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# Annals of Anatomy

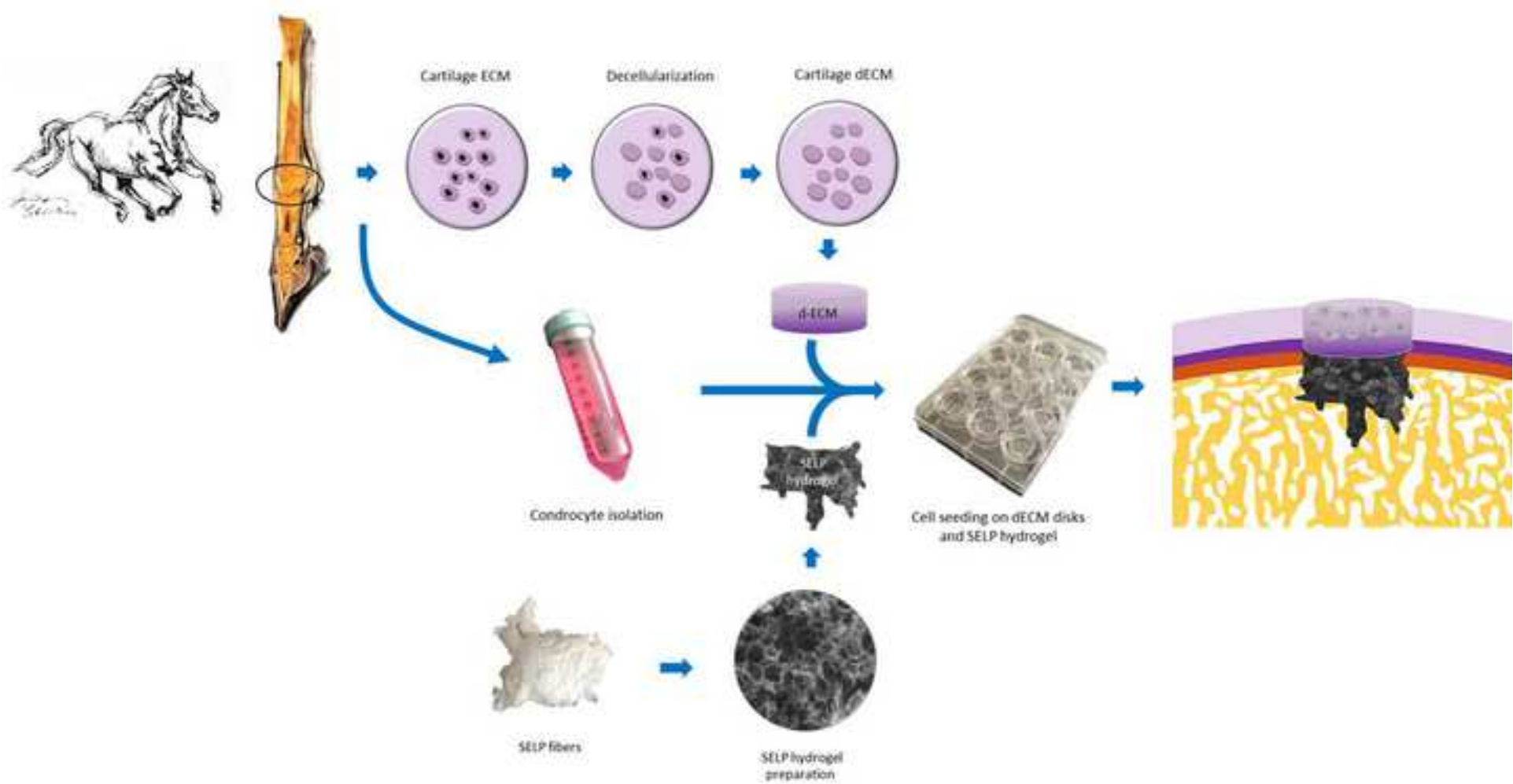
## Biomimetic approach for an articular cartilage patch: combination of decellularized cartilage matrix and silk-elastin-like-protein (SELP) hydrogel

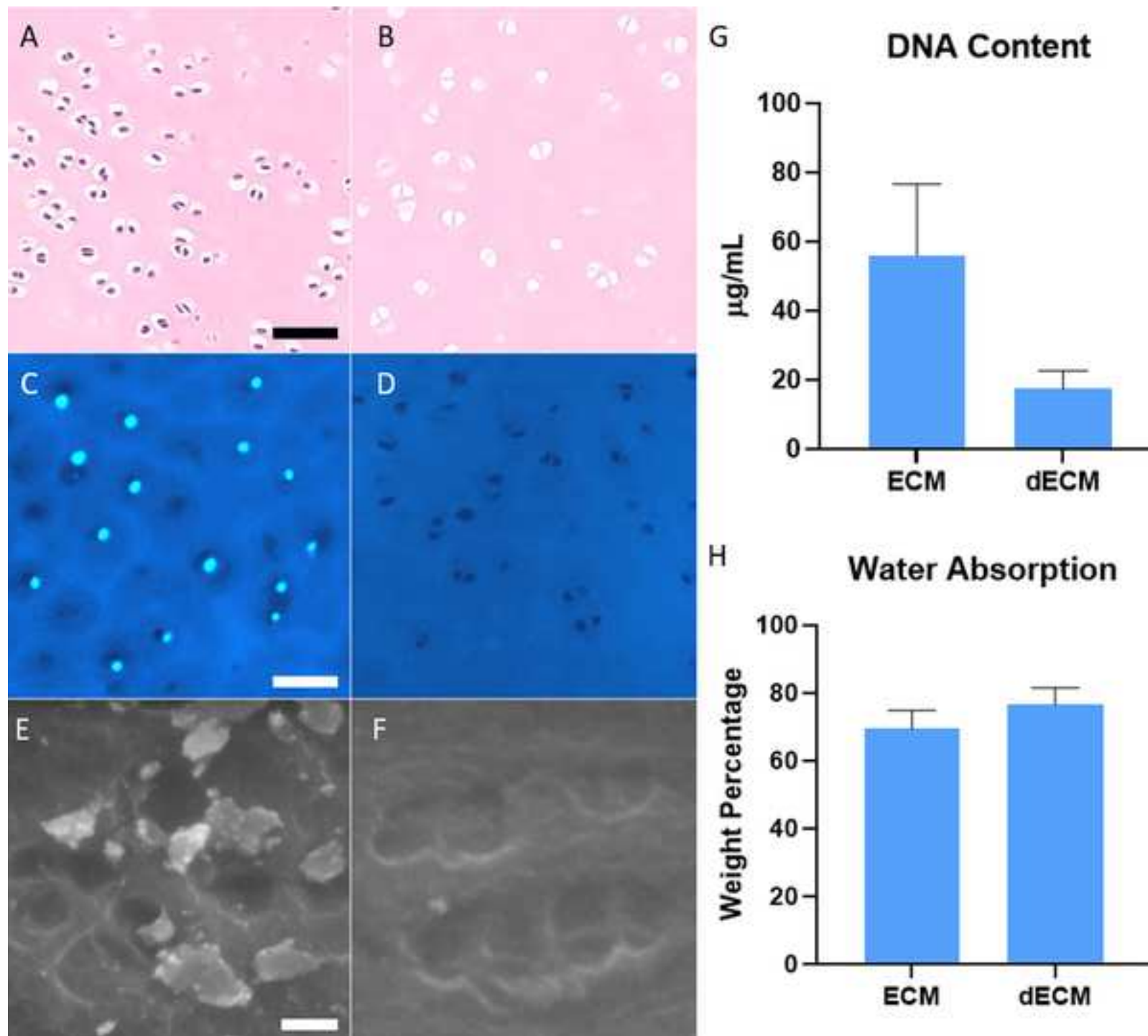
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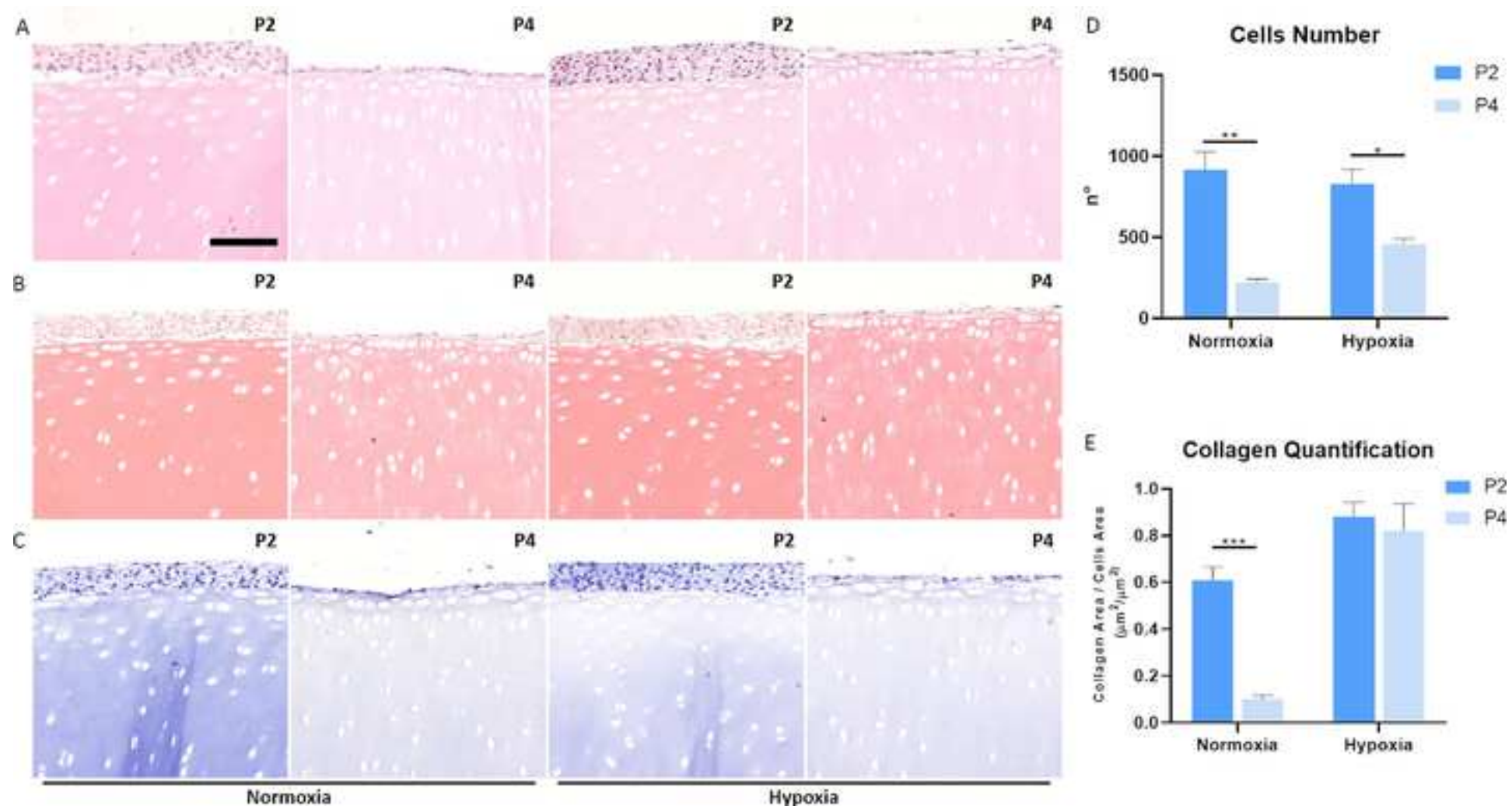
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<b>Abstract:</b>	<p>Articular cartilage degradation due to injury, disease and aging is a common clinical issue as current regenerative therapies are unable to fully replicate the complex microenvironment of the native tissue which, being avascular, is featured by very low ability to self-regenerate. The extracellular matrix (ECM), constituting almost 90% of the entire tissue, plays a critical role in its function and resistance to compressive forces. In this context, the current tissue engineering strategies are only partially effective in restoring the biology and function of the native tissue. A main issue in tissue regeneration is treatment failure due to scarce integration of the engineered construct, often following a gradual detachment of the graft. In this scenario, we aimed to create an adhesive patch able to adequately support cartilage regeneration as a promising tool for the treatment of cartilage injuries and diseases. For this, we produced an engineered construct composed of decellularized ECM (dECM) obtained from horse joint cartilage, to support tissue regeneration, coupled with a Silk-Elastin-Like Proteins (SELP) hydrogel, which acts as a biological glue, to guarantee an adequate adherence to the host tissue. Following the production of the two biomaterials we characterized them by assessing: 1) dECM morphological, chemical, and ultrastructural features along with its capability to support chondrocyte proliferation, specific marker expression and ECM synthesis; 2) SELP microarchitecture, cytocompatibility and mechanical properties. Our results, demonstrated that both materials hold unique properties suitable to be exploited to produce a tailored microenvironment to support cell growth and differentiation providing a proof of concept concerning the in vitro biological and mechanical efficacy of the construct. The SELP hydrogel displayed a very interesting physical behavior due to its high degree of resistance to mechanical stress, which is generally associated with physiological mechanical load during locomotion. Intriguingly, the shear-thinning behavior of the hydrogel may also make it suitable to be applied and spread over non-homogeneous surfaces, therefore, we hypothesize that the hybrid biomaterial</p>

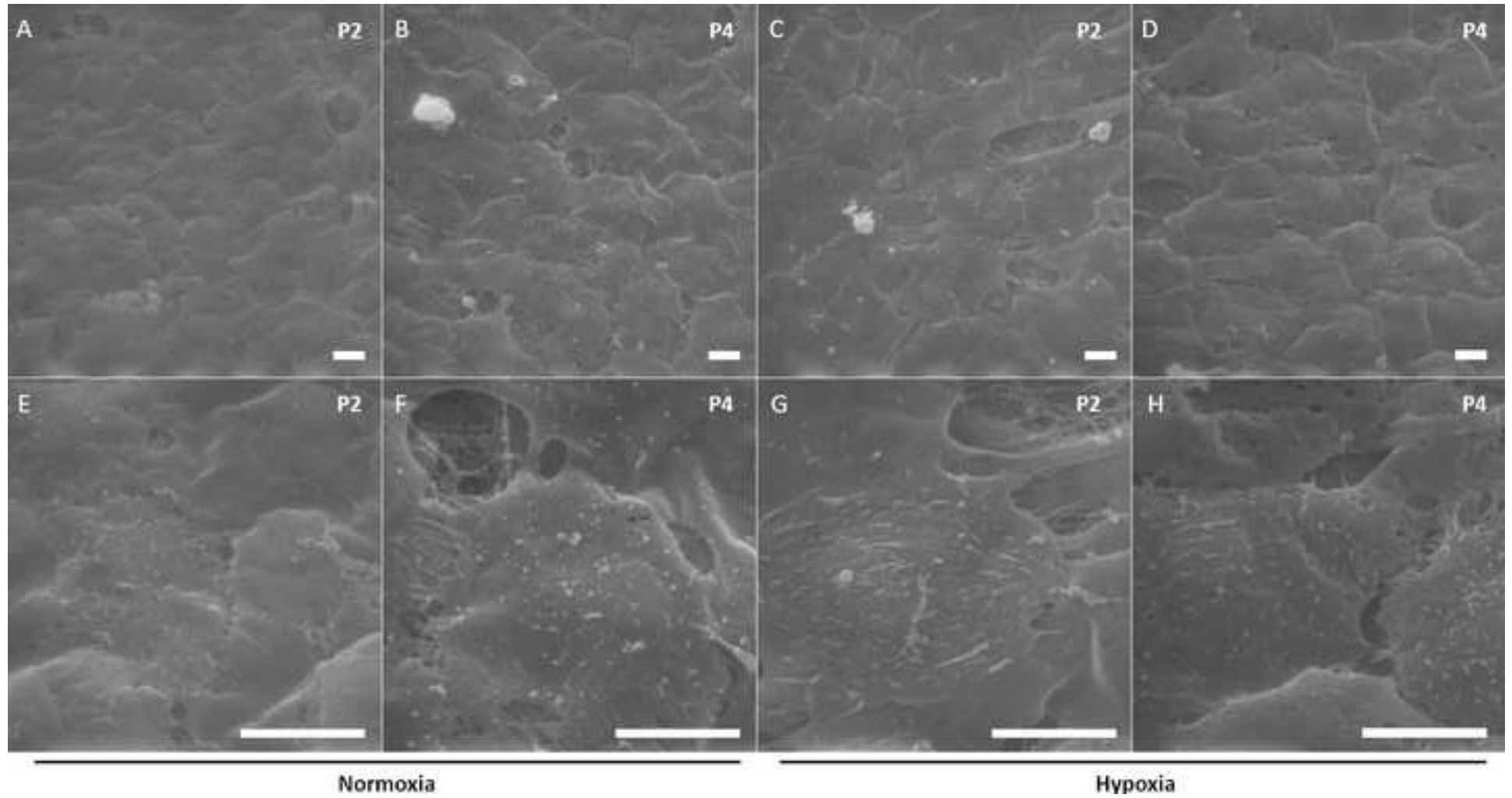
	proposed may be a real asset in the treatment of cartilage defects and injuries.
<b>Suggested Reviewers:</b>	Sivia Clotilde Bianca Modena, Prof University of Milan Faculty of Veterinary Medicine silvia.modina@unimi.it
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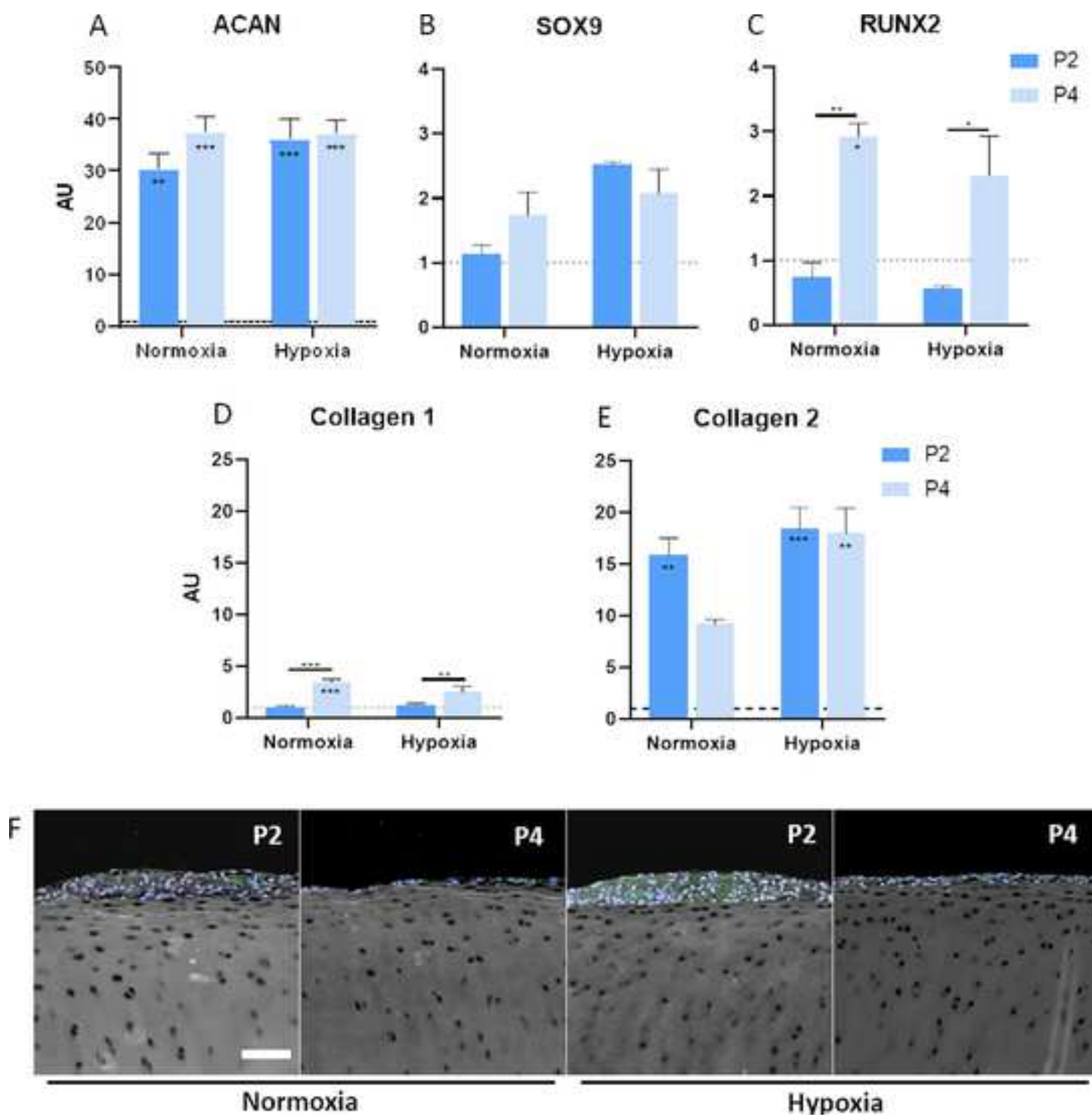
Figure 1



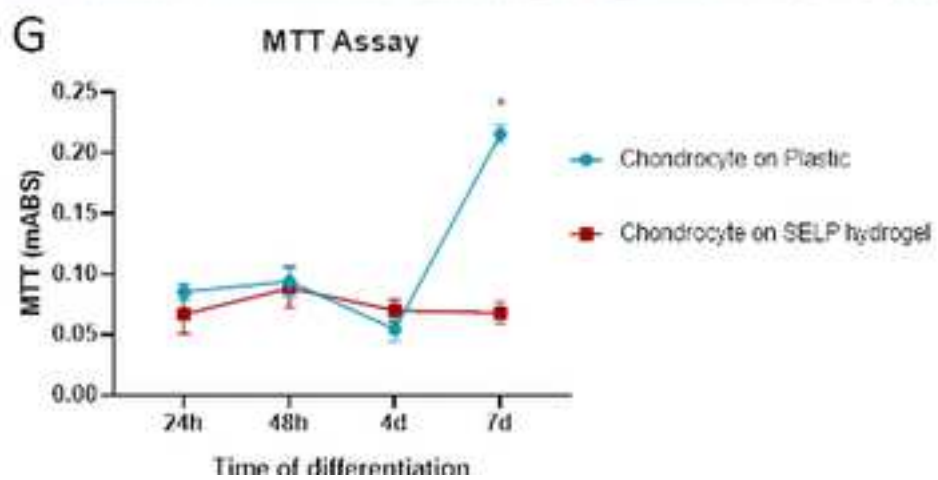
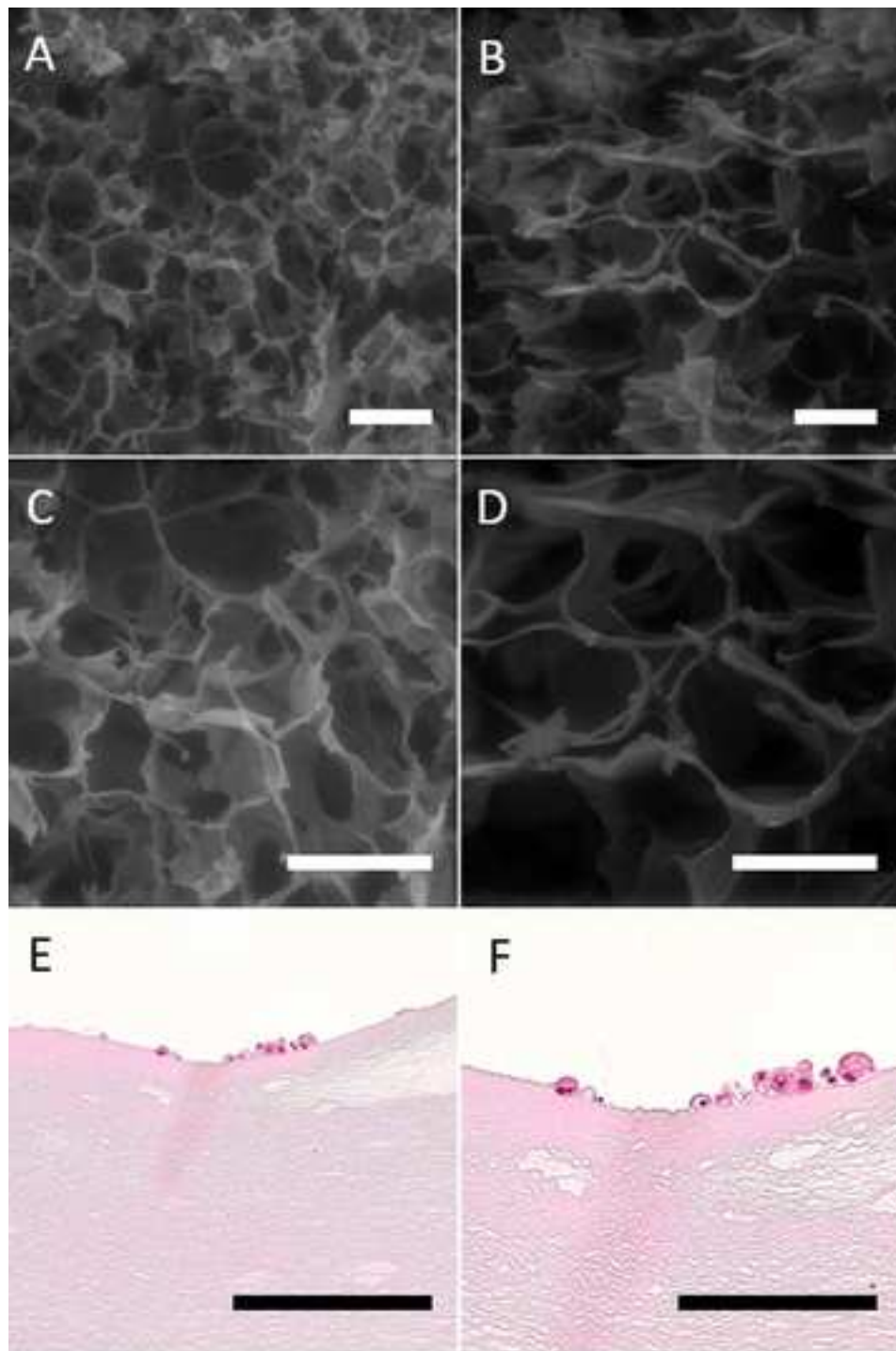


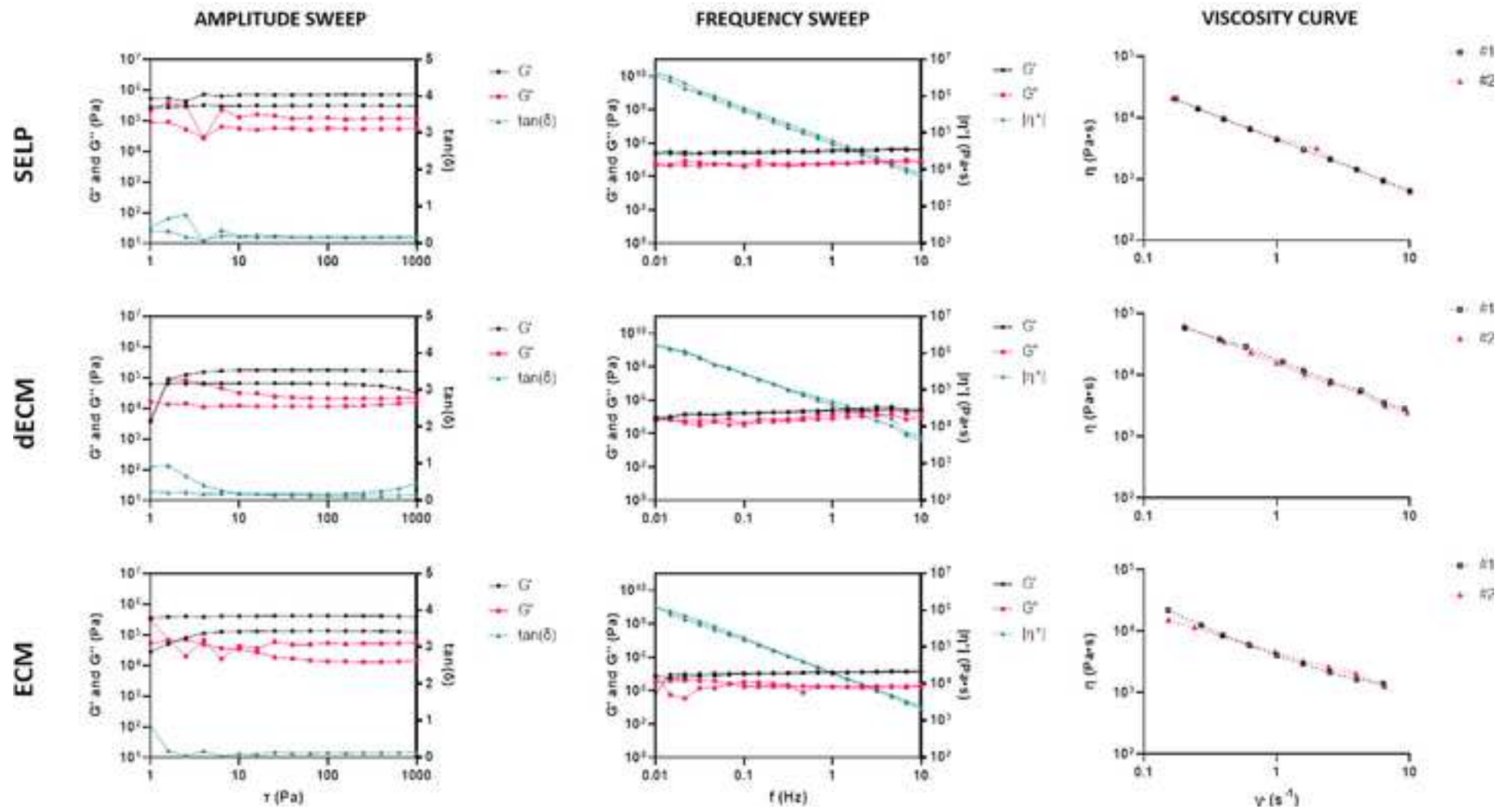


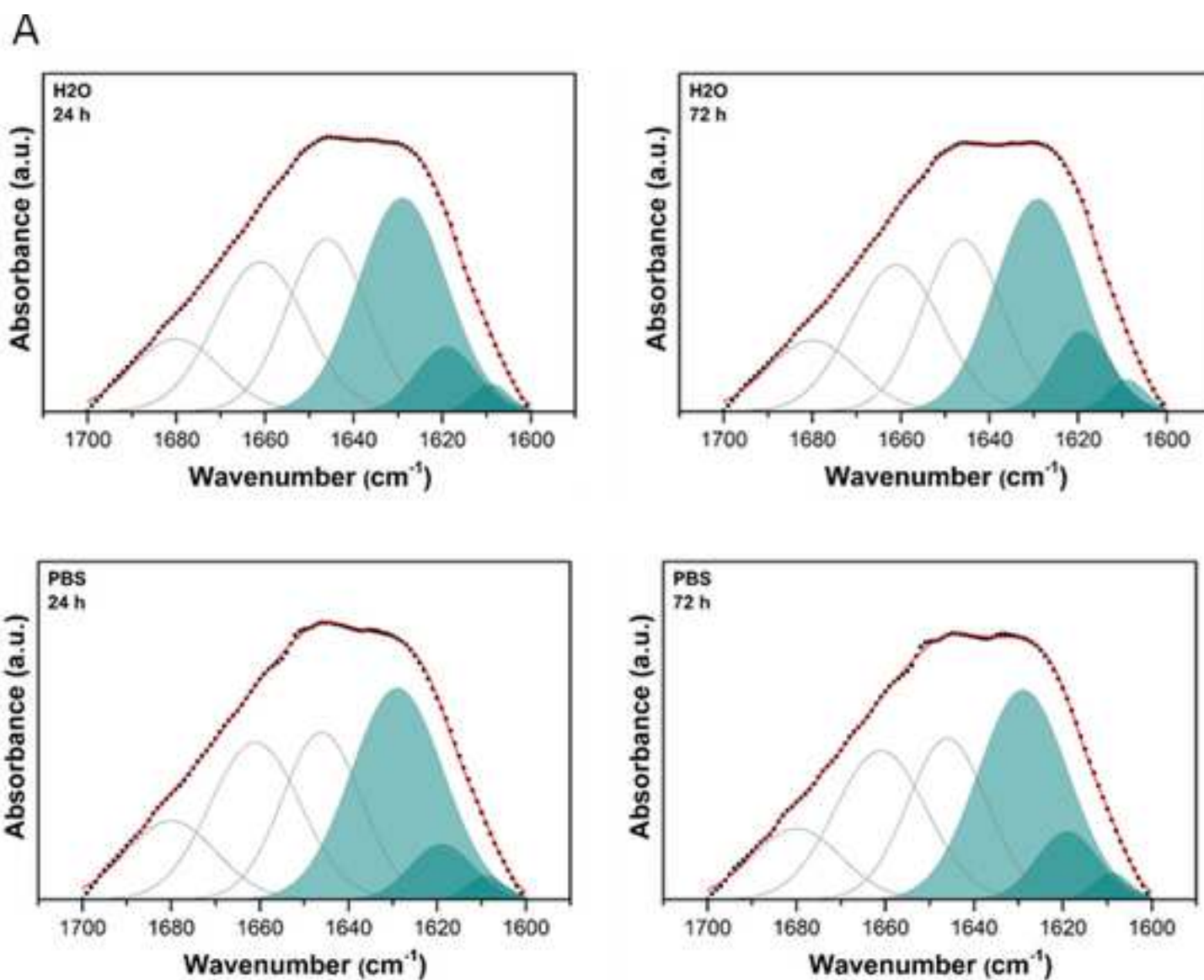












**B**

### Fractional distribution of secondary structures

	$\beta$ - structures	random coils	turns and bends
H <sub>2</sub> O 24 h	52.3	24.0	23.7
H <sub>2</sub> O 72 h	52.8	24.0	23.2
PBS 24 h	52.7	22.8	24.5
PBS 72 h	53.4	22.6	24.1

1 **Biomimetic approach for an articular cartilage patch: combination of decellularized**  
2 **cartilage matrix and silk-elastin-like-protein (SELP) hydrogel**

3

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5 **Ciccimarra<sup>1</sup>, R. Saleri<sup>1</sup>, A. Cacchioli<sup>1</sup>, F. Gazza<sup>1</sup>, R. Machado<sup>2</sup>, L. Ragionieri<sup>1°</sup>, C.**  
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21

22 **Abstract**

23 Articular cartilage degradation due to injury, disease and aging is a common clinical issue as current  
24 regenerative therapies are unable to fully replicate the complex microenvironment of the native tissue which,  
25 being avascular, is featured by very low ability to self-regenerate. The extracellular matrix (ECM), constituting  
26 almost 90% of the entire tissue, plays a critical role in its function and resistance to compressive forces. In  
27 this context, the current tissue engineering strategies are only partially effective in restoring the biology and  
28 function of the native tissue. A main issue in tissue regeneration is treatment failure due to scarce integration  
29 of the engineered construct, often following a gradual detachment of the graft. In this scenario, we aimed to  
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34 adherence to the host tissue. Following the production of the two biomaterials we characterized them by  
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37 cytocompatibility and mechanical properties. Our results, demonstrated that both materials hold unique  
38 properties suitable to be exploited to produce a tailored microenvironment to support cell growth and  
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40 construct. The SELP hydrogel displayed a very interesting physical behavior due to its high degree of  
41 resistance to mechanical stress, which is generally associated with physiological mechanical load during  
42 locomotion. Intriguingly, the shear-thinning behavior of the hydrogel may also make it suitable to be applied  
43 and spread over non-homogeneous surfaces, therefore, we hypothesize that the hybrid biomaterial  
44 proposed may be a real asset in the treatment of cartilage defects and injuries.

45 **Keywords:** Articular Cartilage; Decellularized Extracellular Matrix; Silk-Elastin-Like Protein; Chondrocytes

46

## 47 1 Introduction

48 Articular cartilage damage and degeneration caused by injury, disease and aging are very common clinical  
49 issues. Regenerative therapies are currently unable to replicate the specific microenvironment of the native  
50 articular cartilage (Jiang et al., 2021; Nurul et al., 2021; Xie et al., 2021). This highly hydrated, avascular tissue  
51 displays complex morphological and chemical features, mainly related to the microarchitecture of its  
52 extracellular matrix (ECM), which makes up around 90% of the total volume of the tissue. Articular cartilage  
53 (AC) is divided into four compartments holding a differential microarchitecture comprising a superficial zone,  
54 a transitional zone, a deep zone and a calcified zone. Each of them contributes differently to mechanical  
55 loading, highlighting the critical role of this zonal arrangement for tissue function, particularly for its  
56 resistance to compressive forces, making it essential for the protection of the underlying subchondral bone.  
57 Cartilage is a hypocellular, avascular, aneural, and alymphatic tissue, resulting in limited self-repair capacity  
58 after injuries (Karuppall, 2017). In particular, the lack of vascularization limits the infiltration of progenitor  
59 cells and the consequent ability of the tissue to self-regenerate. Cell-based therapies have demonstrated a  
60 disease-modifying effect on osteoarthritis in preclinical animal models, representing a favorable therapeutic  
61 approach (Boffa et al., 2023). However, lost cartilage is often replaced by fibrocartilage that is mechanically  
62 inferior compared to native tissue (Armiento et al., 2019).

63 Cartilage tissue engineering includes several approaches attempting to address this open challenge through  
64 the use of synthetic materials (Stampoultzis et al., 2021). Although these latter can be modified to mimic the  
65 mechanical and biochemical properties of the cellular microenvironment, they generally fail to fully  
66 reproduce the multitude of interactions occurring in the native tissue. Most likely, this is due to the dynamic  
67 nature of some specific proteins composing the extracellular matrix, which probably underpins a more  
68 nuanced role for ECM in homeostasis and disease (Bonnans et al., 2014). Because of this complexity, research  
69 on cartilage regeneration has not yet reached satisfactory results thus making cartilage repair an open  
70 challenge in the clinical arena (Ngadimin et al., 2021). The decellularized ECM is currently considered a  
71 promising natural substrate for cartilage regeneration (Xia et al., 2019). The use of decellularization  
72 technology preserves natural tissue ECM, while simultaneously eliminating specific homogenous/xenogeneic  
73 cells in tissues or organs, allowing to synthesize minimally immunogenic scaffolds with key biological and  
74 biomechanical properties (Brown et al., 2022; Kasravi et al., 2023; Morris et al., 2017). To date, decellularized  
75 extracellular matrix (dECM) is a well-recognized biomaterial developed to engineer more than 15 types of  
76 tissues and organs, being able to preserve the native environment by promoting cell proliferation and  
77 providing biochemical cues for cell differentiation (Brown et al., 2022). Also, in materials science and tissue  
78 engineering, there is currently a widespread use of natural hydrogels, namely hydrophilic polymers networks  
79 able to absorb large amounts of water while maintaining their structure suitable to be used in different forms  
80 such as films, scaffolds, nanoparticles and drug carriers (Li et al., 2019; Wei et al., 2021; Zhang and  
81 Khademhosseini, 2017). In tissue regeneration approaches, to avoid treatment failure due to the detachment  
82 of components and to promote construct integration with the host tissue during regeneration, it is  
83 mandatory that the biomaterials adhere perfectly to the latter (Sani et al., 2019; Wang et al., 2007; Zhou et  
84 al., 2018). In this scenario, our approach aims to combine the use of dECM and a Silk-Elastin-Like Protein  
85 (SELP) hydrogel for the creation of a cartilage patch with suitable biological and mechanical properties, in  
86 which the SELP hydrogel acts as a powerful bioadhesive glue able to firmly bond the construct to the damaged  
87 cartilage area and to effectively guarantee its integration with the host tissue. SELPs are genetically  
88 engineered protein polymers that combine in the same polypeptide chain the properties of both silk and  
89 elastin (Machado et al., 2015, 2013b). Due to their versatility of processing and biocompatibility, SELPs have  
90 been engineered for a wide variety of biomedical applications (Correia et al., 2019; Machado et al., 2015;  
91 Shen et al., 2022; Varanko et al., 2020). SELP-based therapies for cartilage regeneration are still in the very

92 early stages of development (Cipriani et al., 2018; Haider et al., 2008) but they are certainly very promising  
93 and with the advancement of research in this field, these recombinant protein polymers will likely become  
94 an increasingly important tool in the treatment of cartilage injuries and diseases.

## 95 **2 Materials and methods**

96 The experimental setup is summarized in Figure 1.

### 97 **2.1 Articular Cartilage harvest**

98 Articular cartilage was obtained from metacarpophalangeal and metatarsophalangeal joints of horses (*Equus*  
99 *ferus caballus*) ranging from 5 to 8 years old (n=8), slaughtered for human consumption in a slaughterhouse  
100 certified by the Italian Ministry of Health according to the Regulation (EC) 853/2004 (Zerbini & Ragazzi S.R.L.,  
101 Correggio (RE), Italy; approval nr. CE-IT 798-M). Joints were immediately delivered to our laboratory and then  
102 used to collect the cartilage. After arthrotomy and cartilage exposure, the joints were carefully examined,  
103 and those with macroscopic lesions related to overt osteoarthritis (OA) or with evidence of synovitis were  
104 excluded from the study. Cartilage slices approximately 1 - 1.5 mm thick were cut with a surgical blade; to  
105 obtain standardized disks of cartilage, a punch of 8 mm (n = 192) diameter was used. Cartilage disks were  
106 immediately frozen in liquid nitrogen and stored at -80 °C until use. All the procedures were undertaken in  
107 a sterile environment.

### 108 **2.2 Cartilage Decellularization**

109 Frozen cartilage disks were defrosted at room temperature and incubated for 1 h at 37 °C, under gentle  
110 agitation, in a solution of 0.25% Trypsin + EDTA 1X. After two washing steps (5 min each) in Phosphate  
111 Buffered Saline (PBS), cartilage disks were incubated in 3 % Triton X-100/PBS, under agitation at 4 °C for 48  
112 h. Subsequently, disks were rinsed twice in PBS (30 min each) and then incubated in 1 % Sodium Dodecyl  
113 Sulfate (SDS) at 4 °C under constant stirring for 24 h. After four washing cycles (20 min each), disks were  
114 incubated overnight (O/N) at 37 °C in a solution containing 1.5 mg/mL DNase and RNase in 0.15 M NaCl and  
115 30 mM MgCl<sub>2</sub>·6H<sub>2</sub>O. Cartilage disks were soaked in a cartilage washing solution of PBS, 1% Pen-Strep and 1%  
116 amphotericin for 1 h and then treated with liquid nitrogen and stored at -80 °C. Before use, disks were  
117 sterilized by UV under a laminar flow hood (Thermo Scientific) for 30 min per each side, at 18–21 °C.

118

### 119 **2.3 DNA content evaluation**

120 The effectiveness of cartilage decellularization process was assessed through both nuclear counterstaining  
121 with DAPI (4',6-diamidino-2-phenylindole) and DNA content assay. For nuclear counterstaining, after  
122 deparaffinization and rehydration, sections were covered with 300 nM DAPI (Invitrogen) staining solution for  
123 5 min and rinsed three times in PBS (5 min each). Fluorescent whole slide imaging (WSI) was acquired using  
124 a NanoZoomer S60 scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and DAPI fluorescence was  
125 detected. DNA content was evaluated by DNeasy quantification kit (Qiagen) following the manufacturer's  
126 instructions. DNA concentration (n=3) was measured at 260 nm using a nanodrop spectrophotometer  
127 (Thermo Scientific NanoDrop Lite Spectrophotometer).

128

### 129 **2.4 Water absorption capacity**

130 The initial dry weights of native equine and decellularized cartilage disks were carefully measured (W<sub>0</sub>). The  
131 scaffolds were then immersed in PBS at 37 °C (pH 7.4) for 8 h. Cartilage disks were taken out from PBS and  
132 the wet weights were recorded as W<sub>1</sub>. The water absorption ratio was determined by applying the following  
133 formula:  $(W_1 - W_0)/W_0 \times 100\%$ , where W<sub>0</sub> and W<sub>1</sub> are the initial and final weights of the scaffolds,  
134 respectively.

135

## 136 2.5 SELP preparation

137 The recombinant SELP was biologically produced in microbial cell factories and purified according to  
138 previously described procedures (Machado et al., 2013a). The complete amino acid sequence was previously  
139 reported and comprises 9 tandem repeats of 5 silk blocks (sequence GAGAGS) fused with 9 elastin blocks  
140 (sequence VPAVG) (Fernandes et al., 2018; Machado et al., 2013a). To prepare the SELP hydrogel, 32 mg of  
141 pure lyophilized SELP were weighed and then clipped and frayed using tweezers, to facilitate the subsequent  
142 solubilization in cold deionized water (dH<sub>2</sub>O) or PBS (1X) for a concentration of 8% (v/v). Dissolution was  
143 ensured by maintaining the protein solution overnight at 4 °C under constant stirring (200 rpm). After  
144 dissolving, the gel-like solution was incubated for 24 h and 72 h in an oven at 37 °C and used for subsequent  
145 assays.

## 146 2.6 Rheological characterization

147 The viscoelastic properties of both dECM and SELP were measured using three types of rheological tests  
148 carried out using a rotational HAAKE Mars 60 (Thermo Scientific) measuring system with a gap of 0.5 mm  
149 between plates and described below.

- 150 · Amplitude sweep: tests were performed by applying a stress with increasing amplitudes, ranging from 1 to  
151 1000 Pa, and a constant frequency of 1 Hz, to determine the linear viscoelastic range (LVER) (range where  
152 the elastic and viscous moduli are independent from the strain).
- 153 · Frequency sweep tests: the measurements were performed by applying a stress with increasing  
154 frequencies, ranging from 0.01 to 10 Hz at a constant oscillation amplitude of 50 Pa (within the linear  
155 viscoelastic range) to obtain the elastic and viscous moduli ( $G'$  and  $G''$ , respectively) along with the complex  
156 viscosity ( $\eta^*$ ).
- 157 · Viscosity curve: the measurement was performed by applying a shear rate increasing from 0.1 to 10 s<sup>-1</sup> and  
158 calculating the respective viscosity.

159 All the tests were performed in duplicate at 37 °C to simulate the potential behavior of the biomaterials when  
160 in the body.

## 162 2.7 Structural characterization of SELP by ATR-FTIR

163 The secondary structure of the SELP-based materials was assessed by attenuated total reflection Fourier-  
164 transform infrared (ATR-FTIR) spectroscopy in a range between 4000 – 400 cm<sup>-1</sup> using a Spectrum Two™ from  
165 Perkin Elmer (Perkin Elmer, Waltham, MA, USA) equipped with a diamond ATR. Spectra were collected with  
166 a resolution of 4 cm<sup>-1</sup>. Analysis of the secondary structure content was carried out in the amide I band region  
167 (1700 – 1600 cm<sup>-1</sup>) following the procedures described elsewhere (Correia et al., 2019; Pereira et al., 2021,  
168 2017). Afterwards, the amide I band region was truncated and normalized, followed by baseline correction,  
169 using OriginPro 9.0 software (Or, Northampton, MA). Briefly, for component analysis, the second derivative  
170 spectra of amide I band were smoothed with an eleven-point Savitsky-Golay smoothing function. The number  
171 of components and their peak positions were determined by second derivatization and used as initial  
172 parameters for iteratively fitting with a Gaussian function ( $R^2 > 0.999$ ). To ensure a comparable secondary  
173 structure assignment across all the samples, curve fitting was performed using the same set of parameters.  
174 The structural conformations were assigned by reference to literature (Table 1) and the contribution of each  
175 fitted component to the amide I band was determined by integrating the area under the curve and  
176 normalizing the value for the total area of amide I.

177 **Table 1.** Vibrational band assignments used for the analysis of the secondary structure content (Machado et  
178 al., 2015; Pereira et al., 2021, 2017).



Wavenumber [cm <sup>-1</sup> ]	Secondary structure assignment
1605 – 1615	aggregated strands
1616 – 1621	aggregated beta-strand/beta-sheets (weak) <sup>1</sup>
1622 – 1627	beta-sheets (strong) <sup>1</sup>
1628 – 1637	beta-sheets (strong) <sup>2</sup>
1638 – 1655	random coils
1656 – 1662	alpha-helices
1663 – 1696	turns and bends
1697 – 1703	beta-sheets (weak) <sup>1</sup>

179 <sup>1</sup>Intermolecular beta-sheets    <sup>2</sup>Intramolecular beta-sheets

## 180 **2.8 Scanning Electron Microscopy**

181 Scanning Electron Microscopy (SEM) was performed for SELP and for cartilage disks before and after  
182 decellularization to evaluate disk microstructure, and then, after cell seeding, to evaluate cell adhesion and  
183 interaction with the biomaterial. Samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate  
184 buffer (pH 7.3) for 2 h at 4 °C. After dehydration through a graded series of ethanol, specimens were critical-  
185 point dried with liquid carbon dioxide (CPD 030 Baltec, Wallruf, Germany) and then sputter-coated (Balzers  
186 device) with gold-palladium (Plano, Germany) with a SCD 040 coating device (Balzer Union, Wallruf,  
187 Germany). All the samples were observed using a Zeiss DSM 950 scanning electron microscope with  
188 accelerating voltage set at 10 KV (Zeiss, Jena, Germany).

## 190 **2.9 Cell isolation and culture**

191 Chondrocytes were isolated from the articular cartilage following a protocol previously described (De Angelis  
192 et al., 2020). Freshly isolated chondrocytes were plated (P1: passage 1;  $2 \times 10^4$  cells/cm<sup>2</sup>) in triplicate in a  
193 Petri dish (57 cm<sup>2</sup>) (Sarstedt, Nümbrecht, Germany), cultured in DMEM with 4.5 g/L glucose (SIGMA Aldrich)  
194 and 10% FBS at 37 °C and 5% CO<sub>2</sub> in a humidified atmosphere. Medium was replaced every three days until  
195 monolayer formation when chondrocytes were detached by 0.25% trypsin – 0.02% EDTA (SIGMA Aldrich)  
196 and counted using a Bürker hemocytometer. Then,  $2 \times 10^4$  cells were seeded in a subsequent subculture  
197 (Passage P2, P3, P4). In the present study, isolated chondrocytes at both Passage number 2 (P2) and Passage  
198 number 4 (P4) (n= 3 each in triplicate) were used. A suspension of  $2 \times 10^4$  cells/cm<sup>2</sup> cells were seeded in 6-  
199 well plates (Sarstedt, Nümbrecht, Germany) with and without the dECM disks and gelled SELP support, and  
200 then cultured in medium DMEM 4.5 g/L glucose supplemented with 50 µg/mL 2-phospho-L-ascorbic acid,  
201 with 10% FBS, and changed twice a week. Cells were incubated at 37 °C in normoxic (19% of O<sub>2</sub>) or hypoxic  
202 (5% of O<sub>2</sub>) conditions for the time specified for each single experiment.

## 204 **2.10 Histology**

205 Samples were fixed in 10% formalin for 24 h at 4 °C and histologically processed (Leica, HistoCore PEARLS).  
206 Sections of 5 µm were obtained with a rotary microtome (Slee Cut 6062; Slee Medical, Mainz, Germany) and  
207 stained with Hematoxylin and Eosin (H&E), Picro-Sirius Red Stain Kit (PSR), and Toluidine Blue stain kit (TB),  
208 following the manufacturer's instructions (Histo-Line Laboratories). The sections were acquired as whole  
209 slide images (WSI) by a digital slide scanner (Nanozoomer S-60, Hamamatsu, Japan).

### 210 **2.11 Cells and Collagen Fiber Quantification**

211 After two weeks of culture, the number of chondrocytes on decellularized cartilage was assessed using  
212 QuPath version 0.4.3 (Bankhead et al., 2017). For all samples, cells were detected at the interface between  
213 chondrocytes and cartilage in H&E stain, using the native tool of the software 'Cell Detection'. The connective  
214 component was evaluated on PSR stains using the 'Pixel Classifier' classification algorithm, trained on  
215 representative images, in 10 regions of interest (ROI) per sample. The area occupied by the connective  
216 component was compared to that of the ROI and expressed as a percentage of occupied area.

### 217 **2.12 Cell viability**

218 MTT assay was performed to evaluate chondrocytes viability on the SELP hydrogel. Chondrocytes were  
219 seeded in 24-well plates with a density of  $1 \times 10^4$  cells/well cultured in DMEM and incubated with 20  $\mu$ L (5  
220 mg/mL) of MTT solution. After 4 h of incubation, the medium and the MTT solution were removed and cells  
221 were lysed with 150  $\mu$ L DMSO. The precipitated formazan crystals were solubilized, and detection was  
222 performed at 490 nm using a Victor-3™ 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA).

### 223 **2.13 Gene Expression analysis**

224 Total RNA was extracted from cells seeded on plastic and on dECM using TRIreagent (Ambion, Inc., Austin,  
225 TX), according to the manufacturer's instructions. dECM disks were transferred into liquid nitrogen and  
226 disrupted with mortar and pestle. TRIreagent (1 ml) was then added to the powder and RNA was extracted  
227 according to the manufacturer's instructions. Purity and concentration were assessed by UV  
228 spectrophotometry (GeneQuant Pro, Amersham Pharmacia Biotech-GE Healthcare Life Sciences, Little  
229 Chalfont, UK) at 260/280 nm and 260/230 nm respectively. RNA integrity and quality was evaluated by using  
230 an Agilent Bioanalyzer 2100 and RNA 6000 Labchip kit (Agilent Technologies, Santa Clara, CA, USA).

231 Prior to cDNA synthesis, RNA samples were treated with DNase (Sigma). Total RNA (1  $\mu$ g/20  $\mu$ L) was reverse-  
232 transcribed using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The  
233 reverse-transcription reaction was performed according to the manufacturer's instructions, under the  
234 following thermal conditions: 10 min at 25 °C, 120 min at 37 °C followed by 5 min at 85 °C. cDNA samples  
235 were stored at 20 °C until PCR was performed.

236 cDNA concentration was assessed by UV-spectrophotometry (GeneQuant Pro) and 5 ng of each sample were  
237 used as a template for real-time quantitative PCR (qPCR) performed by using a StepOne thermocycler  
238 (Applied Biosystems, StepOne software v. 2.1). The cDNA (5 ng/20  $\mu$ L) was amplified with Fast SYBR Green  
239 Master Mix (Applied Biosystems) along with specific sets of primers at 300 or 500 nM. The primers were  
240 designed based on published gene sequences or by using Primer Express® software package (Applied  
241 Biosystems) to create oligonucleotides with similar melting temperatures and minimal self-complementary,  
242 purchased from Eurofins MWG Operon (Ebersberg, Germany). Details of each primer set for detection of  
243 gene expression are reported in Table 2. The reference gene HPRT1 was selected as endogenous control  
244 according to minimal intra-/inter-assay variation. Samples were kept at 95 °C for 20 s (hold step) and then  
245 subjected to 40 cycles consisting of a denaturation step at 95 °C for 3 s followed by an annealing/extension  
246 step at 60 °C for 30 s. Fluorescence due to SYBR Green I incorporation was acquired at the end of the  
247 extension step. A no-RT and a no-template control (NTC) were included in each experiment. A melting curve  
248 analysis for specific amplification control was performed (from 60 °C to 95 °C) at the end of the amplification  
249 cycles. NTC controls were assumed as negative and reliable if the quantification cycle (Cq) was  $\geq 35$ . Data  
250 were analyzed according to the  $2^{-\Delta\Delta C_t}$  method (Schmittgen and Livak, 2008) in which expression levels of  
251 each gene are normalized to the HPRT1 cDNA amount and expressed as arbitrary Unit (AU). All experiments  
252 were performed in triplicate.

253 **Table 2.** Sequences of primer pairs used for RT-qPCR analysis.

Gene/Protein designation	Forward (F) and reverse (R) primers	Concentration (nM)
HPRT1/Hypoxanthine phosphoribosyltransferase	F 5'-AATTATGGACAGGACTGAACGG-3' R 5'-ATAATCCAGCAGGTCAGCAAAG-3'	300
RUNX2	F 5'-CTGTGGTTACTGTCATGGCG-3' R 5'-TCGTTGAACCTTGCCACTTG-3'	300
COL1A1/Collagen type I alpha 1 chain	F 5'-AGAAGAAGACATCCCAGCAGTCA-3' R 5'-CAGGGCTCGGGTTCCATA-3'	500
COL2A1/Collagen type II alpha 1 chain	F 5'-GGATGGCTGCACGAAACAC-3' R 5'-CAGGCGGAGGTCTTCTG-3'	300
SOX9/Transcription factor SOX9	F 5'-TACCACCTTGTGCGAATCAGT-3' R 5'-GGTCACACGGTTCTCCATCAT-3'	300
ACAN/Aggrecan	F 5'-GACCACTTTACTCTTGCGTTTG-3' R 5'-GTCAGGGTCTGAAACGTCTACTGA-3'	500

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## 2.14 Immunofluorescence

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Immunofluorescent (IF) reactions were performed on paraffin embedded decellularized cartilage seeded and cultured with chondrocytes to localize collagen type II within the ECM. Briefly, paraffin embedded sections were deparaffinized, rehydrated, and exposed to 10 mM citrate buffer pH 6.0, at a boiling point for antigen retrieval. After cooling, slides were rinsed in a washing buffer and then incubated in blocking buffer (0.3 M glycine, 5% bovine serum albumin in PBS 1X (Sigma-Aldrich, USA)) for 15 min at room temperature (RT). Then, sections were incubated with primary antibody (anti-collagen type II: 2 µg/mL (orb 10436, Byorbit, Cambridge, UK) O/N at 4 °C and with secondary antibody (AffiniPure goat anti-rabbit Alexa Fluor-488 conjugated: 3 µg/ml (111-545-144, Jackson ImmunoResearch, Cambridge, UK) for 45 min at RT. After each step, the sections were washed with PBS.

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Lastly, the nuclei were counterstained with 300 nM of DAPI (Cat#D1306, Invitrogen, USA) solution for 5 min and mounted with ProLong Diamond Antifade Mountant (Cat# S36963, Invitrogen, USA). Fluorescent WSI were acquired using a NanoZoomer S60 scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan).

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## 2.15 Statistical Analysis

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The experimental results are reported as means and standard deviation. In all experiments, each treatment was performed in triplicate. Statistical analyses were performed using Prism 8 software (GraphPad Software Inc., San Diego, CA). One and Two-way analysis of variance (ANOVA) were performed, followed by Dunnett's or Tukey's multiple comparisons post hoc tests. For all the applied tests, a  $P < 0.05$  (\*) was considered as statistically significant.

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## 3 Results

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### 3.1 Decellularized cartilage characterization

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Morphological, chemical and ultrastructural investigations were performed on the cartilage disks to verify the decellularization efficacy. The native equine cartilage used as control showed the classical hyaline cartilage morphology with randomly dispersed lacunae characterized by chondrocytes embedded in an extensive ECM (Figure 2A, C). The decellularized cartilage displayed a well-preserved ECM with empty lacunae without any cellular debris, confirming the decellularization effectiveness (Figure 2B). Nuclear counterstaining with DAPI revealed strong fluorescence in cells from native cartilage, while no cells were

284 observed in the decellularized cartilages (Figure 2C, D). From the ultrastructural standpoint, SEM analysis  
285 showed a preserved ECM microarchitecture, featured by a compact fibrillar matrix, and rounded, empty  
286 lacunae, providing further evidence that decellularization had properly taken place (Figure 2E, F).  
287 Decellularization was also confirmed through the quantitative assessment of DNA content (Figure 2G). In  
288 addition, the quantitative analysis of the water absorption ratio (Figure 2H) showed that the decellularization  
289 process did not alter this parameter, demonstrating comparable results between the decellularized and the  
290 native tissue.

### 291 **3.2 Cell behavior on the decellularized cartilage**

292 Chondrocytes at P2 showed a greater capacity of adhesion to the disk of dECM compared to P4 cells, in both  
293 normoxia and hypoxia conditions. Histological analysis, performed after 2 weeks of culture, showed multiple  
294 layers of P2 cells with a roundish morphology, whereas the P4 chondrocytes formed a monolayer  
295 characterized by a flattened cell morphology (Figure 3A). The histological observation was supported by the  
296 results of cell proliferation assay (Figure 3D), which showed a statistically significant decrease in cell  
297 proliferation at P4. The different oxygen conditions did not substantially affect the proliferation; however,  
298 hypoxia seemed to stimulate the production of extracellular matrix, resulting in a more abundant and  
299 compact matrix as revealed by both Picrosirius red and Toluidine blue stains (Figure 3B, C). This was also  
300 supported by collagen quantification (Figure 3E). Electron microscopy analysis revealed that chondrocytes  
301 were uniformly distributed and formed a monolayer on the surface of the dECM (Figure 4). At both P2 and  
302 P4, cells showed a roundish morphology with a slightly prominent nucleus and formed homogeneous  
303 monolayer sheets with a high degree of cell-cell interaction (Figure 4). ECM synthesis appeared particularly  
304 evident in P2 cells exposed to hypoxic condition (Figure 4G).

### 305 **3.3 Gene expression on decellularized cartilage**

306 When used as substrate for chondrocytes, the dECM significantly improved the expression of typical markers  
307 of chondrocyte maturity, namely collagen II and aggrecan, related to fibrillar and proteoglycan synthesis  
308 (Figure 5A, E). The combination of dECM and hypoxia sustained collagen II expression in both P2 and P4  
309 chondrocytes, whereas P4 cells did not reach the same expression levels under normoxic condition. This  
310 result was confirmed via immunofluorescence analysis (Figure 5F), which displayed an increased level of  
311 collagen II within the extracellular matrix newly deposited by chondrocytes during hypoxia. Aggrecan  
312 expression remained high in all the experimental conditions tested (Figure 5A). Consistent with the data  
313 reported above concerning matrix markers, the expression of the chondrogenic transcription factor Sox9  
314 resulted increased by hypoxia in both P2 and P4 chondrocytes (Figure 5B). However, dedifferentiation  
315 markers, collagen I and Runx2, were significantly increased only in P4 cells under normoxia and hyperoxia  
316 conditions (Figure 5C, D).

### 317 **3.4 SELP structure**

318 SEM analysis of SELP hydrogels showed a 3D porous microarchitecture featured by a well interconnected  
319 structure without morphological differences between the different conditions tested, solvent (dH<sub>2</sub>O or PBS)  
320 and time (24 h and 72 h) (Figure 6A-D). The analysis of histological sections revealed that chondrocytes  
321 cultured onto the SELP maintain the physiological roundish morphology along with the organization in  
322 multicellular clusters (Figure 6E, F). The cytocompatibility of the SELP hydrogel was confirmed by MTT  
323 analysis, which showed no cytotoxicity during the incubation time, characterized by an initial phase of  
324 adaptation up to 4 days, followed by a cell activation phase. After 7 days of incubation, cells started to slightly  
325 lose their ability to proliferate compared to plastic (cell culture plates) seeding ( $P < 0.05$ ), indicating a loss of  
326 differentiation state on the 2D cultures (Figure 6G). To evaluate structural changes deriving from the solvents  
327 and/or incubation time, the secondary structure of SELP was assessed by FTIR (Figure 7). Secondary structure

328 analysis with curve fitting was performed in the amide I band region ( $1600 - 1700 \text{ cm}^{-1}$ ), which originates  
329 from the C=O stretching vibration of the amide group and is the most sensitive spectral region used for  
330 protein studies (Correia et al., 2019; Fernandes et al., 2018; Pereira et al., 2017). For all samples, more than  
331 half of the secondary structures are attributed to  $\beta$ -structures ( $\beta$ -sheets and aggregated strands), which are  
332 characterized by strong hydrogen bonding between the polypeptide chains (Machado et al., 2015; Pereira et  
333 al., 2017). Comparing the different conditions (solvent and incubation time), no relevant changes were  
334 observed in the quantitative structural analysis (Figure 7). Since the hydrogel was proposed as a bioadhesive,  
335 its behavior when subjected to physical stress was preliminary evaluated by an adhesion test over time  
336 (supplementary Figure S1).

### 337 **3.5 Rheological test**

338 To better characterize the proposed materials in terms of mechanical features and to assess the behavior of  
339 the individual parts of the system under physical stress, the rheological analysis was performed on the SELP,  
340 dECM and ECM (Figure 8). The amplitude sweep test showed that from 0 to 1000 Pa of amplitude, the  
341 hydrogel and the ECM were still in the linear viscoelastic region (LVER), as both the storage  $G'$  and loss  $G''$   
342 moduli remained constant, with higher  $G'$ . It is worth noting that some peaks are observed in the  $G'$  and  $G''$   
343 plots for both the ECM and dECM, but this is most likely due to the irregular shape of the pieces analyzed,  
344 which did not allow a consistent gap of 0.5 mm throughout the whole sample. Consistently with the results  
345 of the frequency sweep test, again the  $G'$  and  $G''$  remained constant, indicating the expected viscoelastic  
346 behavior of the hydrogel and the ECM in the frequencies applied. The test also highlighted a decrease in the  
347 complex viscosity  $|\eta^*|$  with increasing frequencies, showing a shear-thinning behavior of all the tested  
348 materials, effectively leading to a more liquid-like state in higher frequencies. This behavior is further  
349 highlighted by the viscosity curve, where the viscosity  $\eta$  decreases linearly as the shear rate increases. Finally,  
350 the viscosity curve showed higher yield stress and viscosity of the SELP hydrogel in comparison to the native  
351 cartilage, which showed lower viscosity, probably because of its structural organization, unlike SELP which,  
352 instead, showed values very similar to those of native tissue.

## 353 **4 Discussion**

354 In this study an ECM stemming from decellularized cartilage of animal origin, specifically from equine  
355 metacarpophalangeal and metatarsophalangeal joints, was evaluated in vitro and proposed as structural  
356 substitute of articular cartilage to enhance tissue regeneration. The use of dECM has revealed great potential  
357 for clinical use and is widely expanding in the fields of tissue engineering and regenerative medicine as a  
358 natural alternative for cartilage repair. Decellularized cartilage can be used to produce scaffolds or three-  
359 dimensional supports that enable cartilage tissue engineering and can be used to provide a favorable  
360 environment for cartilage repair and regeneration by facilitating the adhesion and growth of cartilage tissue  
361 progenitor cells (Cheng et al., 2014; Stone et al., 2023). The decellularization approach displays the advantage  
362 to preserve natural tissue ECM while simultaneously obliterating the specific cells in tissues or organs  
363 allowing to obtain biomimetic scaffolds with promising biological and biomechanical properties (Morris et  
364 al., 2017). A proper decellularization process preserves the complex ECM biomolecular and physical cues,  
365 thus boosting cell growth and viability (Kim et al., 2019) as confirmed by our results in terms of both cell  
366 proliferation and chondrocyte phenotype maintenance, proved by gene expression analysis and newly  
367 formed matrix deposition. The use of dECM as a scaffold material is beneficial over the use of other natural  
368 and synthetic materials, as it inherently keeps the bioactivity and properties of native tissue.  
369 Decellularization is the most effective method to guarantee the biocompatibility of biological derived  
370 scaffold, reducing tissue and organ immunogenicity. With regards to decellularization protocols, several  
371 methods have been proposed to retain as much of the tissue's bioactivity as possible while maximizing the  
372 removal of nuclear material (Xia et al., 2019). To ensure a high quality decellularization process we strictly

373 followed the guidelines published on this topic (Kasravi et al., 2023). Our results showed an optimal removal  
374 of native cartilage cells without altering the biomechanical properties of the tissue. At the same time, no loss  
375 of bioactivity of the native cartilage was evident, as the dECM sustained the proliferation of the chondrocytes  
376 cultured on decellularized cartilage. Furthermore, the ability to secrete extracellular matrix, as particularly  
377 shown in the P2 cells, is clear evidence of the maintenance of cartilage bioactivity after the decellularization  
378 process. The main limitation for the use of adult differentiated chondrocytes in cell-based therapy and tissue  
379 engineering for the repair of articular cartilage is the difficulty of maintaining their state of differentiation  
380 during cell expansion (Ravanetti et al., 2022). In the present study we assessed two dedifferentiation states  
381 of chondrocytes (P2 and P4) onto the dECM as biomatrix in both normoxia and hypoxia culture condition.  
382 Our results demonstrated an increased expression of chondrogenic-related genes when chondrocytes were  
383 cultured on dECM. For both ACAN and Collagen type II genes, high expression levels show chondrogenic  
384 differentiation, except for P4 cells under normoxic conditions, being statistically lower than P2 cells under  
385 the same conditions. The latter result probably reflects the reduced ability of late-stage chondrocytes to  
386 adapt to a normoxic condition. The chondrogenic transcriptional factor SOX9 also shows high expression  
387 under all experimental conditions, except P2-stage chondrocytes in normoxia. These results agree with  
388 previous studies demonstrating the positive effect of microenvironmental factors such as hypoxia on the  
389 maintenance of chondrocyte differentiation in culture (Ravanetti et al., 2022). Finally, the expression levels  
390 of the RUNX2 and Collagen type I genes, which normally are not expressed by cells that are differentiating  
391 towards the chondrocytic lineage, confirmed what has been observed. The low levels of RUNX2 and Collagen  
392 type I in P2 stage chondrocytes compared to P4 show a greater capacity of the first to differentiate. All  
393 together this data clearly indicates that the decellularized biomatrix represents a favorable environment for  
394 the maintenance of the chondrogenic phenotype which is also favored by hypoxic conditions.

395 The mechanical stability at the implantation site is an essential requirement for the use of any biomaterial  
396 for regenerative medicine applications. For our cartilage patch we propose a natural hydrogel to make the  
397 structure adherent to the subchondral bone. Hydrogels for tissue engineering, derived from various sources  
398 of both natural and synthetic origin, have recently attracted huge attention in cartilage regeneration research  
399 thanks to the similarity in their features to those of cartilaginous tissue, such as the capacity to absorb large  
400 amounts of water while maintaining their structure. In view of the ability to mimic the native tissue, hydrogel  
401 development is currently a new frontier in the research addressed to treat cartilage defects (Han et al., 2018;  
402 Wang et al., 2014). The biocompatibility and preliminary outcome of the bio-adhesive properties of SELP  
403 make it promising to be considered as a biological glue. SELP are designed to combine the high mechanical  
404 and tensile strength of silk with the high resilience of the elastin in a single structure (Borrelli et al., 2020;  
405 Katari et al., 2015; Machado et al., 2013a). These artificial block copolymers are based on repetitive amino  
406 acid sequence motifs commonly found in silk and elastin. SELP are biocompatible and can be processed into  
407 different nano- and macrostructures such as hydrogels, fibers, and films (Chang et al., 2011; Huang et al.,  
408 2015; Zeng et al., 2014). SELP fiber mats, beside not being cytotoxic, support cell adhesion and proliferation  
409 of human skin fibroblast confirming their suitability for tissue engineering applications (Machado et al.,  
410 2013b) and have emerged as a promising biomaterial due to their unique properties, including self-assembly,  
411 biocompatibility and tunable mechanical properties. Concerning the preparation of SELP in different  
412 dissolution media, we have demonstrated that both water and PBS are adequate solvents, and do not exert  
413 significant changes at the morphological or structural level. This further supports the versatility of SELP  
414 processing as, depending on the application, solvents with different ionic strengths can be used. For instance,  
415 water can be employed for various applications, such as surface coating and drug delivery (Huang et al.,  
416 2015), while preparation of SELP with PBS provides a pH-balanced environment for specific biological and  
417 medical applications requiring buffering capacity. Furthermore, the buffering capacity of PBS can help

418 maintain a stable pH of the SELP even in the presence of acidic or basic substances (Dandu et al., 2009). This  
419 may be particularly important in biomedical applications where gel-like solutions may be exposed to different  
420 pH levels. In general, the choice of water or PBS for preparing SELP depends on the intended application, the  
421 stability and solubility of the protein, and the pH requirements of the system. In our experiments, the bio  
422 and cyto-compatibility of SELP-based materials showed no cytotoxicity, supporting the adhesion and  
423 proliferation of chondrocytes at different experimental times. This finding confirms the suitability of using  
424 SELPs for tissue engineering applications (Machado et al., 2013b).

425 The rheological tests highlight several interesting properties about the tested materials. First, it is noticeable  
426 that both the hydrogel and the ECM present a viscoelastic behavior, indicating their ability to be subjected  
427 to physical stress and strain without suffering structural ruptures, which is of utmost importance when  
428 considered for a potential scaffold for osteochondral lesions. Moreover, while it seems that higher shear  
429 rates lead to a decrease in viscosity, this should not have an impact on the structural integrity of the gel and  
430 could make it more convenient for its application and spreading on the potential lesion site. Lastly, the  
431 decellularization process does not lead to any significant difference in the physical characteristics of the ECM,  
432 highlighting its effectiveness and suitability for a potential scaffold to treat osteochondral lesions.

433 Given the common structures of silk protein, from which the SELP derives, the high amount of beta sheets  
434 is probably the cause of the viscoelastic behavior of the hydrogel, and its capability to withstand shear stress  
435 without losing structural integrity for the tested conditions.

436 Overall, the hydrogel shows very interesting physical characteristics that could be exploited as a bioadhesive  
437 for tissue engineering, since it seems to be able to resist physical stress associated with tissue or bone  
438 movement, without losing its structural integrity. Interestingly, its shear-thinning behavior could also be of  
439 use when considering that the hydrogel has to be applied and spread over non-homogeneous surface. FTIR  
440 spectra indicates that the high percentage of secondary beta structures could be the main cause of these  
441 physical properties. A previous study demonstrated that SELP-47 K hydrogel can be used as a scaffold to  
442 encapsulate and culture human mesenchymal stem cells in chondrogenic medium, inducing their  
443 differentiation toward a chondrogenic phenotype (Haider et al., 2008). Accordingly, in our study SELP  
444 supports the maintenance of chondrogenic roundish morphology, favoring the aggregation of cells into  
445 clusters. Therefore, the proposed SELP hydrogel could be used with the dual purpose of effectively attaching  
446 the dECM to the osteochondral bone, but also to successfully maintain cell phenotype in the tissue after  
447 construct implantation.

## 448 **5 Conclusions**

449 In the present study we proposed and in vitro evaluation of an engineered construct to foster cartilage  
450 regeneration. Our tissue is composed of decellularized ECM (dECM) obtained from horse joint cartilage, to  
451 support tissue regeneration, coupled with a Silk-Elastin-Like Protein (SELP) hydrogel, acting as a biological  
452 glue, to guarantee an adequate adherence to the host tissue. Both dECM and SELP display unique properties  
453 allowing the creation of tailor-made microenvironments capable of supporting cell growth and  
454 differentiation. Moreover, dECM mimics the natural environment of cartilage, making it an attractive option  
455 for repairing damaged or diseased cartilage supporting, especially if combined with hypoxia, the  
456 differentiation of chondrocytes, even those at P4 displaying a more dedifferentiated phenotype. SELP, alone  
457 or in combination with a dECM, used to create cartilage patch is an interesting tool for tissue engineering  
458 and regenerative medicine. Collectively, the results of the rheological tests emphasize several interesting  
459 properties about the hydrogel, first of all the viscoelastic behavior, indicating hydrogel ability to be subjected  
460 to physical stress and strain without suffering structural alterations, feature of utmost importance  
461 considering its potential application as bioadhesive. Moreover, while it seems that higher shear rates lead to  
462 a decreased viscosity, this should not impact the structural integrity of the hydrogel, making it suitable for

463 the treatment of load bearing tissue. Overall, the hydrogel holds very interesting physical characteristics that  
464 may be a real asset for its application, particularly due to its resistance to the physical stress generally  
465 associated with tissue and bone movement. Intriguingly, the shear-thinning behavior of the hydrogel may  
466 also make it suitable to be applied and spread over non-homogeneous surfaces. The proposed hybrid  
467 biomaterial, while needing to be further tested in preclinical animal models, seems promising for potential  
468 applications in the treatment of cartilage defects and injuries.



469 **Figure Captions**

470 **Figure 1** Graphic summary of the biomimetic approach combining decellularized cartilage matrix and Silk-  
471 elastin-like-protein (SELP) hydrogel for an articular cartilage patch.

472 **Figure 2.** Histology of native (ECM) and Decellularized Cartilage Extracellular Matrix (dECM) stained with H&E  
473 (A, B), counterstained with DAPI (C, D) and analyzed with Scanning Electron Microscopy (E, F). A-D scale bar  
474 = 50  $\mu\text{m}$ ; E-F scale bar = 10  $\mu\text{m}$ . Histograms show the chemical quantification of DNA content (G) and water  
475 adsorption capacity (H) of ECM and dECM.

476 **Figure 3.** Histological microphotographs of dECM cultured with equine chondrocytes at passages P2 and P4  
477 in normoxic and hypoxic culture conditions, stained with H&E (A), Picro Sirius Red (B) and Toluidine Blue (C).  
478 (Scale Bar = 100  $\mu\text{m}$ ). Figure D shows cell count of P2 and P4 chondrocytes cultured in adhesion under 20%  
479 (normoxia) or 5% oxygen (hypoxia) condition for 2 weeks on dECM. Figure E shows the collagen quantification  
480 based on Picrosirius Red staining within the newly deposited ECM. Asterisks indicate significant difference (\* $P$   
481 < 0.05, \*\*  $P$ <0.01, \*\*\*  $P$ <0.001) between normoxia and hypoxia condition at P2 or P4 ( $n$  = 5).

482 **Figure 4.** Scanning Electron Microscopy of chondrocytes cultured on dECM in normoxic conditions at P2 (A,  
483 E) and P4 (B, F) and in hypoxic conditions still at P2 (C, G) and P4 (D, H). Scale bar = 10  $\mu\text{m}$ .

484 **Figure 5.** Gene expression of ACAN (A), SOX-9 (B), RUNX2 (C), types I (D) and type II (E) collagen, in  
485 proliferating P2 and P4 chondrocytes cultured in adhesion under 20% oxygen (normoxia) or 5% oxygen  
486 (hypoxia) for 2 weeks ( $n$  = 5). The gene expression of control (GADPH) is shown as a dotted line. Asterisks  
487 within the bars indicate significant difference referred to control. Asterisks above the lines indicate significant  
488 difference between P2 and P4 (\* $P$  < 0.05, \*\*  $P$ <0.01, \*\*\*  $P$ <0.001). Immunofluorescence panel (F) shows  
489 collagen type II in P2 and P4 equine chondrocytes cultured on dECM in normoxia or hypoxia condition. Scale  
490 bar = 100  $\mu\text{m}$ .

491 **Figure 6** Scanning Electron Microscopy of SELP hydrogel in PBS (A, C) and water (B, D) after 24 hours (scale  
492 bar = 100  $\mu\text{m}$ ). Histology sections (H&E staining) of chondrocytes cultured on the SELP (E, scale bar 50  $\mu\text{m}$ ; F  
493 scale bar =100  $\mu\text{m}$ ). MTT assay of equine chondrocytes cultured on SELP and plastic used as control, after 24,  
494 48 hours and 4, 7 days of culture (G). Asterisks indicate significant difference between substrates (\* $P$  < 0.05).

495 **Figure 7** Amplitude sweep test, frequency sweep tests and viscosity curves for the SELP hydrogel, dECM and  
496 ECM. For the amplitude sweep, the frequency  $f$  was kept constant at 1 Hz, and for the frequency sweep, the  
497 amplitude  $\tau$  was kept constant at 50 Pa. The  $G'$  and  $G''$  moduli are represented in the left Y axis, whereas the  
498  $\tan(\delta)$  and  $|\eta^*|$  are represented in the right Y axis for their respective graph. The viscosity  $\eta$  is represented  
499 as a function of the shear rate  $\dot{\gamma}$ . In all cases, the temperature was kept at 37  $^\circ\text{C}$  and the gap between the  
500 plates was 0.5 mm. All tests were run in duplicate, with both replicas being represented.

501 **Figure 8** Curve-fitted second derivative spectra (solid red line) of SELP-based samples in  $\text{H}_2\text{O}$  and PBS after  
502 24 and 72 h. The bands filled in cyan indicate contributions from  $\beta$ -structures. The Gaussian bands (light gray)  
503 were fitted iteratively to the amide I band (black dots) using peak positions determined from the second  
504 derivative spectrum (A). The fractional distribution of the major secondary structure components is  
505 represented in B.

506 **Supplementary Figure S1:** A SELP hydrogel was spread between two circular-shaped cardboards of 1.5 mm  
507 in diameter. Different loads (50, 150, 300 and 500 g) were applied up to 7 days.  $\checkmark$  Indicates the maintenance  
508 of the adhesiveness for the tested conditions.

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