Supporting Information

Human-based fibrillar nanocomposite hydrogel as bioinstructive matrices to tune stem cell behavior

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Experimental section

Preparation of Platelet lysate: Platelet concentrates were obtained from different platelet collections performed at Serviço de Imunohematologia do Centro Hospitalar de São João (CHSJ, Porto, Portugal), under a previously established cooperation protocol. All the platelet products were biologically qualified according to the Portuguese legislation. The platelet count was performed at the IPS using the COULTER® LH 750 Hematology Analyzer and the sample volume adjusted to 1 million platelet μL^{-1}. Platelet lysates (PL) were prepared according to a previously established protocol[1]. Briefly, the collected platelet concentrate samples were subject to three repeated temperature cycles (frozen with liquid nitrogen at −196 °C and heated at 37 °C water bath), in order to promote platelets lysing and the release of their corresponding protein content. Aliquots of PL were stored at −80 °C. Prior to use, the lysate was centrifuged at 4000 G for 5 minutes and filtered through a 0.45 µm pore filter to remove the platelet membrane fragments.

Total Protein content quantification: Total protein content was quantified by Bicinchoninic Acid Assay (Thermo Fisher Scientific, USA). Briefly, a calibration curve in the range of 25-2000 µg mL^{-1} and PL concentrates at different dilutions were measured at 562 nm on a plate reader according to Pierce BCA Protein Assay Kit instructions.

CNC production: CNC were extracted from microcrystalline cellulose (MCC) powder (Sigma-Aldrich, USA) following the typical sulfuric acid hydrolysis according to Bondeson with minor modifications[2]. In brief, 42 g of MCC was mixed with 189 mL of deionized water (DI). DI/MCC suspension was then put in an ice bath and stirred using a mechanical agitator (500 rpm) during 10 minutes. 188.3 mL of concentrated sulfuric acid (95−98% from Sigma-Aldrich, USA) was added dropwise up to a final concentration of 64 wt.%. The suspension was heated to 44 °C while stirring at 500 rpm for 120 min. The reaction was stopped by diluting the suspension with cold water (5x) and left to decant at 4 °C. The supernatant was discarded and the remaining suspension was centrifuged for 10 minutes at 9000 rpm and 5 °C. The supernatant was successively replaced with DI water and the suspension subjected to centrifugation cycles until the supernatant became turbid. The resulting suspension was collected and extensively dialyzed using cellulose dialysis membrane MWCO: 12-14 kDa (Sigma-Aldrich, USA) against DI water until neutral pH. After dialysis the content was sonicated three times (VCX-130PB-220, Sonics) for 10 minutes using an ultrasound probe at 60% of amplitude output, under ice cooling to prevent overheating. The cloudy suspension was centrifuged one last time to remove big particulates and the final supernatant containing the CNC was stored at 4 °C until further use.

Oxidation of CNC: Aldehyde CNC (a-CNC) were produced by sodium periodate ($NaIO_4$) oxidation[3]. In a typical experiment, $NaIO_4$ (Sigma-Aldrich, USA) was added to CNC aqueous suspension (1.5 wt.%) in a 1:1 molar ratio ($NaIO_4$:CNC). The mixture was allowed to stir at room temperature (RT) for 12 hours preventing from light exposure. Unreacted periodate was quenched by the addition of excess of ethylene glycol (Sigma-Aldrich, USA). The mixture was transferred into a dialysis membrane (cellulose dialysis membrane MWCO: 12-14 kDa) and dialyzed against DI for 3 days with regular water replacement. The final working suspension of modified CNC was collect and stored at 4 °C. The desired

concentration of the working suspension was adjusted by diluting or concentrating it against poly(ethylene glycol) (average MW 20,000 kDa, Sigma-Aldrich, USA) using benzoylated cellulose dialysis membranes (2000 Da NMWCO, Sigma-Aldrich, USA) .

Analysis of chemical modification of CNC: Fourier ransform infrared spectroscopy (FTIR) was used to evalute the chemical modification of CNC in a Shimadzu spectrometer**.** Before FTIR analysis, freeze-dried CNC and a-CNC were oven-dried at 105 °C for 30 minutes preventing from air exposure to avoid water resorption and pelleted in KBr (Sigma-Aldrich, USA). Sectral data were acquired between 4400 and 400 cm^{-1} from 128 averaged scans at a resolution of 4 cm^{-1}.

Morphological characterization of CNC: CNC morphology were analysed by Atomic Force Microscopy (AFM). Drops of the diluted modified CNC suspension (0.0015 wt. %) were deposited on freshly cleaved and carefully washed mica discs (9.9 mm diam. 0.27 thick). The suspension was left to adsorb for 15 minutes and the excess liquid was removed. The disc was washed two times with ultrapure water (Milli-Q, 18.2 M Ω cm^{-1}) and allowed to dry overnight. The samples were imaged in tapping mode with a MultiMode AFM (Bruker, USA) and the particle size distribution was determined with Gwyddion software (n=50).

Quantification of CNC carbonyl content: The carbonyl group content of the oxidized a-CNC was determined by conductometric titration[3]. In a typical run, 3.6 mL of a-CNC aqueous suspension (1.39 wt.%, 0.050 g) and 0.025 g (0.62 mmol) of NaOH were dispersed in a final volume of 10 mL of ultra-pure water. 0.193 g of silver (I) oxide (Sigma-Aldrich, USA) were added to the solution, which was allowed to stir overnight and selectively oxidize the aldehyde groups to carboxylic acids. 5 mL of the oxidized reaction mixture were diluted with 80 mL of ultra-pure water and the pH was adjusted to c.a. 3.5 with hydrochloric acid (Thermo

Fisher Scientific, USA). Finally, the solution was titrated using 0.01M sodium hydroxide (Thermo Fisher Scientific, USA). The total amount of carboxyl groups corresponding to the carbonyl content or degree of oxidation (DO) was calculated from Equation S1:

$$DO = \frac{162C(V_2 - V_1)}{w - 36C(V_2 - V_1)}$$

(S1)

where C is the NaOH concentration (mol L^{-1}), V_1 and V_2 are the amount of NaOH as shown in conductimetric titration curves (Fig S1D), and w (g) is the weight of a-CNC.

Quantification of CNC sulfation degree: The CNC sulfate content was determined following the CNC pre-treatment steps and conductometric titration according to Beck, S. et al[4] with minor modifications. First, CNC suspension was fed from the top of the column that contains a bed of dowex marathon C hydrogen form strong acid cation (Sigma Aldrich, USA) to fully protonate CNC sulfate half-ester groups. Then, sulfate half-ester content was determined via conductometric titration (113 mg of CNC in 200 mL of 1 mM NaCl aqueous solution and 10 mM NaOH as titrant), and sulfate half-ester groups content and the sulfur content (%)[5] was calculated using Equation S2 and S3, respectively:

$$mmol/kg = \frac{V_{NaOH} C_{NaOH}}{m_{CNC}} \quad \text{(S2)}$$

where V_{NaOH} is the inset equivalence point determined from conductometric titration curve, C_{NaOH} is the concentration of titrant used and m_{cnc} is the mass of the CNC suspension.

$$\%S = \frac{V_{NaOH} C_{NaOH} M_W(S)}{m_{susp} C_{susp}}\ x\ 100 \quad \text{(S3)}$$

where m_{susp} and C_{susp} are the mass and concentration (mass %) of the CNC suspension and Mw (S) is the atomic mass of sulfur.

Hydrogel degradation: For quantification of total protein release, at different time points the supernatant was collected and replaced with fresh PBS. Total protein content was quantified by bicinchoninic acid assay (Thermo Fisher Scientific, USA). Briefly, a calibration curve in the range 25-2000 μg mL^{-1} and the protein released from PL-CNC hydrogels were measured at 562 nm on a plate reader according to Pierce BCA Protein Assay Kit instructions. The results are an average of three measurements obtained per formulation (n=4).

Table S1. List of genes under evaluation, forward (F) and reverse (R) primers used in the gene expression analysis of encapsulated hASCs in PL-CNC hydrogels over 1 and 7 days in culture. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), SRY-box 9 (SOX9), runt-related transcription factor 2 (RUNX2), collagen type I alpha 1 chain (COLA1), Mohawk (MKX), alkaline phosphatase (ALP), platelet-derived growth factor-B (PDGF-B), vascular endothelial growth factor (VEGFA), leptin (LEP), lipoprotein lipase (LPL), cartilage oligomeric matrix protein (COMP) and peroxisome proliferator-activated receptor γ (PPARγ).

Gene	***Sequence (5'-3')***
β-Actin	*F:CTGGAACGGTGAAGGTGACA* *R:AAGGGACTTCCTGTAACAA*
GAPDH	*F:GGGAGCCAAAAGGGTCATCA* *R:GCATGGACTGTGGTCATGAGT*
SOX9	*F:TTCATGAAGATGACCGACGC* *R:GTCCAGTCGTAGCCCTTGAG*
RUNX2	*F:TTCCAGACCAGCAGCACTC* *R:CAGCGTCAACACCATCATTC*
COL1A1	*F:CCCCAGCCACAAAGAGTCTAC* *R:TTGGTGGGATGTCTTCGTCT*
MKX	*F: TCGCACAGACACTCTGGAAAA* *R: TGTTAAGGCCATAGCTGCGT*
ALP	*F:GAAGGAAAAGCCAAGCAGGC*

	R:GGGGGCCAGACCAAAGATAG
PDGFB	*F:CCCCACACTCCACTCTGATT*
	R:GCCCTGGCCTCTAGTCTTCT
VEGFA	*F:CCATCCAATCGAGACCCTGG*
	R:TCCGCATAATCTGCATGGTG
LEP	*F:CTCAGGGATCTTGCATTCCC*
	R:CCATGCATTTGGCTGTTCAG
LPL	*F: ACTTGGAGAGGGACGAAGAA*
	R: ATGATGCAGGCCAATGGTAG
COMP	*F: AGGATGGAGACGGACATCAG*
	R: TCTGCATCAAAGTCGTCCTG
PPARγ	*F: TGGGTGAAACTCTGGGAGAT*
	R: GCGATCTCTGTGTCAACCAT

Hematoxylin and eosin (H&E) staining. After 14 days of culture, PL-CNC 0, 0.31 and 0.61 formulations were washed with PBS and then fixed in 10% formalin (Thermo Fisher Scientific, USA) for 30 min at RT. The samples were embedded in Histogel specimen processing gel (Thermo Fisher Scientific, USA), dehydrated through graded ethanol solutions and embedded in paraffin for further sectioning using a microtome (HM355S, Microm, Thermo Scientific). Sections of 5 μm thickness were prepared and stained with hematoxylin and eosin (H&E) for histological evaluation of cell distribution and hydrogel retraction. Sections were observed under a transmitted light Microscope (Zeiss, Germany).

Supplemental results and discussion

Morphological and chemical characterization of modified CNC. CNC were extracted from commercial MCC by the common sulfuric acid hydrolysis procedure[2]. In this reaction, sulfuric acid reacts with the surface hydroxyl groups via an esterification process allowing the grafting of anionic sulfate ester groups that are the basis of their high stability in aqueous solutions. Then, vicinal hydroxyl groups on CNC's surface were converted to carbonyls by periodate oxidation during 12 hours. CNC morphology and dimensions were evaluated by

AFM (Figure S1-A and S1-B). They exhibit a typical rod-like shape morphology with averaged height of 3.5 ± 1.3 nm and length of 168 ± 60 nm, similar to previous works[2,3]. The effective aldehyde functionalization of CNC was confirmed by FTIR (Figure S1-C), demonstrated by the characteristic C=O stretching vibration band at 1740 cm^{-1} from the aldehyde groups visible on modified CNC and not in initial CNC formulation. Conductometric titration was performed to quantify the corresponding degree of oxidation (Figure S1-D). It was estimated that 9.1 ± 0.95 carbonyl groups per 100 anhydroglucose units have been oxidatively introduced. The degree of chemical modification achieved guarantee sufficient reactivity while preserving the integrity of crystalline structure of CNC, since higher degrees of oxidation showed to affect its' original crystalline properties[6]. Conductometric titration was also used to determine the charged sulfate half-ester groups at the CNC surface (Figure S1-E). The estimated content of sulfate groups in CNC is 296.78 ± 10.62 $mmol.kg^{-1}$ (0.95 ± 0.03 %S). These results are in agreement with those reported for CNC isolated from different cellulose sources by sulfuric acid hydrolysis that typically contains 80-350 $mmol.kg^{-1}$ of anionic sulfate half-esters introduced at some of the surface hydroxyl groups[7].

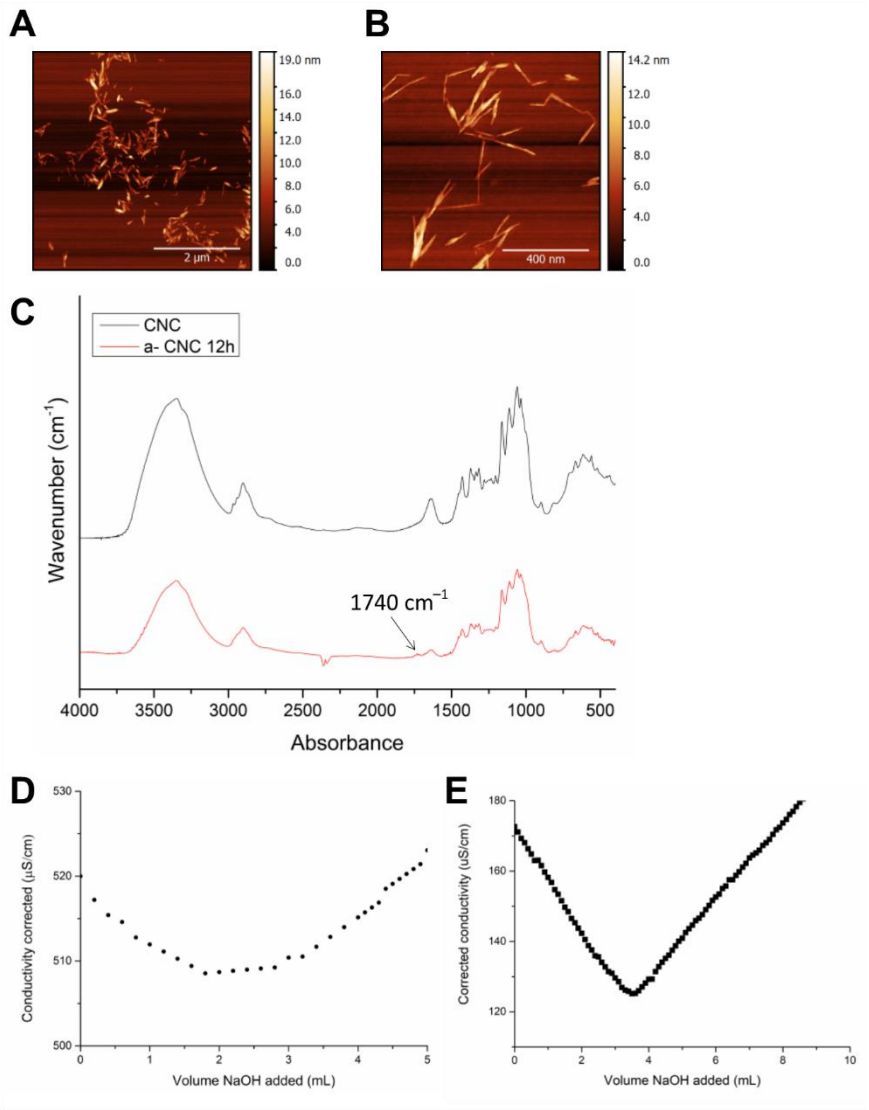


Figure S1. Morphological and chemical characterisation of CNC and modified CNC. AFM images of modified CNC at 5x5 µm (A) and 0.5x0.5 µm (B). FTIR analysis of CNC and modified CNC (C). Conductometric titration curve of modified CNC to calculate the oxidation (D) and sulfation degree (E).

Physical characterization of hydrogels. The impact of clotting factors in fibrin network formation was performed using different concentrations of thrombin (low, L, - 1 $U.mL^{-1}$ and high, H, 2 $U.mL^{-1}$) and calcium (L – 2.5 mM and H - 5 mM), (HH, HL and HH formulations – thrombin and calcium), Figure S2-A. According to the rheological analysis, LH formulation led to faster polymerization and superior storage modulus (G') in comparison with HH and

HL formulations. These results are consistent with previous studies reporting that softer clots are obtained at higher thrombin and lower calcium levels[8]. The incorporation of CNC (at 0.61 wt.% concentration) did not hamper PL matrix polymerization and resulted in an increase of G' in all formulations (HH, HL and LH). Based on these results, we developed PL-CNC hydrogels using 1 $U.mL^{-1}$ of thrombin, 5 mM of $CaCl_2$ and variable CNC concentration (0 – 0.61 wt.%).

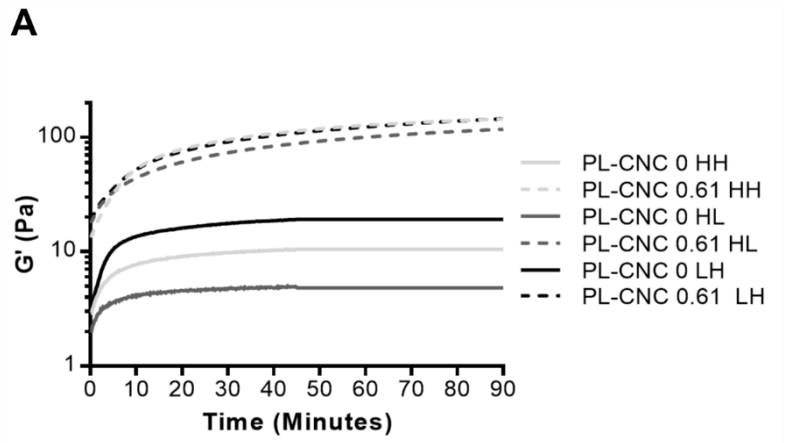


Figure S2. Characterization of hydrogel gelification kinetics. Storage modulus (G') versus polymerization and crosslinking time at 1% strain and frequency of 1 Hz for fibrillar hydrogels with different concentrations of thrombin and calcium (HH, HL and HH formulations), and without (PL-CNC 0) or with (PL-CNC 0.61) CNC incorporation (A).

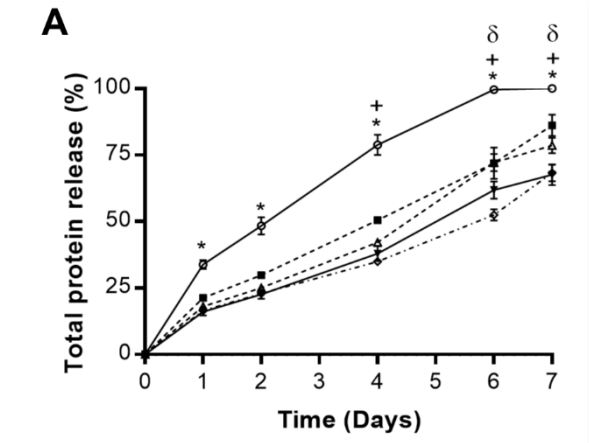
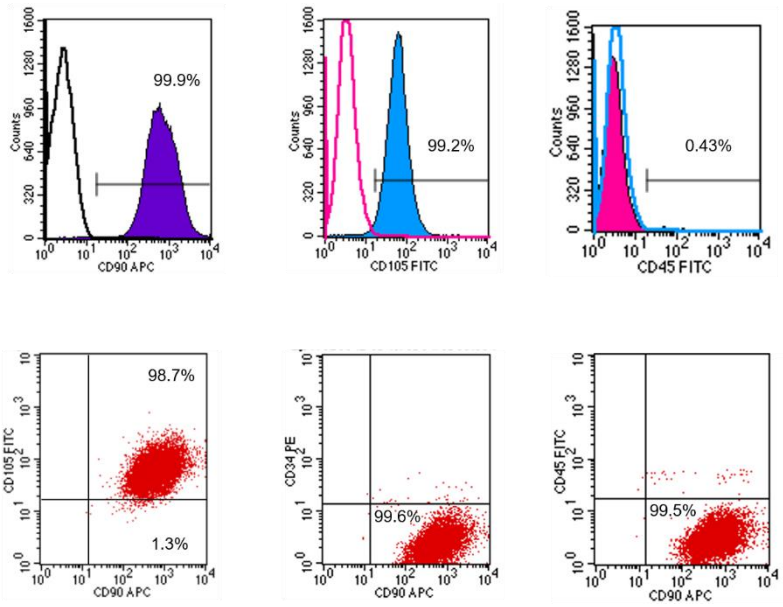


Figure S3. Characterization of hydrogel degradation. Percent of total protein release from PL-CNC hydrogels over 7 days immersed in PBS (A). * PL-CNC 0 vs PL-CNC (0.15-0.65); + PL-CNC 0.15 vs PL-CNC (0.46-0.61) and δ PL-CNC 0.31 vs PL-CNC (0.46-0.61).



	%
CD90+	99.9%
CD105+	99.2%
CD45-	0.43%
CD90+/CD105+	98.7%
CD45-/CD90+	99.5%

Figure S4. Flow cytometry analysis of stemness markers, CD45 (≤2%), CD90 (≥95%) and CD105 (≥95%).

Hematoxylin and eosin (H&E) staining. Eosin stains extracellular and intracellular proteins while hematoxylin stains cell nuclei and some carbohydrates. Eosin is more intense in PL-CNC 0 hydrogels than in CNC-loaded hydrogels, indicating the shrinkage of these hydrogels (Figure S5-B). Furthermore, cells are tightly packed in PL-CNC 0 while cells on hydrogels with CNC are spread and form well-developed cellular networks.

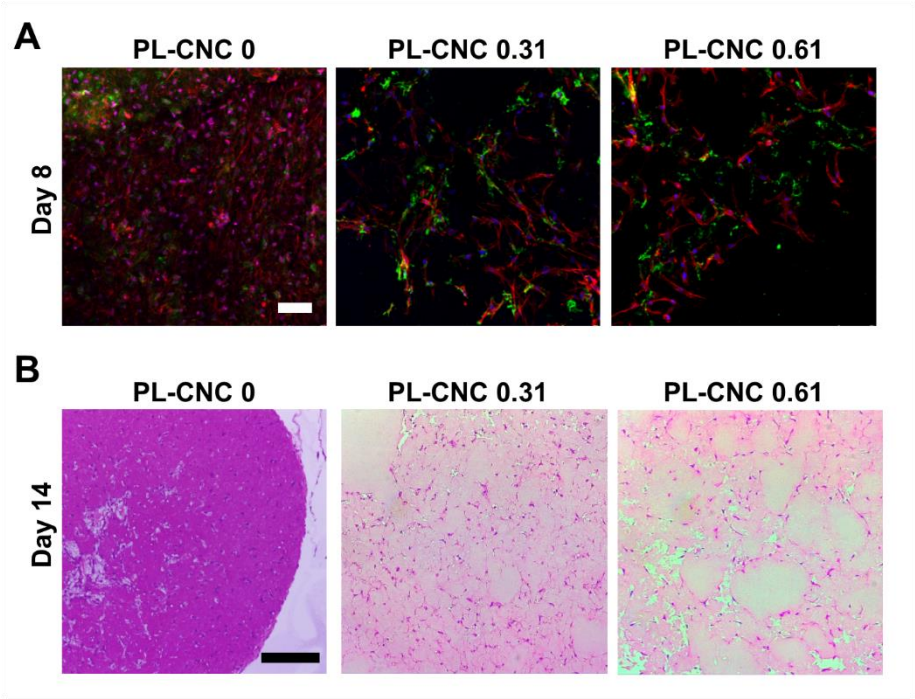


Figure S5. In vitro evaluation of matrix production, cell morphology and hydrogels retraction upon cell encapsulation. Fluorescence microscopy images showing cytoskeleton organization and matrix production after 8 days in culture (A). H&E staining after 14 days in culture showing cellular distribution and (B). Staining collagen III (green), actin (red) and nuclei (blue) (A). Cell nuclei and some carbohydrates (purple) and extracellular and intracellular proteins (pink) (B). Scale bars: 75 µm (A) and 200 µm (C).

References:

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