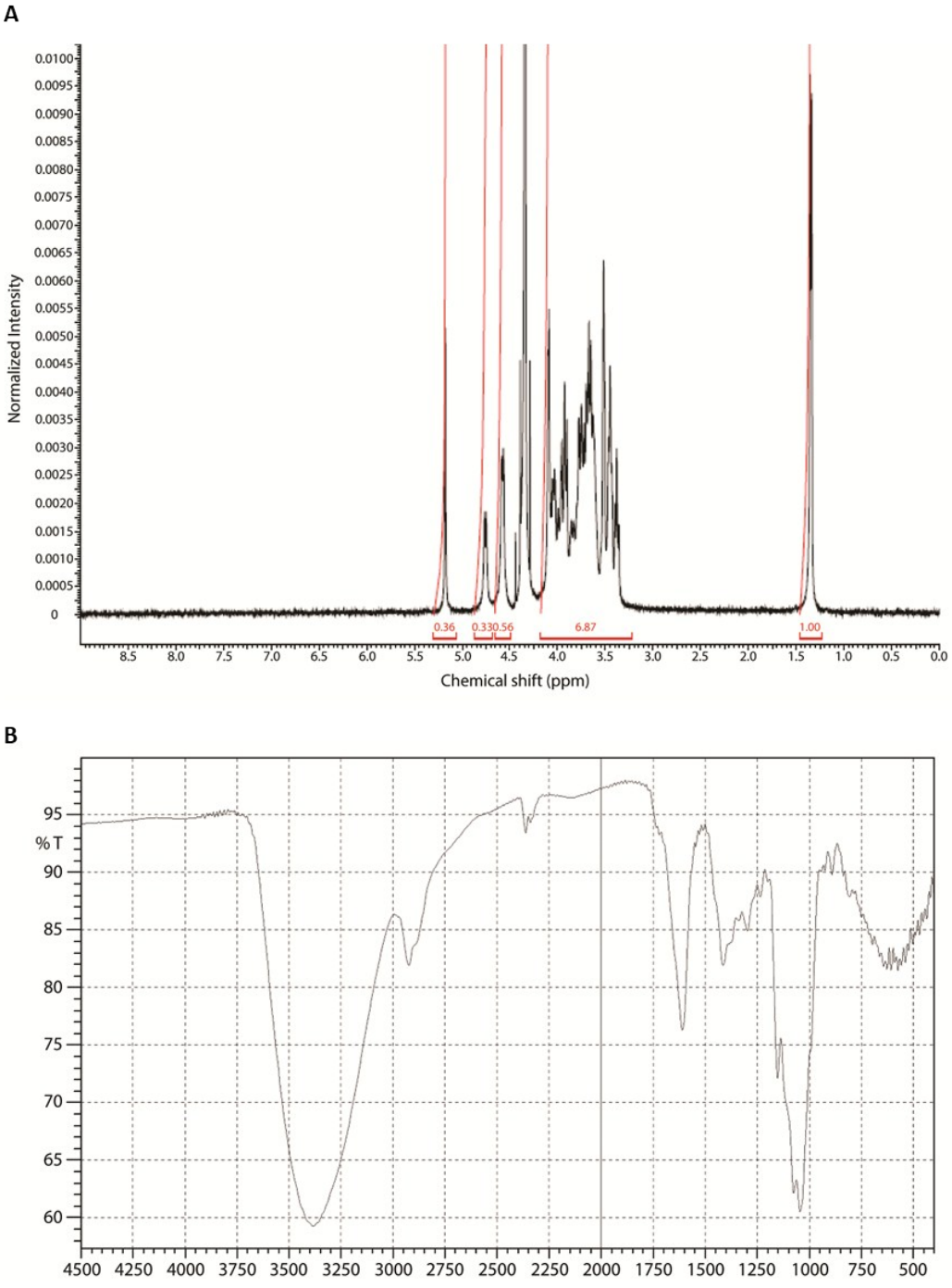

Fig. S1 Structural characterisation of GGp. (A) ^{1}H NMR (D_2O, 1 % w/V, 70 ºC) and (B) FTIR spectra. Both spectra were obtained under conditions described in section 2.4 Physicochemical characterisation.

Table SI. ^{1}H NMR peak assignments for GGp.

Chemical shift (ppm), Multiplicity	Assignment
1.34 - 1.35, doublet	CH_3, rhamnose
4.49 - 4.56, doublet	CH, glucose
4.74 - 4.76, doublet	CH, glucuronic acid
5.15, singlet	CH, rhamnose

Table SII. FTIR peak assignments for GGp.

Wavelength (cm^{-1})	Functional group
3500-3200	OH
2950	aliphatic C-H
1600	C=O

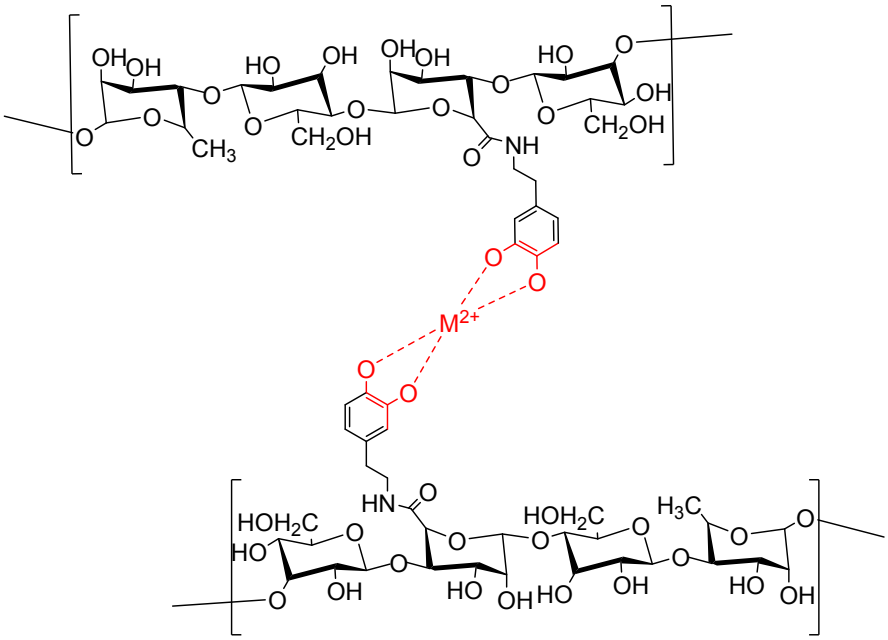

Fig.S2 Ionically-mediated crosslinking of catechol groups of the dopamine substituent of STM-148B.

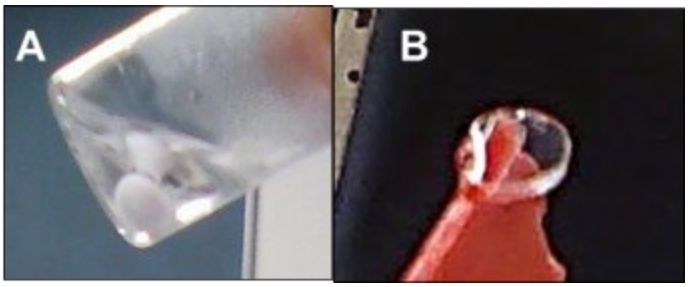

Fig. S3 Representative images of the crosslinked STM-148B solution (A) before being placed on the rheometer and (B) STM-148B hydrogel formed at end of measurement.

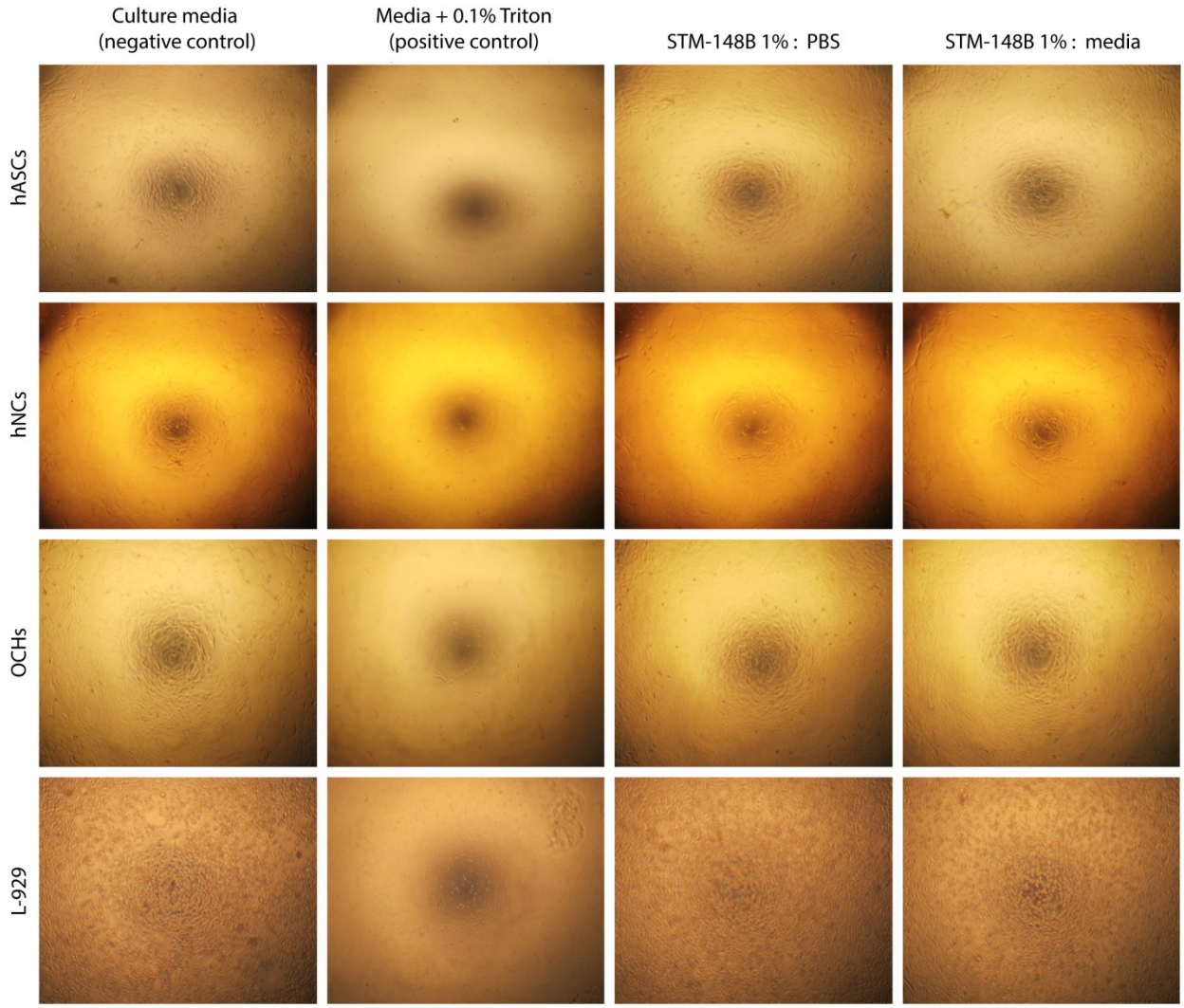

Fig. S4 Representative images of cultured cells 24 hours after incubation with STM-148B extracts. Cells (hASCs, hNCs, OCHs and L-929) were incubated for 24 hours with extracts of STM-148B hydrogels crosslinked by PBS Ca^{2+}/Mg^{2+} or culture media to a final polymer concentration of 1 % (w/V). Control conditions included cells incubated with culture media (negative control) and cells incubated with 0.1 % Triton X-100 in culture media (positive control). Subsequently, cells were observed under a light microscope (4x magnification) for assessment of changes in morphology and density.

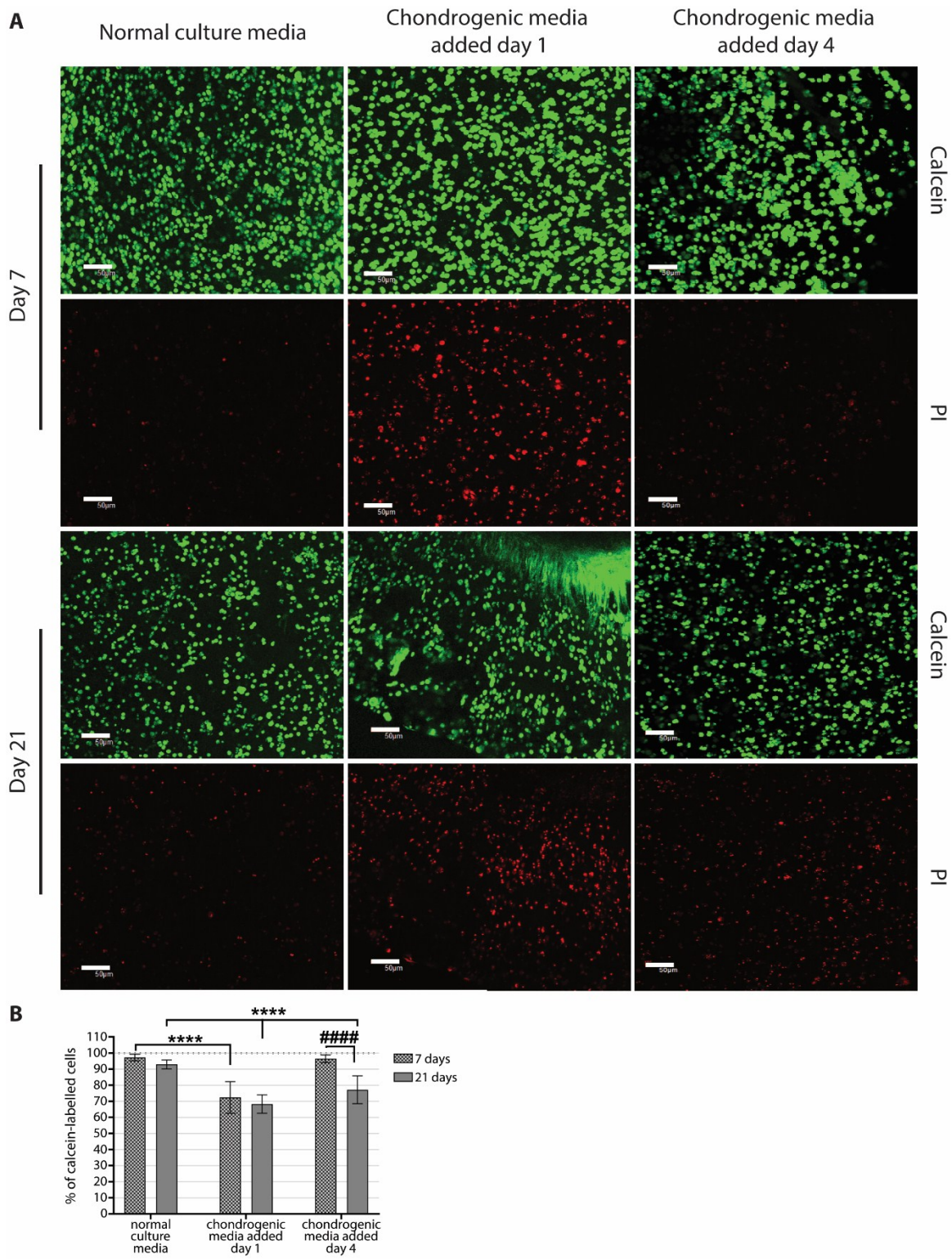

Fig. S5 Confocal microscopy of hASCs encapsulated in STM-148B hydrogels and cultured in normal culture media or chondrogenic differentiation media added at day 1 or day 4 post-encapsulation. Representative images of cells at day 7 or 21 post-encapsulation at 10x

magnification. Live cells were stained with calcein (green) and dead cells were stained with PI (red); scale corresponds to 50 µm. Representative images of cells at day 1, 7 or 21 post-encapsulation at 10x magnification. The percentage of cells labelled with calcein or PI was calculated by dividing the number calcein/PI-labelled cells by the total number of cells (cells labelled with calcein plus cells labelled with PI). Quantification was performed using ImageJ software. **** $p < 0.0001$ compared to cells cultured in normal culture media, #### $p < 0.0001$ compared to cells cultured for 7 days, using two-way ANOVA with Sidak's post hoc test (for comparison between conditions at day 7 and day 21) or Tukey's post hoc test (for comparison between conditions within the same day); outliers (2/23 values for hASCs cultured in normal media for 7 days; 3/21 values for hASCs cultured for 7 days in chondrogenic differentiation media added at day 4) were identified and removed using the ROUT method (Q = 10 %).

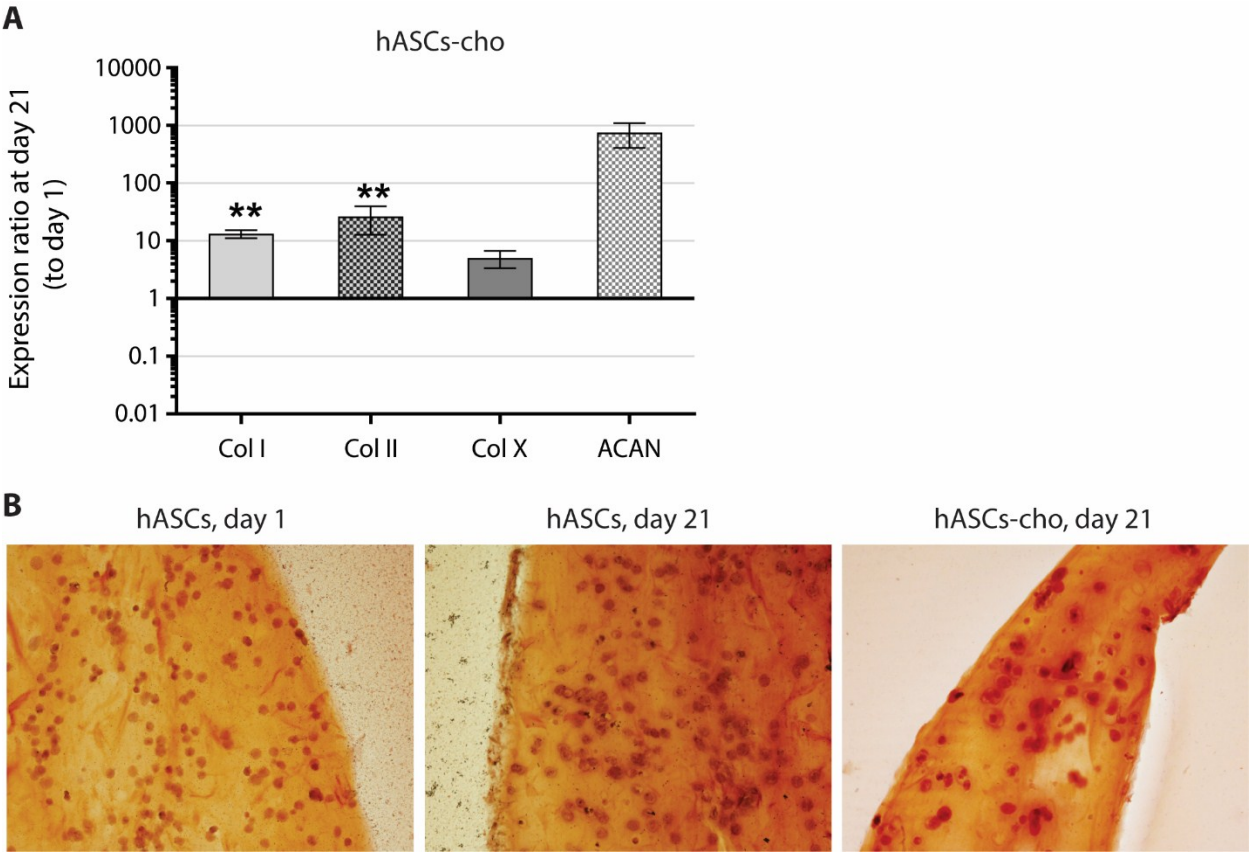

Fig. S6 Expression of ECM markers in hASCs encapsulated in STM-148B hydrogels and cultured for 21 days in chondrogenic differentiation media added at day 4. Cells (hASCs) were encapsulated in STM-148B, cultured for 21 days in normal media or chondrogenic differentiation media added at day 4 (hASCs-cho) and then processed for qPCR or safranin-O staining analysis. (A) The relative mRNA expression of collagen I (Col I), collagen II (Col II), collagen X (Col X) and aggrecan (ACAN) in hASCs was assessed at day 21 (to day 1) by qPCR using the ΔΔCT method. ** $p < 0.01$ compared to hASCs cultured for 1 day in normal culture media, using Welch's unequal variances t test[45]; no outliers were identified using the ROUT

method (Q = 10 %). (B) Representative images of hASCs processed for proteoglycan detection via safranin-O staining and observed under bright-field microscopy (10x amplification).

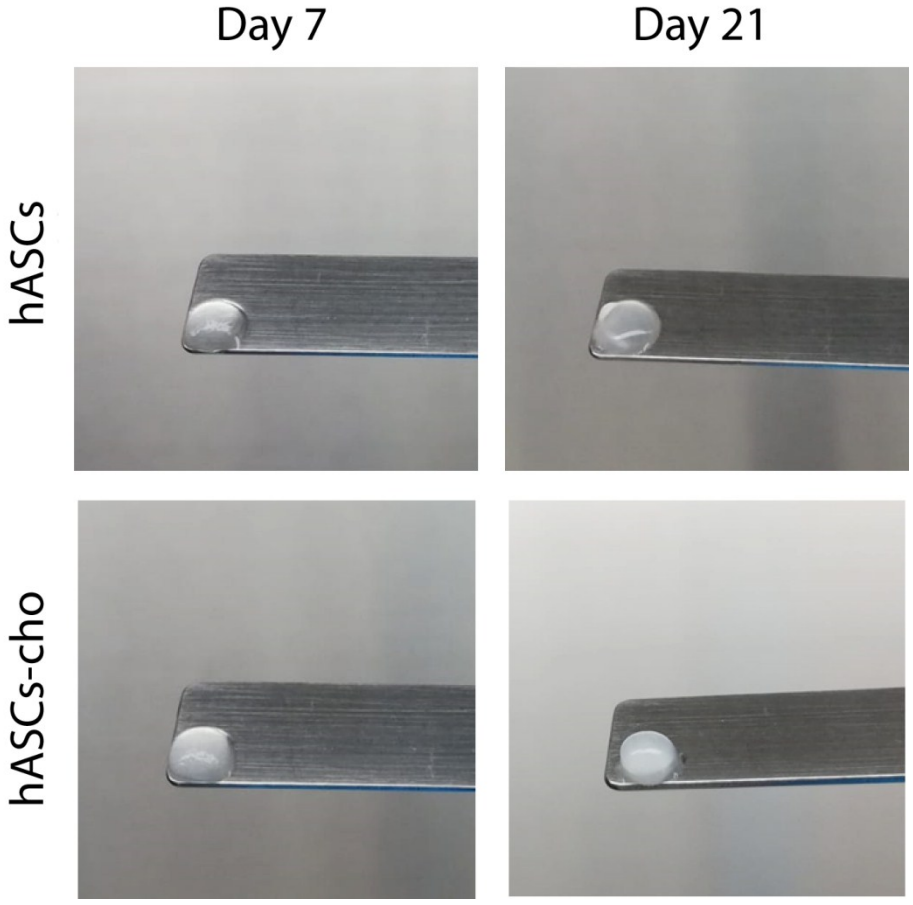

Fig. S7 Representative images of STM-148B hydrogels encapsulating hASCs and cultured in normal culture media or chondrogenic differentiation media. Cells (hASCs) were encapsulated in STM-148B and cultured for 21 days in normal media or chondrogenic differentiation media added at day 1 (hASCs-cho). Representative images of hydrogels at 7 and 21 days of culture.

Supplementary Notes

1. Native HA-GG contains two acyl substituents, namely acetate (one acetate group for every two repeat units) and glycerate (one glycerate group for every repeat unit), both located on

the same glucose residue, while LA-GG (Figure 1A, 1) is obtained through alkaline hydrolysis of the former and typically contains less than 5 % acyl.

HA-GG hydrogels are soft and elastic, while LA-GG hydrogels are harder, less elastic, relatively stable towards mild heat and dilute acid, but prone to gradually lose mechanical strength due to the diffusion and exchange of the divalent cations over time *in vivo*.

2. The ionic crosslinking mechanism of gellan gum by cations has been discussed extensively in cited reference 16 thus: "*Formation of a true gel network is reliant upon aggregation of helical sequences, which on the basis of current evidence can occur via different mechanisms dependent on the cation used in cross-linking; divalent cations, such as* Ca^{2+} *and* Mg^{2+} *form direct bridges by site binding between pairs of double helices, monovalent ions, such as* Na^{+} *and* K^{+} *suppress repulsion by binding to the surface of the helices balancing the negative charge of the carboxyl groups. The concentrations required to induce gelation are therefore different and depend on the gellan concentration, but typically, concentrations of 5mM* Ca^{2+} *and* Mg^{2+}*, 100 mM for* Na^{+} *and* K^{+}*, would be required for cross-linking."*

Further discussion of the crosslinking mechanism of gellan gum and its methacrylated derivative can be found in references 17-19. Notwithstanding, a further crosslinking mechanism specific to STM-148B beyond the electrostatic shielding of the carboxyl groups of gellan gum involves ionic crosslinking of the catechol moiety of the dopamine substituent via coordination of the hydroxyl groups with ions as shown in **Fig. S2**.